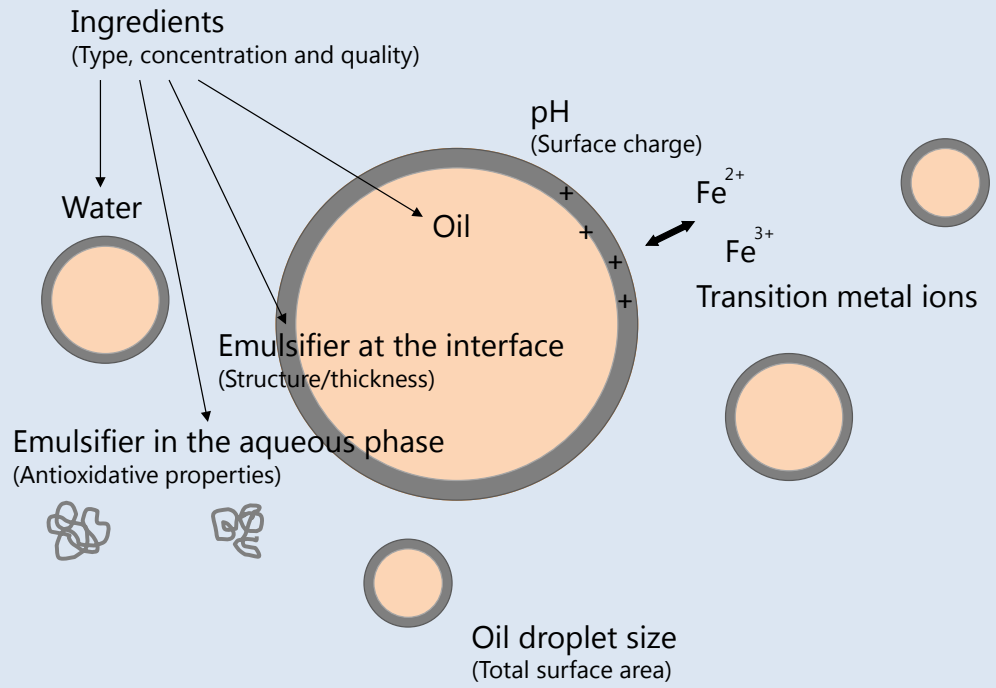
Factors Influencing the Effect of Milk-based Emulsifiers on Lipid Oxidation in Omega-3 Emulsions



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PhD Thesis
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DTU Food
National Food Institute

Factors Influencing the Effect of Milkbased Emulsifiers on Lipid Oxidation in Omega-3 Emulsions

PhD Thesis by Anna Frisenfeldt Horn

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Preface

The present thesis concludes my PhD project which has been carried out as a part of the research project "Omega-3 food emulsions: Control an investigation of molecular structure in relation to lipid oxidation". The research project was financed by the Danish Food Ministry (DFFE) and DTU globalization funds, and carried out in collaboration with Arla Foods amba, and DTU Mechanical Engineering.

My PhD project was conducted at the Division of Industrial Food Research, National Food Institute, Technical University of Denmark during the period April 2008 to January 2012. The PhD project was interrupted by two periods of leave, where I was assigned other tasks, for a total of 10 months. My main supervisor on the project was Professor Charlotte Jacobsen. Furthermore, Senior research scientist Nina Skall Nielsen and Associate professor Peter Szabo acted as co-supervisors.

During the PhD project period I have been on two research stays. The first one in the Food Process Engineering Group, Wageningen University, The Netherlands under supervision by Associate professor Karin Schröen (August 2008) and the second at the Department of Food Science, University of Massachusetts, Amherst, MA, USA under supervision by Professor Julian McClements and Professor Eric Decker (February – June 2009). The research stay in Amherst was supported by a research grant from FOOD Denmark.

Many people have supported me along the way, and these people deserve my sincere thanks.

First of all I am deeply grateful to my main supervisor Professor Charlotte Jacobsen for always believing in me, for continuously encouraging me, and for taking the time for discussions and advising me whenever needed. Most importantly, for teaching me that in research there is no such thing as problems, only consecutive challenges that have to be concurred.

I would also like to thank my two co-supervisors Nina Skall Nielsen and Peter Szabo along with the other participants in the project, Lene Vognsen and Ulf Andersen from Arla Foods amba, and Andy Horsewell and Louise Søgaard from DTU Mechanical Engineering. I appreciate all our discussions of my experimental plans and results throughout the project. I am also grateful for the supervision by Associate Professor Karin Schröen, Professor Julian McClements and Professor Eric Decker during my research stays, and for the experimental work carried out by Nathalie Barouh (Master student from France) and Marie Nouard (Master student from KU Life).

I owe my sincere thanks to Lis Berner for her outstanding technical work in the lab, and for her critical "blue" approach to the work, which have led to valuable discussions on the experimental strategies. In addition, she has always been there for me and listened when times were tough. I also want to thank technicians Jane Jørgensen, Trang Vu and Inge Holmberg for being kind, helpful and always fun to spend time with.

My heartfelt thanks go to Mette for her invaluable help and guidance, and for always being there. Furthermore, my thanks go to a great number of other people whom have made my years at the Division of Industrial Food Research a wonderful experience - you know who you are - I have enjoyed your company every single day.

Finally, I would like to thank my family and my friends, for helping me out through the period of thesis-writing, and for listening to all my fish oil-talk.

Mette, Maria and Line Marie - you each have a special place in my heart.

My nieces and nephews - you are the stars in my life.

Anna Frisenfeldt Horn

Kgs Lyngby, January 31., 2012

Summary

Intake of fish oil, and in particular the long chained polyunsaturated omega-3 fatty acids, has over the last centuries been associated with a wide range of health beneficial effects. Nevertheless, the intake of these healthy lipids is still lower than recommended in most Western populations. An interest in omega-3 enriched foods has therefore developed. The challenge when the polyunsaturated omega-3 fatty acids are added to foods is their sensitivity towards heating, metal ions and oxygen, as these factors can lead to lipid oxidation. To avoid this, a possible approach is to incorporate and thereby protect the fatty acids in an emulsion before they are added to the food product. However, the use of these so-called delivery emulsions in different food products has shown contradictory results.

On this background, the overall goal of the present PhD work was to increase our knowledge about factors related to the choice of emulsifier, homogenization equipment and emulsification conditions that could influence lipid oxidation in simple fish oil-in-water emulsion systems. The main focus was on the use of milk proteins alone or in combination with phospholipids as emulsifiers. In addition, the aim was to utilize this knowledge for designing delivery emulsions for the addition of fish oil to foods, and thereby achieve oxidatively stable fish oil enriched products.

In simple emulsions, sodium caseinate, whey protein isolate, soy lecithin and combinations of milk proteins and milk phospholipids were investigated as emulsifiers in both 5% and 70% fish oil-in-water emulsions. The effects of the individual emulsifiers were evaluated at different pH values, emulsifier concentrations and with or without the addition of iron. Generally, protein stabilized 5% oil-in-water emulsions were more oxidatively stable at low pH than at neutral pH, whereas the opposite was observed for 70% oil-in-water emulsions. It was shown that emulsions prepared with the highly flexible milk protein casein were the least oxidized at the varying conditions, followed by emulsions with whey protein isolate. The use of soy lecithin or a combination of milk protein and milk phospholipids as emulsifier in these 5% and 70% emulsions was shown only to be advantageous in 70% emulsions at low pH. Moreover, a good quality of the emulsifier was shown to be crucial for obtaining a better oxidative stability of emulsions prepared with phospholipids than with milk proteins.

The oxidative stability of 10% oil-in-water emulsions prepared with varying ratios of individual whey protein components, α-lactalbumin and β-lactoglobulin, was furthermore investigated at different pH values. Similarly to the 5% emulsions, the oxidative stability of these 10% emulsions was better at low pH than at neutral pH, independent of the type of emulsifier. No difference was observed in the antioxidative effect of the whey protein components when emulsions were prepared at pH 4. Nevertheless, at neutral pH the highest antioxidative effect during the emulsification process was achieved when using the emulsifier with the highest concentration of β-lactoglobulin, whereas during storage the best oxidative stability was observed in the emulsions with the highest concentration of α-lactalbumin. These differences were ascribed to the partitioning of α-lactalbumin and β-lactoglobulin between the interface and the aqueous phase in the emulsion.

It was demonstrated that the use of different high pressure homogenizers influenced lipid oxidation in emulsions prepared with whey protein isolate as emulsifier, but not emulsions prepared with sodium caseinate. Moreover, it was shown that that the applied pressure during high pressure homogenization influenced the resulting oxidative stability of the emulsion dependent on the emulsifier used. Overall, it was concluded, that the partitioning of proteins between the interface and the aqueous phase, and the

composition of protein components at the interfacial layer played an important role for the oxidative stability of emulsions prepared on different equipments and under various conditions.

In two case studies, fish oil-in-water emulsions prepared with different milk-based emulsifiers were used as delivery emulsions in milk and cream cheese. Unexpectedly, results showed that a better oxidative stability was achieved when the fish oil was added as neat oil to the milk than as a 10% delivery emulsion. Furthermore, no difference was observed on the oxidative status of the milks dependent on the type of emulsifier used for preparing the delivery emulsions.

Independent of the introduction method of fish oil to cream cheese (neat oil vs a 70% delivery emulsion), the fish oil enriched cream cheese oxidized during a 20 weeks storage period to a degree where the sensory quality of the product was significantly impacted. However, in contrast to the fish oil enriched milks, differences in the oxidative stability were observed between cream cheeses containing delivery emulsions prepared with different emulsifiers. The use of a combination of milk proteins and milk phospholipids for preparing the delivery emulsion was shown to change the macro structure of the cream cheese. Furthermore, this cream cheese was less oxidized than the cream cheeses added delivery emulsions with whey protein isolate or sodium caseinate but similarly oxidized as the cream cheese added neat fish oil. Interestingly, the use of sodium caseinate as emulsifier in the delivery emulsions was shown to result in the least oxidatively stable fish oil enriched cream cheese.

Overall, this PhD work showed that factors related to both the choice of emulsifier, homogenization equipment and emulsification conditions influence the oxidative stability of simple fish oil-in-water emulsions. These factors include the oil concentration, the type of milk protein or phospholipid used as emulsifier, the pH, the addition of iron, preheating of the protein prior to homogenization, the equipment used for homogenization and the pressure applied during high pressure homogenization. In addition, lipid oxidation in simple fish oil-in-water emulsions was shown to depend on combinations of these factors, and not any one of them alone. Moreover, it was shown that despite an attempt to optimize the above-mentioned and thereby create an oxidatively stable fish oil-in-water delivery emulsion, this was not enough to ensure a protection of the fish oil when the delivery emulsion was added to milk or cream cheese.

Resumé

Indtag af fiskeolie, og især de langkædede polyumættede omega-3 fedtsyrer, er over de seneste årtier blevet sat i sammenhæng med en lang række helbredsmæssige effekter. Flere vestlige befolkningsgrupper har dog stadig et lavere indtag af disse sunde fedtsyrer, end det der anbefales for at opnå de sundhedsgavnlige effekter. Interessen for omega-3 berigede fødevarer er derfor stigende. Udfordringen med de polyumættede fedtsyrer er dog, at de er meget oxidationsfølsomme, når de kommer i forbindelse med f.eks. varme, metalioner og ilt, der som oftest er til stede i eller under produktionen af fødevarer. I nogle fødevarer har det vist sig, at man kan opnå en beskyttelse af fedtsyrerne mod oxidation ved at inkorporere dem i emulsioner inden tilsætning til fødevaren. Det modsatte er dog også vist, og effekten af at bruge de såkaldte "delivery emulsioner" er derfor ikke entydig.

På denne baggrund har dette ph.d. projekt haft til formål at øge vores viden om lipidoxidation i simple fiskeolieberigede emulsioner gennem en grundig undersøgelse af faktorer relateret til valget af emulgator, homogeniseringsudstyr og emulgeringsbetingelser. Fokus var på mælkeproteiner som emulgatorer, enten anvendt alene eller i kombination med phospholipider. Desuden var det et mål at bruge den opnåede viden til at fremstille "delivery emulsioner" med henblik på at opnå oxidativt stabile fiskeolieberigede fødevarer.

For simple emulsioner blev natriumkaseinat, valleproteinisolat, to kombinationer af mælkeproteiner og mælkephospholipider samt sojalecithin undersøgt som emulgatorer i både 5% og 70% emulsioner. Emulgatorernes effekt på den oxidative stabilitet af de fremstillede emulsioner blev evalueret afhængig af pH, emulgatorkoncentration og jerntilsætning. Generelt blev det vist, at mælkeproteinbaserede 5% emulsioner var mere stabile ved lavt pH end ved neutral pH, mens det modsatte gjorde sig gældende for 70% emulsioner. Det blev desuden vist, at emulsioner fremstillet med det meget fleksible mælkeprotein kasein stort set ved alle de undersøgte betingelser oxiderede mindst, efterfulgt af emulsioner fremstillet med valleproteinisolat. Brugen af sojalecithin eller en kombinationen af mælkeprotein og mælkephospholipid som emulgator i disse emulsioner viste sig kun at være fordelagtig i 70% emulsioner ved lavt pH. En god kvalitet af emulgatoren viste sig desuden at være altafgørende for at opnå en bedre oxidativ stabilitet af emulsioner fremstillet med phospholipider sammenlignet med mælkeproteiner.

Den oxidative stabilitet af 10% olie-i-vand emulsioner fremstillet med forskellige ratioer af de individuelle valleproteinkomponenter α-lactalbumin og β-lactoglobulin blev endvidere undersøgt ved forskellige pH-værdier. Uafhængigt af emulgatortype, og i lighed med 5% emulsionerne var også disse emulsioner mere oxidativt stabile ved lavt pH. Det blev endvidere vist, at der ingen forskel var på den antioxidative effekt af emulsionerne ved pH 4. Ved neutral pH var den antioxidative effekt under produktionen derimod størst i emulsionen med den højeste koncentration af β-lactoglobulin, mens den mest oxidative stabile emulsion under lagring var den, der blev fremstillet med højest koncentation af α-lactalbumin. Forskellene blev tilskrevet fordelingen af α-lactalbumin og β-lactoglobulin mellem vandfasen og grænsefladen.

Det blev for første gang vist, at brugen af forskellige typer homogeniseringsudstyr kan influere på lipidoxidationen i emulsioner produceret med valleproteinisolat som emulgator men ikke i emulsioner produceret med natriumkaseinat. Det blev desuden vist, at homogeniseringstrykket havde forskellig betydning for oxidationsstabiliteten afhængig af typen af emulgator, der blev brugt. Samlet set blev det konkluderet, at fordelingen og sammensætningen af proteiner på grænsefladen og i vandfasen var vigtige for oxidationen i emulsioner homogeniseret på forskelligt udstyr og under forskellige betingelser.

I to afsluttende fødevareforsøg blev forskellige emulsioner udvalgt og afprøvet som "delivery emulsioner" i hhv. mælk og flødeost. Mod forventning viste det sig, at den oxidative stabilitet af fiskeolieberiget mælk blev forringet, når fiskeolien blev tilsat i en 10% "delivery emulsion" i stedet for som ren olie. "Delivery emulsionens" emulgatortype påvirkede ikke den oxidative stabilitet af mælken.

Ved tilsætning af fiskeolie til flødeost blev det vist, at uanset tilsætningsmetoden (ren olie vs en 70% "delivery emulsion") oxiderede osten over en 20 ugers lagringsperiode i en grad, så den sensoriske kvalitet blev signifikant påvirket. I modsætning til fiskeolieberiget mælk afhang den oxidative stabilitet i flødeosten dog af typen af emulgator brugt til fremstilling af "delivery emulsionen". Ved brug af en kombination af mælkeproteiner og mælkephospholipider som emulgator i "delivery emulsionen" ændredes makrostrukturen af flødeosten. Samtidig var denne flødeost mindre oxideret end de to flødeoste, der blev tilsat "delivery emulsioner" med hhv. natriumkaseinat og valleproteinisolat, men ligeså oxideret som osten tilsat ren fiskeolie. Overraskende viste det sig, at brugen af natriumkaseinat som emulgator resulterede i den højeste grad af lipidoxidation i osten.

Dette ph.d. projekt viste, at faktorer relateret til både valget af emulgator, homogeniseringsudstyr og emulgeringsbetingelser påvirkede den oxidative stabilitet af simple fiskeolie-i-vand emulsioner. De undersøgte faktorer var oliekoncentrationen, typen af mælkeprotein eller phospholipid som emulgator, pH, jerntilsætning, opvarmning af proteinet før homogenisering, typen af homogeniseringsudstyr og det anvendte tryk under højtrykshomogenisering. Det blev desuden vist at den oxidative stabilitet af en emulsion afhænger af kombinationer af disse faktorer og ikke af enkelte faktorer alene. Endelig blev det vist, at på trods af en optimering af disse faktorer i fht. at opnå en oxidativt stabil olie-i-vand emulsion, var dette ikke tilstrækkeligt til at opnå en beskyttelse af fiskeolien når denne "delivery emulsion" blev tilsat til mælk eller flødeost.

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List of Publications

PAPER I

Horn AF, Nielsen NS, Andersen U, Søgaard LH, Horsewell A & Jacobsen C (2011) Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH.

European Journal of Lipid Science and Technology 113, p.1243-1257

PAPER II

Horn AF, Nielsen NS & Jacobsen C (2012) Iron-mediated lipid oxidation in 70% fish oil-in-water emulsions: effect of emulsifier type and pH.

International Journal of Food Science and Technology 47, p.1097-1108

PAPER III

Horn AF, Nielsen NS, Jensen LS, Horsewell A & Jacobsen C (2012) The choice of homogenisation equipment affects lipid oxidation in emulsions.

Food Chemistry 134, p.803-810

PAPER IV

Horn AF, Wulff T, Nielsen NS & Jacobsen C (2012) Effect of α-lactalbumin and ß-lactoglobulin on the oxidative stability of 10% fish oil-in-water emulsions depends on pH.

Food Chemistry (submitted)

PAPER V

Horn AF, Barouh N, Nielsen NS, Baron CP & Jacobsen C (2012) Homogenization pressure and temperature affect protein partitioning and oxidative stability of emulsions.

Journal of the American Oil Chemist´s Society (corrected and resubmitted)

PAPER VI

Nielsen NS, **Horn AF** & Jacobsen C (2012) Effect of emulsifier type, pH and iron on oxidative stability of 5% fish oil-in-water emulsions.

European Journal of Lipid Science and Technology (submitted)

PAPER VII

Horn AF, Nielsen NS & Jacobsen C - Lipid oxidation in milk enriched with neat fish oil or pre-emulsified fish oil.

Draft intended for Food Chemistry

PAPER VIII

Horn AF, Green-Petersen D, Nielsen NS, Andersen U, Hyldig G, Jensen LHS, Horsewell A & Jacobsen C (2012) Addition of fish oil to cream cheese affects lipid oxidation, sensory stability and microstructure.

Agriculture 2, p.359-375

List of Abbreviations

(Only abbreviations used in the text are listed. Abbreviations in figures, tables and schemes are given in the legends. Please refer to Table 4 (p.26) for interpretation of sample code names)

α-lac:	α-lactalbumin
ALA:	α-linolenic acid, C18:3n-3
β-lg:	β-lactoglobulin
Cryo-SEM:	Cryo-scanning electron microscopy
Cryo-TEM:	Cryo-transmission electron microscopy
DHA:	Docosahexaenoic acid, 22:6n-3
DHS:	Dynamic headspace
EFSA:	European Food Safety Authority
EPA:	Eicosapentaenoic acid, 20:5n-3
GC-MS:	Gas chromatography-mass spectrometry
HS:	High speed
L•:	Lipid radical
LC omega-3 PUFA:	Long chain omega-3 polyunsaturated fatty acids
LH:	Unsaturated lipid
LOO•:	Lipid peroxyl radical
LOOH:	Lipid hydroperoxide
MIC:	Microfluidizer
o/w:	Oil-in-water
PC:	Phosphatidylcholine
PE:	Phosphatidylethanolamine
PI:	Phosphatidylinositol
PUFA:	Polyunsaturated fatty acids
PV:	Peroxide value
SDS-page:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM:	Sphingomyelin
SPME:	Solid phase microextraction
TBARS:	Thiobarbituric acid reactive substances
VH:	Valve homogenizer
w/o:	Water-in-oil
WPI:	Whey protein isolate
w/w:	Weight/weight

Chapter 1:
Introduction and Hypotheses

1.1 INTRODUCTION

Long chain polyunsaturated omega-3 fatty acids (LC omega-3 PUFA) were first recognized for their health beneficial effects in the 1970´s, when Bang and Dyerberg (1972) studied plasma lipids and lipoproteins linked to the development of cardiovascular diseases in populations with very different intakes of marine omega-3 fatty acids. Since then, the evidence for health beneficial effects of LC omega-3 PUFA has increased tremendously, and today these fatty acids are not only associated with an improved cardiovascular health (Riediger et al., 2009; Yashodhara et al., 2009). The intake of LC omega-3 PUFA has also been shown to play a role in mental health (Hegarty and Parker, 2011; Perica and Delas, 2011) and inversely correlate with the development of certain cancer types (Caygill et al., 1996). In addition, LC omega-3 PUFA have been related to the development of the fetal brain and to the visual and cognitive development in infants (Koletzko et al., 2008). An overall improvement of a number of human body functions such as the immune system and the reproductive system has furthermore been suggested (Riediger et al., 2009).

On this background, recommendations have been set by different organisations on the intake of omega-3 fatty acids and especially on the two LC omega-3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (EFSA, 2010; ISSFAL, 2004). Nevertheless, the intake is still too low in most Western populations to meet these recommendations (Meyer et al., 2003; Sioen et al., 2007; Sioen et al., 2006). The interest and the market for omega-3 enriched foods have therefore developed rapidly during the last decade. The highly unsaturated omega-3 fatty acids are though invariably in risk of oxidation, whereby the quality of the food product to which they are introduced may be compromised. Strategies for protecting these healthy but oxidatively susceptible lipids have therefore to be developed.

One reasonable approach is the addition of a fish oil-in-water (o/w) emulsion, a so called delivery emulsion, as opposed to adding neat fish oil to the food product. In a delivery emulsion a membrane is created around the oil droplet, which may shield the lipids from its surroundings. Nevertheless, the success of this approach has been shown to be system dependent. Thus, in food products such as milk and cheese it has been shown to be advantageous to use a delivery emulsion (Let et al., 2007a; Ye et al., 2009), whereas in yoghurt and salad dressing fish oil delivery emulsions have been shown to decrease the oxidative stability (Let et al., 2007a). To understand these observations and to improve delivery emulsions for future use, more knowledge is needed about lipid oxidation in emulsion systems. For this purpose, simple o/w emulsions must be utilized to limit the complexity of influencing factors and thereby increase the possibility of scrutinizing the oxidation mechanisms in more detail.

1.2 AIM AND HYPOTHESES

The overall aim of my PhD work was to increase the knowledge about if and how the choice of emulsifier, homogenization equipment and emulsification conditions influence lipid oxidation in simple emulsion systems. A second aim was to use this knowledge for preparing oxidatively stable delivery emulsions for the addition of fish oil to foods.

For this purpose, both o/w emulsions with low (5-20%) and high oil concentrations (70%) were investigated. Emulsions with low oil concentrations are well described in the literature (McClements, 2005; Waraho et al.,

2011) and considered good systems for investigating a wide range of factors related to their production conditions. In contrast, oxidation studies on simple 70% o/w emulsions have never previously been published and little is therefore known about these emulsions. Nevertheless, these emulsions were selected for an exploratory investigation, because it is preferential to have as high an oil content as possible in delivery emulsions, particularly when added to food products with a low water content.

As emulsifiers, milk proteins were included in all studies due to their common use in food products and their potential antioxidative effects (Elias et al., 2005; Faraji et al., 2004; Tong et al., 2000) (elaborated upon in section 3.2.1). In addition, phospholipids of various origin were investigated either alone or in combination with milk proteins. Phospholipids were selected as they have shown potential antioxidative effects (Bandarra et al., 1999; Hamilton et al., 1998; Hildebrand et al., 1984; Judde et al., 2003) and an ability to change the thickness of the interfacial layer in combination with caseinate (Fang and Dalgleish, 1993a) (elaborated upon in section 3.2.1 and 2.4.2, respectively). For homogenization both high shear systems, high pressure systems and a membrane system were employed, due to their different emulsification principles (Jafari et al., 2008), as elaborated upon in section 2.3. For the high pressure systems, different emulsification conditions were moreover applied.

The PhD work set out to test the following hypotheses in simple emulsion systems:

- The type of milk protein used as emulsifier affects the oxidative stability of simple o/w emulsions differently due to varying amino acid compositions and thereby different **antioxidative properties**. Due to the different properties of milk proteins, they will therefore be differently affected by pH and addition of transition metal ions (Paper I-VI).
- Phospholipids can be used as emulsifier in simple o/w emulsions, and be used as an alternative to milk proteins. By having different **antioxidative properties** than milk proteins, phospholipids will protect o/w emulsions against lipid oxidation differently (Paper I, II, VI).
- An increase in emulsifier concentration reduces lipid oxidation as a result of **the thickness of the interfacial layer** and/or its **presence in excess in the aqueous phase** (Paper I, II, VI, Appendix I).
- Combinations of milk proteins and phospholipids can improve the **coverage of the interfacial layer** by emulsifier, through the creation of protein-phospholipid complexes, and thereby increase the oxidative stability of o/w emulsions (Paper I, II, VI, Appendix II).
- Different emulsification techniques and homogenization conditions (temperature and pressure) will affect the oxidative stability of o/w emulsions by influencing the **partitioning of milk proteins** between the interface and the aqueous phase and the **protein conformation** at the interfacial layer and (Paper III, V).
- Since lipid oxidation is an interfacial phenomenon, **the oil droplet size** and thus the total surface area might influence lipid oxidation in o/w emulsions (Paper I, II, VI and Appendix III).
- A positive oil droplet **surface charge** can lead to a repulsion of cationic transition metal ions and thereby a reduction in lipid oxidation in o/w emulsions (Paper I, II, IV, VI).

Two case studies were also included to investigate the use of delivery emulsions in more complex real food systems (Paper VII, VIII). In these case studies, the following hypothesis was tested:

- The oxidative stability of a fish oil enriched food product can be improved by the addition of an o/w delivery emulsion instead of neat oil, if the delivery emulsion is optimized to be oxidatively stable.

Chapter 2:
Emulsions, Emulsifiers and Emulsification Techniques

In this chapter, an introduction to emulsions, emulsifiers and emulsification techniques is given, with special emphasis on emulsifiers and equipment utilized in the present PhD work. In addition, important issues regarding physical stability of emulsions will be covered.

2.1 DEFINITION OF AN EMULSION

Essentially, an emulsion consists of three phases; a dispersed phase present as droplets in a continuous phase and separated by an interfacial region. Emulsions can either be o/w emulsions where oil droplets are dispersed in an aqueous phase, or water-in-oil (w/o) emulsions where water exists as droplets in an oil phase, as illustrated in Figure 1.

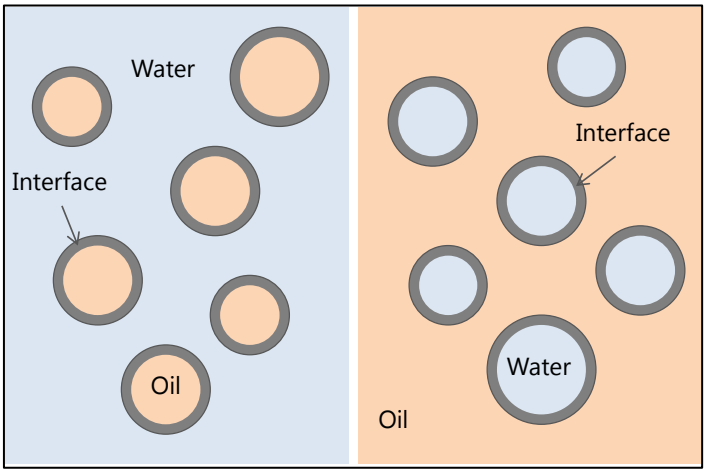


Figure 1. A schematic illustration of an oil-in-water emulsion with oil droplets dispersed in an aqueous phase (left) and a water-in-oil emulsion with water droplets dispersed in an oil phase (right). The oil and water are in both cases separated by an interface of emulsifier.

Emulsions can be very simple, when prepared from only a few ingredients or more complex when prepared with many different ingredients, e.g. food emulsions. Food emulsions such as milk, mayonnaise and cream cheese are examples of o/w emulsions, whereas butter and spreads are examples of w/o emulsions. The present thesis will exclusively deal with o/w-emulsions.

2.2 EMULSION FORMATION

Emulsion formation is the process in which two separate liquids (oil and water) are converted into an emulsion. This process covers

- homogenization, which requires a homogenization device, that can supply energy for the deformation and disruption of oil droplets, and
- emulsification, which is dependent on an emulsifier to facilitate droplet disruption and stabilize the newly formed droplets.

Several homogenization devices have been designed, including high shear, high pressure, membrane and ultrasonic systems (Jafari et al., 2008). These equipments differ in whether they can both perform primary and secondary homogenization or mainly the latter. Primary homogenization is defined as the process where the starting materials are two separate liquids, and secondary homogenization is a size reduction in an already existing emulsion (Figure 2).

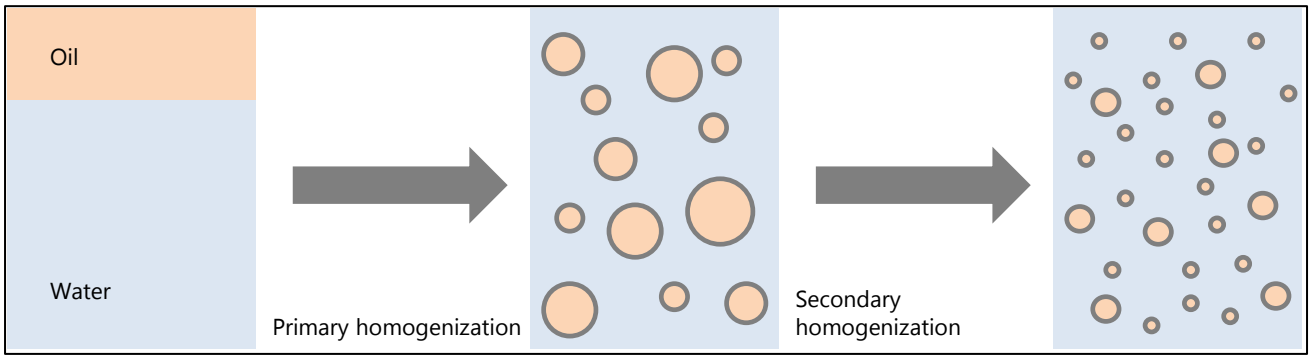


Figure 2. The process of primary and secondary homogenization. Primary homogenization is the process of converting separate oil and water phases to an emulsion. Secondary homogenization is a size reduction in an already existing emulsion.

The homogenization equipments vary in their emulsification principles, and thereby in the product throughput, the physical and chemical stress the product encounters during homogenization, the droplet sizes that can be obtained, and whether their distributions are mono- or poly-disperse (Schultz et al., 2004). Except from ultrasonic systems, all the other emulsification systems have been included in the present PhD work, and described in section 2.3.

The presence of an emulsifier during droplet disruption in the homogenization device, serves two purposes. Firstly, it can lower the interfacial tension whereby droplet disruption is facilitated through a reduction in the energy needed for this process (Walstra, 1993). Secondly, it can adsorb to the surface of the oil droplet and thereby reduce the risk of droplet coalescence.

To maintain the emulsion after formation and avoid that it separates into a layer of oil and water, the emulsion must be stabilized. Some emulsifiers have both emulsifying and stabilizing properties (e.g. milk proteins), whereas others have mainly emulsifying properties (e.g. low molecular weight surfactants). Hence, the addition of a stabilizer (e.g. a protein or a polysaccharide) is necessary. The emulsifiers included in the present thesis are milk proteins and components hereof and various phospholipids of different origin. These emulsifiers are described in section 2.4, and their properties related to lipid oxidation are outlined in section 3.2.1.

2.3 EMULSIFICATION TECHNIQUES – PRINCIPLES, ADVANTAGES, AND DISADVANTAGES

An overview of the main differences, advantages and disadvantages between the homogenization systems used in the present PhD work is given in Figure 3, and further elaborated upon in the following.

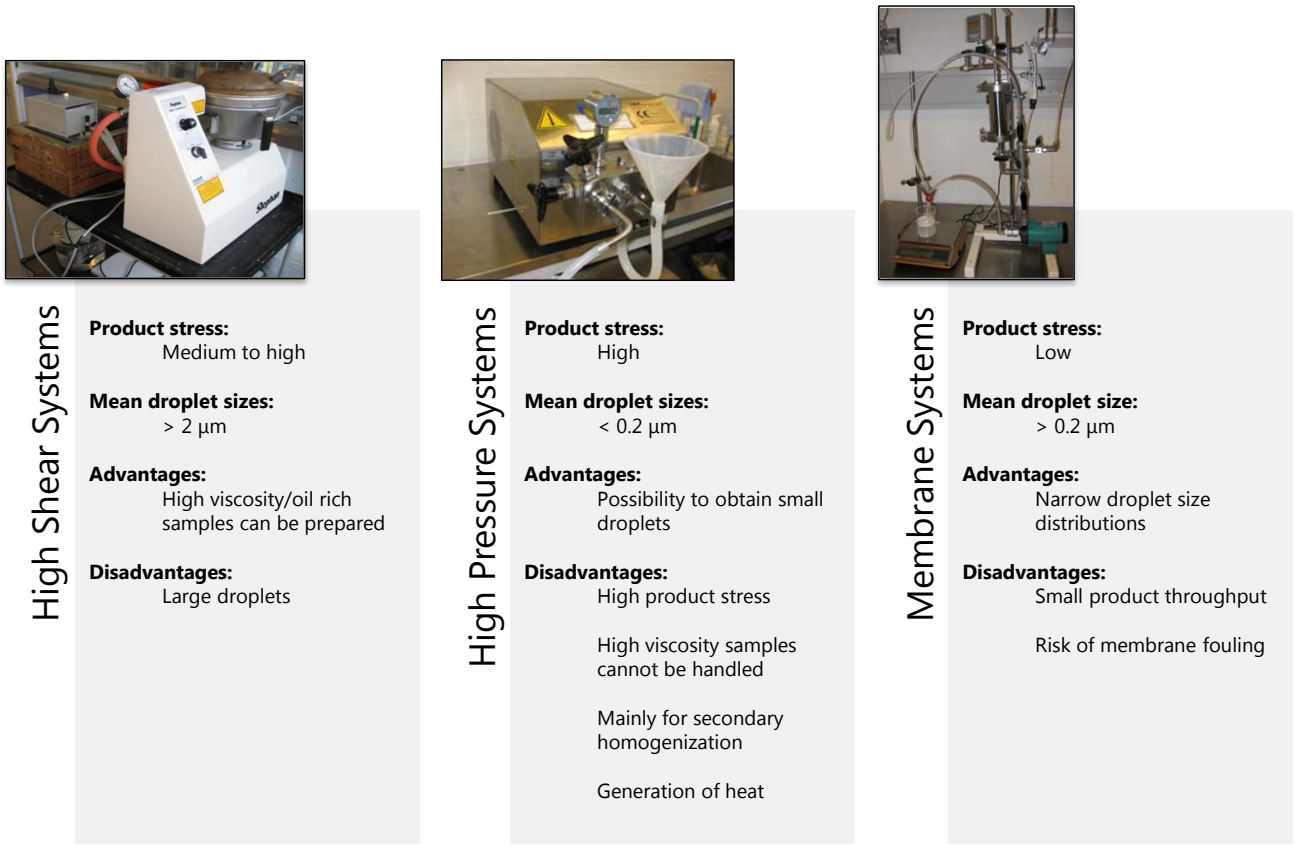


Figure 3. Main differences between three types of emulsification equipment used in the present PhD work, and their advantages and disadvantages. High shear systems are described in section 2.3.1, high pressure systems in section 2.3.2, and membrane systems in section 2.3.3.

2.3.1 HIGH SHEAR SYSTEMS - BLADE AND ROTOR/STATOR

Blade systems are the most simple homogenization systems, consisting of a mixing bowl and rotating blades to create high shear. One advantage of the blade mixer is that very viscous and oil-rich samples can be produced. Thus, in the present PhD work a Stephan mixer was used for preparing 70% o/w emulsions. The disadvantage of the blade mixer is that it produces fairly large oil droplets (> 1µm) and rather broad droplet size distributions.

Another mechanical high shear homogenization system is the rotor-stator device. In this system the liquid flows through a narrow gap between a rotating disk (the rotor) and a static disk (the stator). In the present PhD work handheld mixers with a rotor-stator head were used for primary homogenization prior to high pressure homogenization. When used alone, the handheld rotor-stator device has the same disadvantages as the blade systems.

2.3.2 HIGH PRESSURE SYSTEMS

A wide variety of high pressure systems exist for mainly secondary homogenization. High pressure systems make use of a high pressure pump to force liquids through a narrow tube/valve. In the present PhD work two high pressure systems have been used, namely a two-stage valve homogenizer and a microfluidizer.

In the valve homogenizer a pump pulls the emulsion into a chamber on its backstroke, and then forces it through a narrow valve at the end of the chamber on its forward stroke. In the valve, intense disruptive forces cause the larger droplets to break into smaller ones. The pressure drop across the valve is a result of adjusting the size of the gap through which the emulsion is passed. In a two-stage valve homogenizer the first valve is set at a high pressure for droplet disruption and the second valve is set at a low pressure for disrupting flocs created in the first valve (Schultz et al., 2004).

A microfluidizer works similarly by the force of a pump. However, in contrast to the valve homogenizer, droplet disruption occurs when droplets collide at the end of a very small passage in the interaction chamber. This collision makes droplets reduce in size (Schultz et al., 2004).

In both high pressure systems the pressure applied and the number of passes through the homogenization valve/chamber are highly influencing the resulting oil droplet size distributions, and in comparison to other homogenization devices the obtainable mean droplet size is very small (Jafari et al., 2007; Qian and McClements, 2011).

The disadvantages of using high pressure systems are the very high product stress due to the high pressure gradients and flow rates, as well as the possible generation of heat during homogenization (Mao et al., 2010; Schultz et al., 2004). In addition, this equipment cannot handle emulsions with a very high viscosity.

2.3.3 MEMBRANE SYSTEMS

The principle of membrane homogenization is that the dispersed phase is pressed through a microporous membrane into the continuous phase holding a water soluble emulsifier (Figure 4).

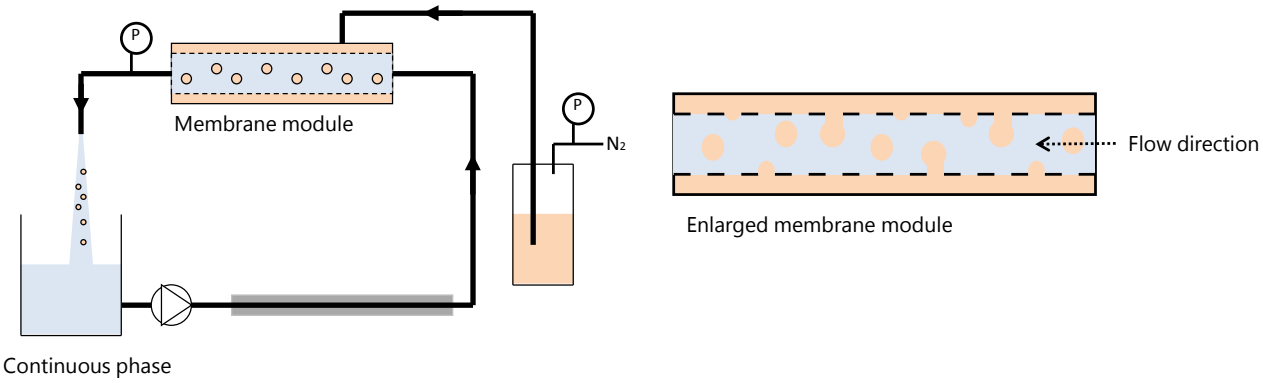


Figure 4. Schematic illustration of a membrane homogenizer (left) and droplet formation in a membrane (right). In the membrane homogenizer oil is pressed through the membrane into the buffer. Droplets are created at the pores of the membrane, and the flow of buffer through the membrane ensures droplet detachment. P: Pressure valve.

Small droplets are thereby created directly at the micropores of the membrane, without the stress of deformation and disruption in zones of high energy density (Nakashima et al., 1991; Schroder and Schubert, 1999). To ensure that the droplets detach regularly from the membrane pores, shear stress is created at the interface between the membrane and the continuous phase by recirculating the latter constantly past the membrane (Vladisavljevic and Williams, 2005), as illustrated in Figure 4.

The main advantages of membrane systems in comparison to the other homogenization systems are the very low product stress, and an ability to create very narrow oil droplet size distributions. The disadvantages of membrane homogenization are the very low dispersed phase flux and possible fouling phenomena at the membrane (Charcosset, 2009). The slow process and thereby the low product throughput makes this equipment useful mainly in lab-scale. Nevertheless, scale-up is possible by adding more membranes.

2.4 EMULSIFIERS – COMPOSITION AND ADSORPTION BEHAVIOUR

2.4.1 MILK PROTEIN AND MILK PROTEIN COMPONENTS

Bovine milk contains approximately 3.2% proteins, whereof around 80% are caseins and 20% are whey proteins (Fox and Mulvihill, 1982). Depending on how they are processed, various protein products can be produced with varying emulsifying and stabilizing properties. The whey protein products used in the present PhD work are very gently prepared, thus they have not been denatured and they have structural properties very similar to the original whey proteins in milk. However, the caseinate used is a sodium salt, which will most likely behave differently in an emulsion than the original casein does in milk.

Bovine milk caseins consist mainly of four different components, α_{s1}, α_{s2}, β and κ, and all four are present in sodium caseinate in an almost similar ratio as in the original milk. The four components differ in their number and composition of amino acid residues and thereby in their structural abilities (Table 1). Caseins lack secondary and tertiary structures, thus, they are considered very flexible molecules with a high surface activity (Creamer, 2003).

In contrast to caseins, whey proteins are globular, with both a more organized secondary and tertiary structure due to less proline and more cysteine residues (Table 1). The term whey proteins cover various different compounds, e.g. α-lactalbumin (α-lac), β-lactoglobulin (β-lg), serum albumin and immunoglobulins. Since α-lac and β-lg constitute the major part of commercial available whey protein products, the focus in the present thesis are on these, whereas the other compounds will not be discussed any further.

Table 1. Characteristics of the amino acid compositions of major proteins in bovine milk (Ng-Kwai-Hang, 2003).

		Approximate concentration in milk [g/L]	Σ amino acid residues	Proline	Cysteine (Sulfhydryl residues)	Phosphoseryl residues
Caseins	α_{s1}	10	199	17	0	8
	α_{s2}	3	207	10	2	10-13
	β	9	209	35	0	5
	κ	3	169	20	2	1
Whey proteins	α-lac	1	123	2	8	0
	β-lg	3	162	8	5	0

When adsorbed at an interface, milk proteins as well as other proteins form a strong viscoelastic film around the oil droplets by arranging themselves in "trains", "loops" and "tails" as visualized in Figure 5.

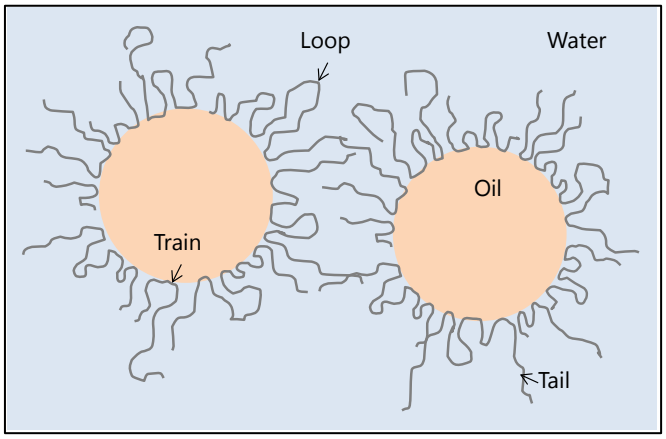


Figure 5. Schematic illustration of the adsorption of milk proteins to an oil droplet interface in a loop and train manner due to distinct hydrophilic and hydrophobic domains in the amino acid structure of the protein.

The "trains", "loops" and "tails" exist as a result of the distinct hydrophilic and hydrophobic domains in the proteins amino acid structure. Hence, upon adsorption at an oil-water interface, the hydrophilic amino acid domains will project into the water phase, whereas the hydrophobic amino acid domains will face the oil phase (Krog, 2004). Consequently, the structurally disordered caseins are expected to possess a higher surface activity and emulsifying capacity than the compact and highly ordered whey proteins. However, the structural conformations and the emulsifying properties of whey proteins are very sensitive towards different treatments, such as homogenization, a change in pH or heating (Fang and Dalgleish, 1997; Fang and Dalgleish, 1998; Hunt and Dalgleish, 1994b; Lee et al., 2007a; Stapelfeldt and Skibsted, 1999).

The thickness of the interfacial layer has been investigated by Dalgleish and co-workers in several studies (Dalgleish, 1993; Fang and Dalgleish, 1993b; Hunt and Dalgleish, 1994a), and these studies can be used to discuss the influence of the distance between the lipids and the possible pro-oxidants in the aqueous phase. In a study on 20% soy o/w emulsions with caseins obtained from skim milk (0.2-2.0%), it was suggested that casein had different conformations depending on the concentration used. At low concentrations casein was expected to stretch over the surface, whereas in high concentration the casein molecules were expected to attend a more compact structure. A compact structure of the casein molecules would provide additional space for more casein at the surface, whereby individual molecules would protrude further out into the aqueous phase and create a thicker interfacial layer (Fang and Dalgleish, 1993b).

To further investigate the thicknesses of the interfacial layers provided by different casein components, a model system of latex particles covered with caseins was used. Using this system it was observed that the thinnest interfacial layer was provided by α_{s1}-casein (5.4 nm) and the thickest by β-casein (11.1 nm). The other two, α_{s2}- and κ-casein, were found to provide thicknesses of the interfacial layers in between (8.5 and 8.3 nm, respectively) (Dalgleish, 1993).

In comparison, the droplet surface adsorption behaviour of whey proteins was suggested to be a little different, owing to the globular nature of the whey proteins (Hunt and Dalgleish, 1994a). Since more whey protein was needed to obtain a stable emulsion (1.5 mg m^{-2} compared to 1 mg m^{-2} for the casein), these proteins were not expected to be able to stretch over the droplet surface to the same extent as caseins.

However, later studies on the adsorption of individual whey protein components (β-Lg or α-lac prepared from whey protein isolate (WPI) products) by Fourier transform infrared spectroscopy revealed some concentration dependent structural changes upon adsorption (Fang and Dalgleish, 1997; Fang and Dalgleish, 1998). In the study on β-lg emulsions, the authors suggested that in low concentration (1% β-lg to 20% oil) the proteins were stretched over the interface whereby they changed conformation. In contrast, when proteins were present in excess (2% β-lg to 20% oil) they did not have to stretch to cover the interface, and therefore did not differ in conformation from the native protein in solution (Fang and Dalgleish, 1997). Thus, a concentration dependent conformational behaviour of whey proteins was suggested similarly to the one suggested for caseins.

In 30% soy o/w emulsions prepared with a combination of sodium caseinate and whey protein concentrate (1:1), whey proteins adsorbed in preference to caseins at total protein concentrations below 3%, whereas the opposite was observed at total protein concentrations above 3% (Ye, 2008). In homogenization studies on milk, the adsorption of the different milk proteins and their conformations at the interface have been shown to depend on the homogenization equipment used (Dalgleish et al., 1996).

2.4.2 PHOSPHOLIPIDS

Phospholipids are compounds with a specific hydrophilic (the head) and a hydrophobic part (the tail) (Figure 6, middle). The head group contains the negatively charged phosphate group whereas the tail group consists of fatty acids (Wang, 2008). The chemical structure for the most common phospholipids is shown in Figure 6, left.

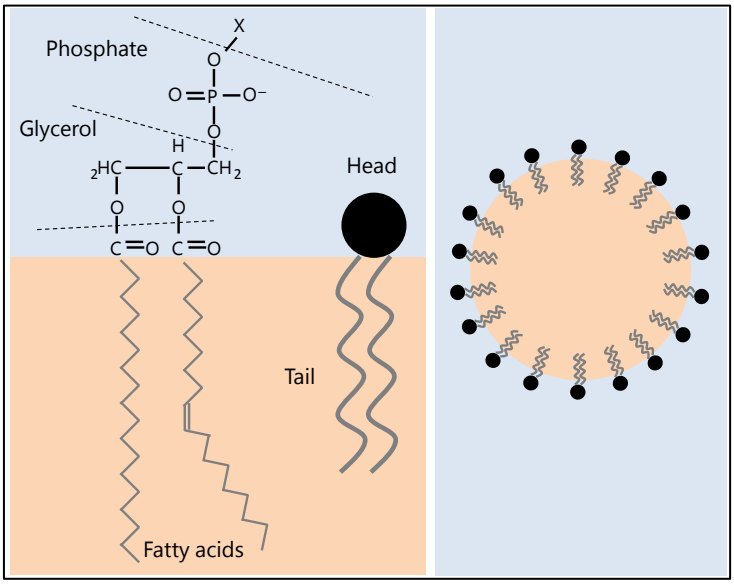


Figure 6. Chemical structure of a phospholipid (X is choline, ethanolamine, serine, inositol or glycerol) (left), depiction of the head and tail part in a phospholipid (middle) and a schematical illustration of the orientation of phospholipids at the interface of an oil droplet (right).

The phosphate group is either esterified with amino alcohols (choline, ethanolamine or serine) or polyols (inositol or glycerol), as depicted by "X" in the figure. Sphingomyelin (SM) which is an important milk phospholipid differs a bit in the structure, as the glycerol part is substituted by an amino alcohol, sphingosine. Commercially available phospholipid products from e.g. milk or soy contain a combination of the different phospholipids. In soy lecithin phosphatidylcholine (PC), phosphatidylethanolamine (PE) and

phosphatidylinositol (PI) are the major constituents, in milk it is PC, PE and SM (Wang, 2008). As visualized in Figure 6 (right), phospholipids adsorb to the oil droplet surface with the hydrophilic head facing the water phase, and the fatty acid tails projecting into the oil droplet.

A few studies have been carried out on emulsions with combinations of milk proteins and phospholipids. Using a combination of caseins and lecithin in 20% o/w emulsions, lecithin was found to enhance the emulsion stability when casein concentration was low (< 0.3%). This was suggested to be due to a better coverage of the oil droplet surface by emulsifier, since the phospholipids supplemented the caseins at the interface, whereby the caseins could protrude more into the water phase and stretch less over the surface (Fang and Dalgleish, 1993a). However, when protein concentrations were ≥0.4%, a displacement of protein at the interface rather than a supplementation occurred (Courthaudon et al., 1991; Dickinson and Iveson, 1993; Fang and Dalgleish, 1993a).

2.5 PHYSICAL STABILITY OF OIL-IN-WATER EMULSIONS

The physical stability of an o/w emulsion is highly dependent on the emulsifier and the droplet size distribution. Emulsion instability includes different processes such as droplet aggregation or gravitational separation.

Droplet aggregation covers two processes: Coalescence and flocculation. Coalescence is the process where two droplets meet, and merge into a bigger droplet, as illustrated in Figure 7 (McClements, 2005). Coalescence occurs if the emulsifier concentration is not high enough whereby oil droplets become large, or if the emulsifier used does not have the properties to sufficiently stabilize the emulsion.

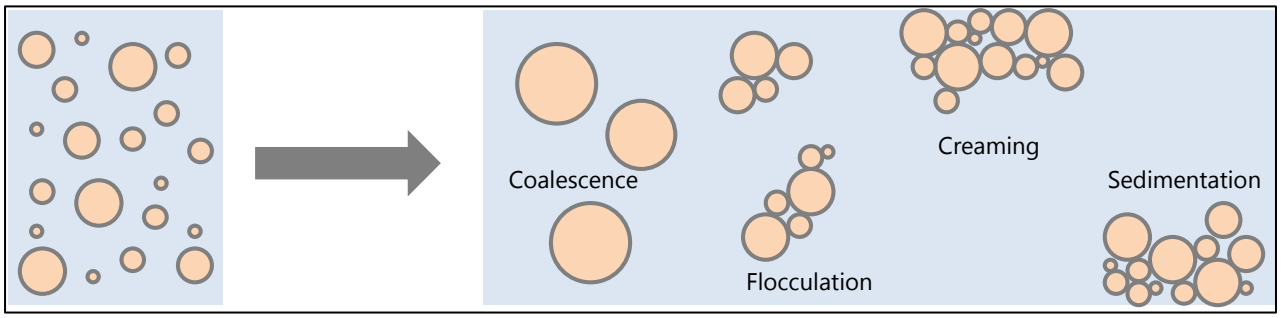


Figure 7. Schematic illustration of physical instability in oil-in-water emulsions. Coalescence: Collision and merger of oil droplets. Flocculation: Collision of oil droplet without merger. Creaming: Droplets with lower densities than their surroundings accumulate at the top of the emulsion. Sedimentation: Droplets with higher densities than their surroundings accumulate at the bottom of the emulsion.

The stability against coalescence in peanut oil emulsions stabilized with various milk proteins was shown to significantly depend on the oil droplet diameter, and to a lesser extent of pH (Klemaszewski et al., 1992). The authors suggested that protein emulsifiers with strong disulfide-bonds (e.g. α-lac) would tend to spread more slowly at the interface during homogenization and thereby result in a relatively weaker film than the more flexible proteins (e.g. β-lg and caseinate). Thus, α-lac stabilized emulsion droplets would be in higher risk of coalescence than β-lg and caseinate droplets. If coalescence continues to occur, the oil and water phase will eventually separate in two, with the oil layer on top of the water due to the density difference.

Similar to coalescence, flocculation is also a process where two droplets collide, but instead of merging, the droplets maintain their individual integrity (Figure 7) (McClements, 2005). In whey protein emulsions

polymerization reactions between individual whey proteins has been linked to emulsion stability (Damodaran and Anand, 1997). Polymerization reactions were shown to occur as a result of protein adsorption to an interface, but also during storage of an emulsion. Molecules of β-lg were able to polymerize both with other β-lg molecules and molecules of α-lac through sulfhydryl-disulfide or disulfide-disulfide interchange reactions. However, α-lac was not able to polymerize with other α-lac molecules due to the lack of free sulfhydryl groups. The authors suggested that the emulsion stability was dependent on whether these polymerization reactions occurred as intra-particle reactions or inter-particle reactions. Inter-particle polymerization reactions were shown to increase flocculation, which could eventually lead to coalescence.

In 30% soy o/w emulsions stabilized by sodium caseinate in concentrations from 0.5% to 3.0%, a concentration dependent tendency for oil droplets to flocculate was observed (Srinivasan et al., 2001). When prepared with 2.0% caseinate, large irregular flocs appeared in the emulsion, and a further increase in caseinate concentration resulted in a network structure of flocs. Over time the flocculation led to a gravitational separation.

Gravitational separation covers two phenomena: Creaming and sedimentation. Gravitational separation occurs due to a difference in density between the oil droplets and the surrounding liquid (Figure 7) (McClements, 2005). If the droplets have a lower density than the surrounding liquid as is normally the case in o/w emulsions, the oil droplets will tend to cream at the top of the emulsions. On the other hand, if the droplets have a higher density than the surrounding liquid, sedimentation occurs, with droplets accumulating at the bottom of the emulsion.

Chapter 3:
Lipid Oxidation in Emulsions

This chapter will summarize the current knowledge on lipid oxidation in emulsions. Lipid oxidation can be divided into autoxidation, enzyme catalyzed oxidation and photooxidation. The studies described in the present thesis have been designed to focus on lipid autoxidation, and this chapter will therefore solely concern this type of oxidation. The main focus in this chapter will be on the effects of emulsifiers and emulsification conditions on lipid autoxidation.

3.1 GENERAL AUTOXIDATION CHEMISTRY AND MECHANISMS

Lipid autoxidation is a complex series of reactions that can be summarized in an initiation stage, a propagation stage and a termination stage.

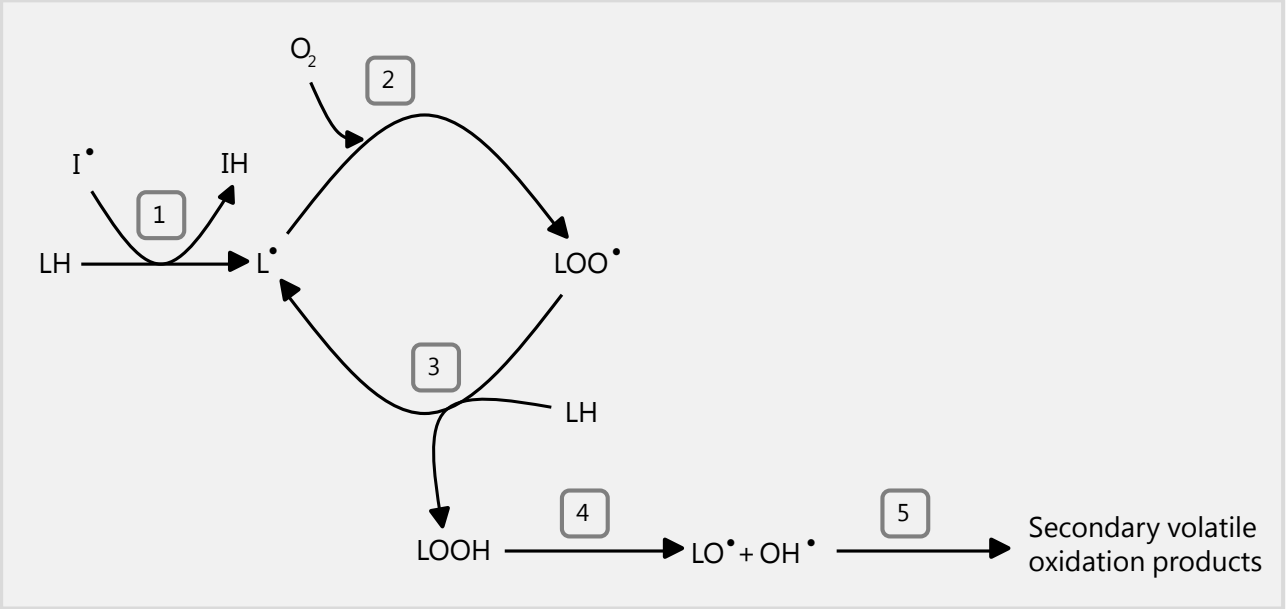


Figure 8. Mechanisms of initiation and propagation of lipid autoxidation. I•: Initiator radical; LH: Unsaturated lipid; L•: Lipid alkyl radical; LO•: Lipid alkoxyl radical; LOO•: Lipid peroxyl radical; LOOH: Lipid hydroperoxide; OH•: Hydroxyl radical.

The initiation of autoxidation is dependent upon an initiator such as a free radical or a transition metal ion. By extraction of hydrogen from an unsaturated lipid (LH), a lipid radical (L•) is formed (Figure 8, 1). This lipid radical immediately reacts with atmospheric oxygen and generates a lipid peroxyl radical (LOO•) (Figure 8, 2), and onsets the propagation of lipid autoxidation. The reaction between the lipid peroxyl radical and an unsaturated lipid leads to the formation of a new lipid radical (Figure 8, 3), whereby the propagation can be continuously repeated. The other product of propagation is a lipid hydroperoxide (LOOH), which is recognized as a primary oxidation product. The type of lipid hydroperoxides generated is dependent on the initial lipid subjected to autoxidation. Thus, the autoxidation of e.g. α-linolenic acid (ALA, C18:3n-3) leads to the formation of four 9-, 12-, 13- and 16-hydroperoxides as illustrated in Figure 9.

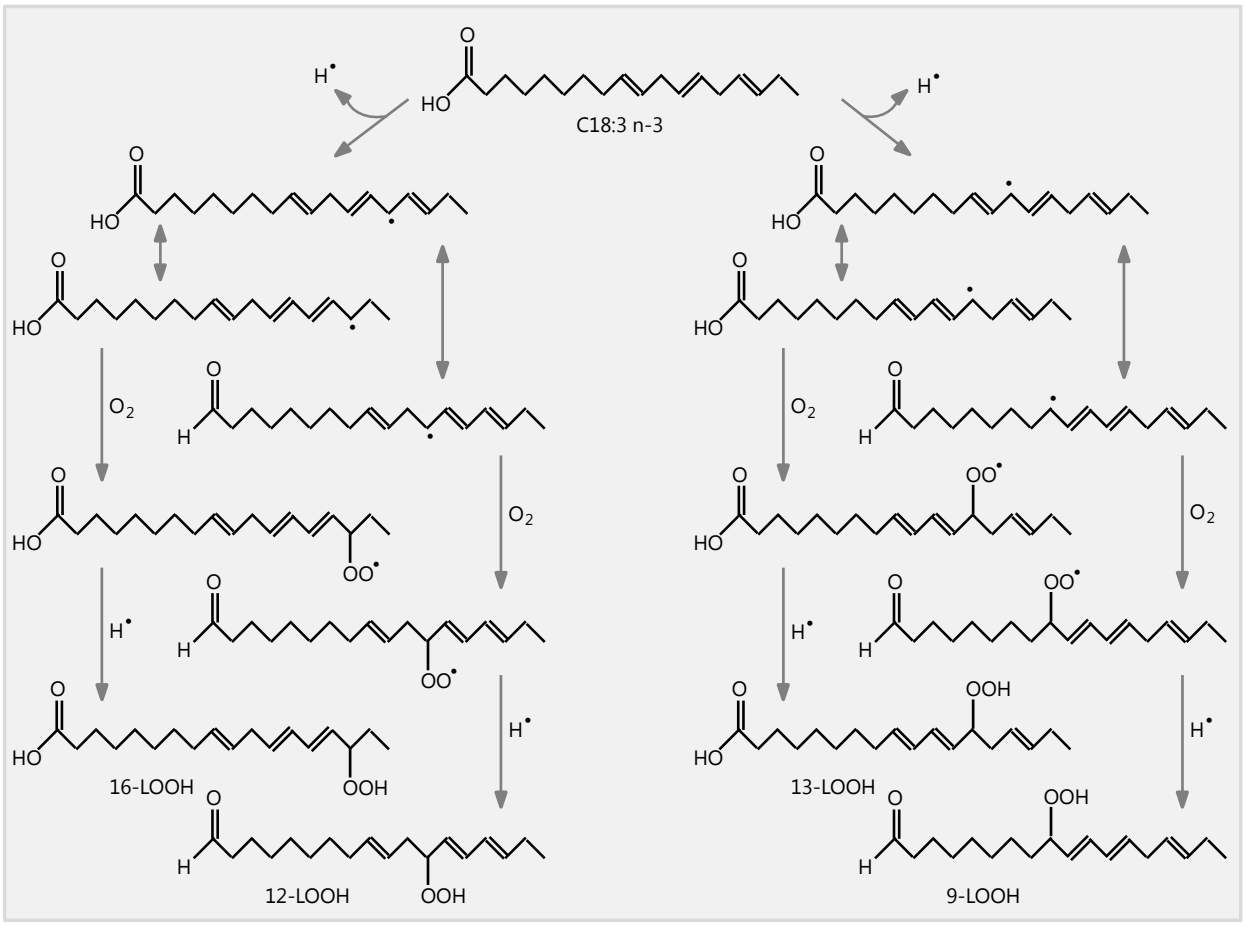


Figure 9. Autoxidation of α-linolenic acid (C18:3n-3), resulting in the formation of the four 9-, 12-, 13- and 16-hydroperoxides. For simplicity all double bonds are shown in trans conformation. Adapted from Frankel (2005).

Likewise, autoxidation of the LC omega-3 PUFA EPA generates eight 5-, 8-, 9-, 11-, 12-, 14-, 15- and 18-hydroperoxides, and DHA ten 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17- and 20 hydroperoxides (Frankel, 2005). Lipid hydroperoxides are relatively unstable, and can be degraded to volatile secondary oxidation products, by homolytic cleavage (Figure 8, 4) followed by β-scission of the lipid alkoxyl radical produced (Figure 8, 5). The volatile secondary oxidation products are described in section 3.1.1.

The propagation is terminated when radicals react with each other to generate non-radical products, as listed in Scheme 1.

$LOO\bullet + LOO\bullet \rightarrow LOOL + O_2$	6
$LOO\bullet + L\bullet \rightarrow LOOL$	7
$LO\bullet + LO\bullet \rightarrow LOOL$	8
$LO\bullet + L\bullet \rightarrow LOL$	9
$L\bullet + L\bullet \rightarrow L\text{-}L$	10

Scheme 1. Termination reactions that eliminate lipid radicals formed during autoxidation. L•: Lipid alkyl radical; LO•: Lipid alkoxyl radical; LOO•: Lipid peroxyl radical; L-L, LOL and LOOL: Lipid dimers. Adapted from Frankel (2005) and Chaiyasit et al. (2007).

In addition, reactions between radicals and antioxidative compounds can terminate the propagation step, as will be elaborated upon in section 3.1.3.

3.1.1 VOLATILE SECONDARY OXIDATION PRODUCTS

As illustrated for ALA in Figure 9, autoxidation results in a range of lipid hydroperoxides, which upon degradation lead to a myriad of volatile secondary oxidation products (mainly alcohols, aldehydes and ketones) of various chain lengths and degree of unsaturation. In contrast to lipid hydroperoxides, which are both taste- and odourless, most volatiles contribute to the flavour deterioration of an oxidized product. In fish oil enriched milk, a total of 60 volatiles were identified as compared to 14 in pure milk without fish oil (Venkateshwarlu et al., 2004a). In this study it was shown, that none of the individual volatile compounds could be distinguished as having a specific fishy or metallic odour, even though the fish oil enriched milk was described as such by a sensory panel (selected volatiles are listed in Table 2 with their specific odour impressions). Hence, the authors concluded that the development of off-flavours as a result of lipid oxidation in fish oil enriched milk was ascribed a combination of volatiles.

Table 2. Selected volatile secondary oxidation products related to lipid oxidation in a: fish oil (Karahadian and Lindsay, 1989), b: fish oil enriched milk (Venkateshwarlu et al., 2004a) and c: mayonnaise (Hartvigsen et al., 2000), and their odour impressions as reported by Hartvigsen et al. (2000) and Venkateshwarlu et al. (2004).

Volatile compounds	Odour impression	Reported in paper/appendix
1-penten-3-one[abc]	plastic[b], leather[b], pungent[c], rancid[c], green[c], glue[c]	III-VII; App II
1-pentanol[ab]	fruity[b]	VIII
1-penten-3-ol[abc]	milky[b], butter[b], sweet[c]	V-VIII: App II
2-penten-1-ol[abc]	(E) grass[b], green[bc]	App II
	(Z) musty[c], compost-like[c]	
Propanal[b]	plastic[b]	III
Butanal[bc]	sour[b]	V, VIII; App II
Pentanal[abc]	rubber[b], green[c], glue[c]	I-III, V, VII, VIII; App II
Hexanal[abc]	green[bc], fresh[b], pungent[c], grassy[c],	III-V, VII, VIII; App II
Heptanal[abc]	chemical[b], burnt[b]	I-III, VIII, App II
Nonanal[ac]	green plant-like[c], compost-like[c]	VI
2-butenal[abc]	(E) plastic[b], old cheese[c]	III, IV, VIII
2-pentenal[abc]	(E) oily[b], soapy[b], pungent[c], glue[c], green[c], grassy[c]	VII-VIII
	(Z) fruity[c]	
2-hexenal[abc]	(E) green[c], sour[c]	III-VIII; App II
2-heptenal[abc]	(E) mushroom[b], earthy[b]	III, VIII; App II
	(Z) fishy[c], sweet[c]	
4-heptenal[abc]	(Z) sweet[b]	IV
2,4-hexadienal[abc]	(E,E) vegetable[b], green[bc], burnt[c]	III, IV; App II
2,4-heptadienal[abc]	(E,Z) fishy[c], fatty[c], burnt[c]	I-VIII; App II
	(E,E) rancid[b], fatty[bc], nasty[c], green[c]	
2,6-nonadienal[abc]	(E,Z) green[b], cucumber[bc]	III, IV; App II
2-ethyl-furane[bc]	sweet[b], flower[c]	VIII
2-pentyl-furane[ac]	green[c]	VIII

As the development of individual volatile secondary oxidation products is highly dependent on the oxidizing matrix, a range of volatiles were quantified in the different studies of this thesis. In Table 2, volatiles that has

previously been related to lipid oxidation and furthermore been shown to increase in the emulsions prepared in the present PhD work are listed. The odour impressions of individual volatile compounds as reported by Venkateshwarlu et al. (2004a) and Hartvigsen et al. (2000) are furthermore given.

3.1.2 PRO-OXIDATIVE COMPOUNDS – TRANSITION METAL IONS

Transition metal ions are ubiquitously present in foods in various amounts, and as mentioned previously they can influence the initiation of lipid autoxidation through the extraction of a hydrogen atom from an unsaturated lipid (Scheme 2, 11). In addition transition metal ions can also promote propagation of lipid oxidation by catalyzing the decomposition of lipid hydroperoxides (Scheme 2, Reactions 12-13).

$$Me^{(n+1)+} + LH \rightarrow Me^{(n)+} + L\bullet + H^{+} \quad 11$$
$$Me^{(n)+} + LOOH \rightarrow Me^{(n+1)+} + LO\bullet + OH^{-} \quad 12$$
$$Me^{(n+1)+} + LOOH \rightarrow Me^{(n)+} + LOO\bullet + H^{+} \quad 13$$

Scheme 2. Reactions between transition metal ions and unsaturated lipids/lipid hydroperoxides. $Me^{(n)/(n+1)+}$: Transition metal ions; LH: Unsaturated lipid; L•: Lipid alkyl radical; LO•: Lipid alkoxyl radical; LOO•: Lipid peroxyl radical; LOOH: Lipid hydroperoxides. Adapted from Frankel (2005).

The decomposition of linoleic acid hydroperoxides as affected by transition metal ions was studied by O´Brien (1969). He observed an activity in the order $Fe^{2+} > Fe^{3+} > Cu^{2+}$. Moreover, he showed that all transition metal ions exhibited a higher activity in this system when lowering pH from 7.0 to 5.5.

3.1.3 ANTIOXIDATIVE COMPOUNDS

The presence of antioxidative compounds can interfere with the autoxidation process at different stages by e.g. metal chelation (whereby reactions 11-13 are inhibited) or by free radical scavenging (as outlined in Scheme 3). Reactions 14-16 retard initiation and propagation, whereas reactions 17-20 terminate the autoxidation process.

$$LOO\bullet + AH \rightarrow LOOH + A\bullet \quad 14$$
$$LO\bullet + AH \rightarrow LOH + A\bullet \quad 15$$
$$L\bullet + AH \rightarrow LH + A\bullet \quad 16$$
$$LOO\bullet + A\bullet \rightarrow LOOA \quad 17$$
$$LO\bullet + A\bullet \rightarrow LOA \quad 18$$
$$L\bullet + A\bullet \rightarrow LA \quad 19$$
$$A\bullet + A\bullet \rightarrow A\text{-}A \quad 20$$

Scheme 3. Reactions between antioxidative compounds/radicals and lipid radicals. L•: Lipid alkyl radical; LO•: Lipid alkoxyl radical; LOO•: Lipid peroxyl radical; LH: Unsaturated lipid; LOH: Lipid alcohol; LOOH: Lipid hydroperoxides; A•: Antioxidant radical; LA, LOA and LOOA: Lipid conjugates with antioxidant; A-A: Antioxidant dimer. Adapted from Chaiyasit et al. (2007).

Antioxidative compounds can either exist as individual molecules, such as tocopherols or as part of a component, e.g. an amino acid residue in milk protein. In the present thesis the effect of added antioxidants has not been evaluated. Hence, of individual molecules only tocopherols will briefly be touched upon as these are original constituents in fish oil and also to a minor degree present in milk and cream cheese. However, both milk proteins and phospholipids are known to exert some antioxidative effects, and these will be further elaborated upon in section 3.2.1.

3.2 LIPID OXIDATION IN SIMPLE EMULSION SYSTEMS

Several studies have been carried out to investigate how different factors affect the oxidation of lipids in emulsions. These factors are visualized in Figure 10, and elaborated upon in the following sections.

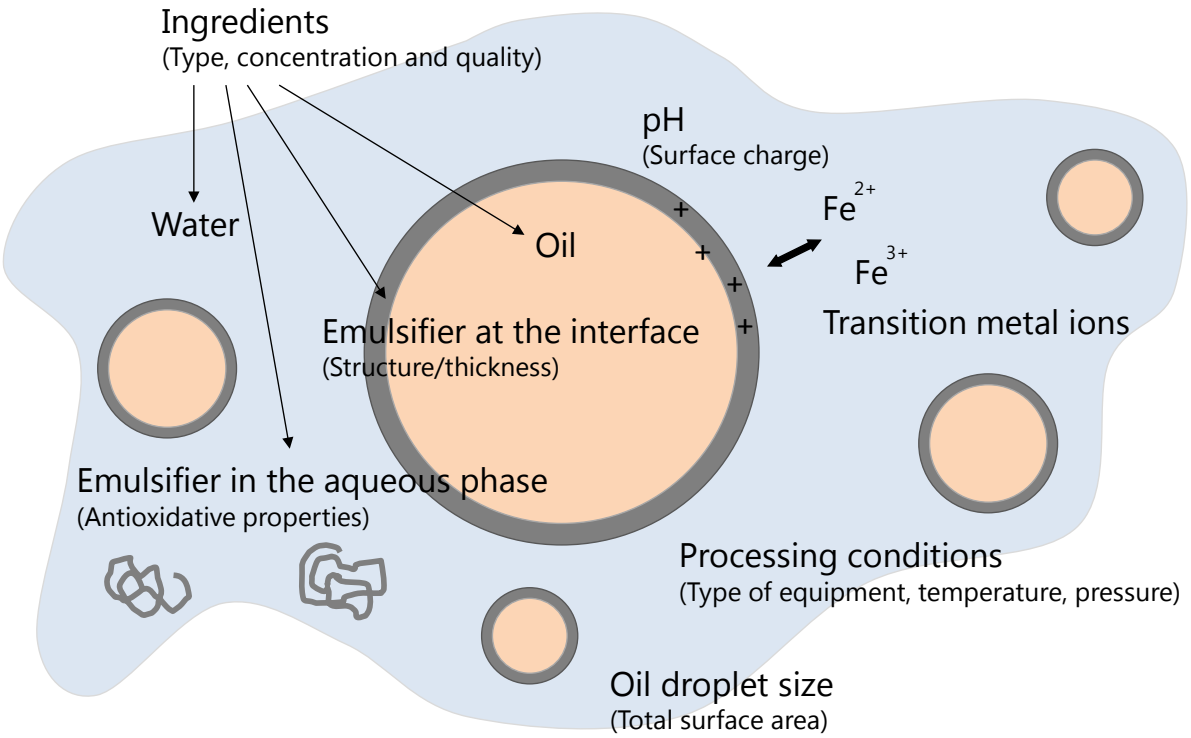


Figure 10. Parameters influencing lipid oxidation in emulsions. Elaborated upon in the following sections (3.2.1-3.2.4)

Most of these parameters are somehow related to the interfacial layer, thus, lipid oxidation in emulsions is to a great extent an interfacial phenomenon.

3.2.1 THE INFLUENCE OF INGREDIENTS

The oxidative stability of an emulsion is highly dependent on the type, concentration and quality of the oil phase. A highly unsaturated oil will be more susceptible to oxidation than a less unsaturated oil (Frankel, 1993; Kamal-Eldin, 2006), and the presence of inherent tocopherols in e.g. fish oil can serve to reduce lipid oxidation (Kamal-Eldin, 2006). Studies on protein stabilized o/w emulsions with varying volumes of the oil fraction have shown that a high oil fraction decreases lipid oxidation. These findings have been related to differences in oil droplet size and thereby the protein availability for each oil droplet (Kargar et al., 2011; Sun and Gunasekaran, 2009). In addition, oil quality might have an influence on the oxidative stability of emulsions, since a low quality oil with a high concentration of lipid hydroperoxides already present will oxidize faster than a good quality oil. In fish oil enriched milk, even a slightly increased peroxide value (PV) in the fish oil added during production resulted in a less oxidatively stable final product (Let et al., 2005a).

Likewise, the type, concentration and quality of the emulsifier also influence the resulting lipid oxidation in emulsions. Overall, the type of emulsifier influences the oxidative stability of emulsions in two ways. Firstly

through its ability to create a protective membrane around the oil droplets that shields the lipids from pro-oxidants in the aqueous phase, and secondly by having different reactive groups with antioxidative properties.

It has been shown, that milk protein components create interfacial layers of different thicknesses (Dalgleish, 1993; Fang and Dalgleish, 1993b; Hunt and Dalgleish, 1994a). However, a direct link between the thickness of the interfacial layer and protection of the lipids still does not exist. The antioxidative activities of various milk protein components have been ascribed to their amino acid compositions. The major differences between caseins and whey proteins are their contents of phosphoseryl (e.g in serine) and sulfhydryl residues (e.g. in cysteines) as stated in Table 1.

Phosphoseryl groups have been suggested to mainly exhibit metal chelating properties, whereas sulfhydryl groups can scavenge free radicals (Elias et al., 2005; Faraji et al., 2004; Tong et al., 2000). The metal chelating properties of caseins have mainly been associated with their presence in the continuous phase (Berton et al., 2011; Faraji et al., 2004; Ries et al., 2010). It has however, been suggested that metal ions could also bind to the casein tails that extend out into the continuous phase (Richards et al., 2011). Nevertheless, the effect of such a binding is debatable. It could be argued that it would promote oxidation due to the close proximity of transition metal ions to the oil phase. On the other hand, it could potentially shield the oil against lipid oxidation if the metal ions are considered to be sufficiently far from the lipid surface. The radical scavenging properties of whey proteins have been shown to be highly dependent on the unfolding of proteins, since the reactive groups might otherwise be deeply buried within the core of the protein molecule (Elias et al., 2007). However, dephosphorylation of the phosphoseryl groups in casein, as well as blocking of sulfhydryl groups in whey protein, does not completely eliminate the antioxidative activity of the proteins (Cervato et al., 1999; Hu et al., 2003a). Hence, the antioxidative properties of caseins and whey proteins cannot solely be ascribed to their phosphoseryl and sulfhydryl groups.

Before I initiated my PhD work no lipid oxidation studies were available on emulsions prepared with combinations of milk proteins and phospholipids, neither is the antioxidative properties of individual phospholipids well described in the literature. In addition, the studies available on antioxidative effects of phospholipids are generally difficult to compare as lipid oxidation is accelerated by different methods. However, one antioxidative mechanism, which has been studied by several research groups, is the ability of phospholipids to work in synergy with tocopherols (Bandarra et al., 1999; Hildebrand et al., 1984; Judde et al., 2003). Some phospholipids, such as PE, has also been suggested to possess antioxidant activity by an ability to interact with free radicals (Hamilton et al., 1998). The successful use of phospholipids as antioxidants in oils or emulsions has been shown to depend on the fatty acid composition of both the oil and on the individual phospholipids used (Judde et al., 2003; Nwosu et al., 1997; Wang and Wang, 2008). Hence, if the fatty acids in the phospholipids used are highly unsaturated, there is a risk, that the emulsifier oxidizes itself.

The above-mentioned antioxidative effects of milk proteins and phospholipids might be influenced by e.g. the pH of the emulsion or the way lipid oxidation is accelerated. In addition, especially the structure of the milk proteins is influenced by e.g. emulsifier concentration, pH and homogenization conditions whereby also the antioxidative sites of the molecule become more or less exposed for reactions.

3.2.2 PH EFFECTS

The influence of pH on lipid oxidation in emulsions has been investigated in several studies, but its effect is still not fully understood. In emulsions with proteins, a pH below the pI of the proteins will result in a positive surface charge, whereas at a pH above the pI of the proteins a negative surface charge exists.

The relation between pH and the oxidative stability of emulsions prepared with whey proteins are more or less consistent and show an increase in oxidative stability with decreasing pH independent of the oil type used or the method for determining lipid oxidation (Berton et al., 2011; Donnelly et al., 1998; Hu et al., 2003a; Kellerby et al., 2006). The better oxidative stability at low pH has in some of these studies been suggested to be related to the positive surface charge of the oil droplets and thereby a possible repulsion of transition metal ions. In addition at neutral pH the iron is less soluble whereby it might precipitate at the oil droplet surface and promote lipid oxidation (Mancuso et al., 1999). Fewer studies have been carried out with caseinate, since caseinate emulsions are difficult to prepare at low pH because of protein precipitation. However, one study observed a better oxidative stability of caseinate emulsions at low pH similarly to the results for whey proteins, whereas another study observed the opposite (Guzun-Cojocaru et al., 2011; Haahr and Jacobsen, 2008). The lower oxidative stability at low pH could be related to the higher activity of iron for reactions with hydroperoxides at this pH (O´brien, 1969).

3.2.3 OIL DROPLET SIZE

Emulsions with large oil droplets have a smaller total surface area of the droplets exposed to pro-oxidants in the aqueous phase than emulsions with smaller droplets. Hence, it is hypothesized that larger oil droplets are less susceptible to lipid oxidation than smaller droplets. However, conclusions on the relation between oil droplet size and lipid oxidation are not clear since obtaining different droplet sizes often requires that other parameters are varied during emulsion formation. These parameters might influence lipid oxidation themselves, e.g. the type and concentration of emulsifier used or the homogenization conditions.

In mayonnaise, lipid oxidation was observed to progress faster in smaller droplets than in larger ones in the initial part of the storage period, whereas no dependence of droplet size was observed on oxidative flavour deterioration in the later part of the storage period (Jacobsen et al., 2000). Similarly, smaller droplets were observed to oxidize faster than larger droplets in the initial part of the storage of o/w emulsions stabilized by bovine serum albumin when the oxygen was not limited (Lethuaut et al., 2002). However, after 24 hours no difference was observed in the development of volatile secondary oxidation products. In accordance with these studies an increase in the oil volume fraction of caseinate and Tween20 stabilized o/w emulsions, resulted in a better oxidative stability (Kargar et al., 2011). This observation was explained by a concomitant decrease in oil droplet surface area through an increase in droplet size and thereby a reduction in the exposure to iron in the aqueous phase.

In contrast, other studies have shown no correlation between oil droplet size and lipid oxidation (Gohtani et al., 1999; Hu et al., 2003b; Sun and Gunasekaran, 2009). Most of the studies mentioned here obtained different oil droplet sizes by varying the emulsifier type or concentration, the oil volume fraction or the homogenization conditions. Hence, lipid oxidation might not only exist as a result of oil droplet size, but rather as a result of a combination of factors involved in the macrostructure of the emulsion. For example in milk, where oil droplet size was decreased by an increase in homogenization pressure, the protein composition at the interface was

shown to be influenced by the pressure as well (Let et al., 2007b; Sørensen et al., 2007). Thus, lipid oxidation was shown to be more influenced by the protein composition at the interface than of the actual droplet size.

The complexity of influencing factors was also shown in a study where emulsification was done in a microchannel system, and different oil droplet sizes were obtained by using channels of different sizes (Azuma et al., 2009). Emulsions were prepared with either soy oil or fish oil and different non-ionic emulsifiers. In soy o/w emulsions a decrease in droplet size decreased the oxidative stability, whereas in fish o/w emulsions a decrease in oil droplet size increased the oxidative stability. In general, the varying outcomes of these studies imply that in most cases other factors than the actual droplet size affects lipid oxidation more, or at least lipid oxidation is a result of a combination of factors and not solely the droplet size.

3.2.4 HOMOGENIZATION EQUIPMENT AND PROCESSING CONDITIONS

No systematic studies have been carried out in which lipid oxidation has been compared in emulsions produced under similar conditions in different homogenization devices. As mentioned previously the obtainable droplet sizes in different homogenization equipments varies. Hence, the choice of homogenization device might indirectly affect lipid oxidation through the oil droplet sizes produced as elaborated upon in section 3.2.3. Furthermore, different high pressure homogenization equipments have been shown to differ with respect to their generation of heat (Mao et al., 2010), which is another factor that can potentially influence lipid oxidation. Finally, studies in milk have shown that the protein structure at the interface differs depending on the type of high pressure homogenizer used, due to differences in the geometries of the interaction chambers (Dalgleish et al., 1996). Hence, lipid oxidation studies in this area are needed.

Apart from the emulsification principle also homogenization conditions could potentially influence lipid oxidation. In mechanical homogenization devices the only parameter that can be varied is the speed of rotation, which will eventually influence the resulting oil droplet size. However, some mechanical devices such as the Stephan mixer used in the present thesis can be operated under reduced pressure and/or cooling. A reduced pressure will extract air bubbles otherwise incorporated in the emulsion, and thus potentially reduce lipid oxidation.

In membrane homogenizers, parameters can be varied such as the membrane pore size and the flow of the aqueous phase past the membrane or the flow of the oil phase through the membrane (Nakashima et al., 1991; Vladisavljevic and Schubert, 2002). Once again these parameters are closely related to the resulting droplet size whereby they can indirectly affect lipid oxidation, as elaborated upon in section 3.2.3.

In high pressure homogenizers the main parameter that can be varied is the pressure applied. Increasing the pressure or the number of passes through the interaction chamber reduces droplet size (Qian and McClements, 2011). Nevertheless, lipid oxidation studies on emulsions prepared with caseinate, Tween20 or whey protein concentrate have not been able to confirm a relationship between oxidative stability, pressure and droplet size (Dimakou et al., 2007; Kiokias et al., 2007). In contrast, studies on fish oil-enriched milk has shown that an increasing pressure during homogenization increases the oxidative stability due to an exchange of milk protein components between the aqueous phase and the interfacial layer (Let et al., 2007b; Sørensen et al., 2007).

Some studies have shown that, it can be beneficial to pre-treat milk proteins such as whey by heating prior to homogenization, as this could potentially unfold the protein, increase its emulsifying capacity and potentially expose antioxidative components that would otherwise be buried within the core of the protein (Kiokias et al.,

2007). Kiokias et al. (2007) showed a decrease in conjugated diene formation as a result of using pre-heated whey protein as emulsifier in 30% sunflower o/w emulsions. Accordingly, Elias et al, 2007 also observed a decrease in lipid hydroperoxides and TBARS formation upon heating of β-lg to 95°C when added to the aqueous phase of 5% Brij-stabilized menhaden o/w emulsions in a concentration of 500 µg protein/g oil. Interestingly, heating to 95°C reduced the ability of β-lg to bind iron, but increased the ability of β-lg to scavenge peroxyl radicals. Regarding amino acid exposure an increase in tryptophan was observed, while a reduction in cysteine exposure was observed when the protein was heated above 70°C. Thus, it was suggested that the observed enhancement in the antioxidant activity of thermally denatured β-lg (95°C, 15 min) was related to an improved accessibility of radical scavenging amino acids.

3.3 LIPID OXIDATION IN FISH OIL ENRICHED FOOD EMULSIONS

Dairy products are in general considered healthy, and with their natural contents of potentially antioxidative milk proteins these food products are considered good vehicles for fish oil enrichment.

A fish oil enriched dairy product that has been extensively studied is milk. Milk has been shown to be an efficient carrier with regards to fat absorption and to facilitate the biological actions of omega-3 fatty acids even at low doses (Visioli et al., 2000). Furthermore, a range of human intervention studies have shown that intake of milk enriched with EPA and DHA have several desirable cardiovascular effects through an improvement of the blood-lipid profile (mainly by low-density lipoprotein-cholesterol reduction), especially in subjects with elevated values (Lopez-Huertas, 2010).

With regards to results obtained on lipid oxidation in fish oil enriched milk, both the influence of oil type (Let et al., 2003; Let et al., 2004), oil quality (Let et al., 2005a), storage temperature (Let et al., 2005a), presence of tocopherols and other antioxidants (Let et al., 2003; Let et al., 2004; Let et al., 2005b), homogenization conditions (Let et al., 2007b), and the addition of fish oil as neat oil versus in a delivery emulsion (Let et al., 2007a) have been investigated. The best results were obtained either by mixing the fish oil with rapeseed oil or by adding antioxidants. However, both these approaches had some drawbacks. Mixing the fish oil with rapeseed oil decreased the total amount of EPA and DHA incorporated and the use of antioxidants is a relatively expensive approach. The addition of fish oil in a delivery emulsion instead of as neat oil also improved the oxidative stability of the resulting milk, however not to an extent where lipid oxidation was avoided (Let et al., 2007a). Hence, in the present PhD work, milk was considered an appropriate choice for investigating the effect of different delivery emulsions as delivery systems for fish oil and their possible improvements to the oxidative stability compared to neat oil.

In contrast to fish oil enriched milk, fish oil enriched cream cheese is much less studied. However, Kolanowski and Weiβbrodt (2007) have reported the sensory quality of various cheeses upon fish oil addition in different concentrations. The authors observed that it was possible to add 15 g fish oil/kg spreadable fresh cheese (Philadelphia type) before reaching a level where the sensory quality was significantly impacted. In comparison it was possible to add 3 g fish oil/kg processed fresh cheese and 40 g fish oil/kg processed cheese. The spreadable fresh cheese with 15 g fish oil/kg could be stored for up to 5 weeks before the sensory quality decreased. Another study on processed cheese examined the use of a delivery emulsion for fish oil enrichment, and this was shown to increase the oxidative stability as compared to the addition of neat oil throughout storage (Ye et al., 2009). Despite the better oxidative stability, the sensory perception of the cheese was though still different from the control upon storage when fish oil was added in a concentration of 30 g or more per kg cheese (corresponding to approximately 89 mg fish oil in a serving size of 30 g cheese).

Thus, improvements of the delivery emulsion used could be advantageous, as could a better understanding of lipid oxidation in this type of food product. On this background cream cheese was selected for the second case study in the present PhD work.

Chapter 4:
Experimental Approach

The experimental studies in the present PhD work focused on increasing our knowledge about factors related to the choice of emulsifier, emulsification equipment and homogenization conditions influencing lipid oxidation in simple emulsion systems. In addition, this knowledge should be utilized for producing oxidatively stable delivery emulsions for the addition of fish oil to foods.

Seven studies of lipid oxidation in simple emulsions were carried out as part of this PhD work, and these are reported in Papers I-VI and Appendix II. Furthermore, two studies were planned with the aims of investigating the influence of the thickness of the interfacial layer and the oil droplet size. However, due to different circumstances these studies could not be put into practice. A range of pre-experiments were though carried out and these are reported in Appendix I and III, as they still served to increase our understanding of the emulsions prepared in the other studies. Details on the experimental approaches of these two studies are not included in the following, but described separately in the appendices. However, the most important results will be discussed along with the results reported in the papers in Chapter 5. In addition, to the studies of simple emulsions, two studies were also carried out on fish oil enriched food products, namely milk and cream cheese (Paper VII, VIII).

4.1 SIMPLE EMULSION SYSTEMS

Essentially, all emulsions were prepared from 3-4 ingredients; Fish oil as the dispersed phase, 10 mM sodium acetate imidazole buffer as the aqueous phase and 1-2 emulsifiers. These ingredients will be further described in the following.

4.1.1 INGREDIENTS

The fish oil used for production of all emulsions was commercially available refined cod liver oil without antioxidants added after refining. Fish oil from two different batches were used, however variations were small. Details on PV, tocopherol contents and fatty acid compositions are stated in Table 3. The fish oil was stored at -40°C until use.

Table 3. Peroxide value, tocopherols and fatty acid composition of the fish oil used in the various studies.

Cod liver oil	PV	< 0.1 meq/kg	Σ SFA		~ 14 %
	α-tocopherols	~ 200 mg/kg	Σ MUFA		~ 48 %
	γ-tocopherols	~ 100 mg/kg	Σ PUFA		~ 30 %
			Σ n-3		~ 27 %
			ALA	18:3 n-3	~ 1 %
			EPA	20:5 n-3	~ 9 %
			DPA	22:5 n-3	~ 1 %
			DHA	22:6 n-3	~ 12 %

PV: Peroxide value; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated Fatty acids; ALA: α-linolenic acid; EPA: Eicosapentaenoic acid; DPA: Docosapentaenoic acid; DHA: Docosahexaenoic acid

Only fatty acids that could be identified are summarized (A total of 92 %).

The concentration of fish oil used for preparing the simple emulsions was either low (5-20%) or high (70%). Emulsions with low oil concentrations were selected, as these are well described in the literature and considered good systems for investigating a wide range of factors related to their production conditions.

Knowledge on simple emulsions with high oil concentrations is on the other hand scarce. Nevertheless, when the aim is to use the emulsion as a delivery emulsion, a high oil concentration is preferable, particularly in food products where addition of water changes its texture in an unwanted way. Hence, these emulsions were exploratory investigated to increase our knowledge about the effect of different emulsifiers in this system at low and neutral pH and in the presence of added iron. Furthermore, they were selected with the purpose of using them as delivery emulsions in food products with a low water content.

In all oxidation studies milk proteins or milk protein components were included (Paper I-VI, Appendix II). Furthermore, phospholipids from milk (Paper I, II, VI), soy (Paper I, II, VI) and egg (Appendix II) were used in selected studies. Milk proteins were selected due to their common use in food products and their potential antioxidative effects. Phospholipids were selected as they have shown potential antioxidative effects and an ability to change the thickness of the interfacial layer in combination with caseinate. The emulsifiers used and their abbreviations in the present thesis are listed in Table 4.

Table 4. Abbreviations, trade names and details on the emulsifiers used in studies described in the various studies.

Abbreviation	Trade name	Details
CAS	Miprodan® 30	Sodium caseinate
EggPC	37-0100 Phosphatidylcholine	Phosphatidylcholine from eggs (> 98 % dry weight)
LEC	SolecTM E-40-B	Soy lecithin with > 56 % phospholipids†
Lg	---	Non commercial purified β-lactoglobulin
MPL20	Lacprodan® PL-20	Milk phospholipid with approximately 20 % PL
MPL75	Lacprodan® PL-75	Milk phospholipid with approximately 75 % PL
WPIα	Lacprodan® ALPHA-20	Whey protein isolate enhanced with α-lactalbumin
WPI	Lacprodan® DI-9224	Whey protein isolate with mainly β-lactoglobulin
CASPC	---	A combination of CAS and EggPC, ratio 0.6:1, 1:1 or 2:1
LgCAS	---	A combination of Lg and CAS, ratio 1:9
WP	---	A combination of WPI and WPIα, ratio 1:1

†As acetone insolubles

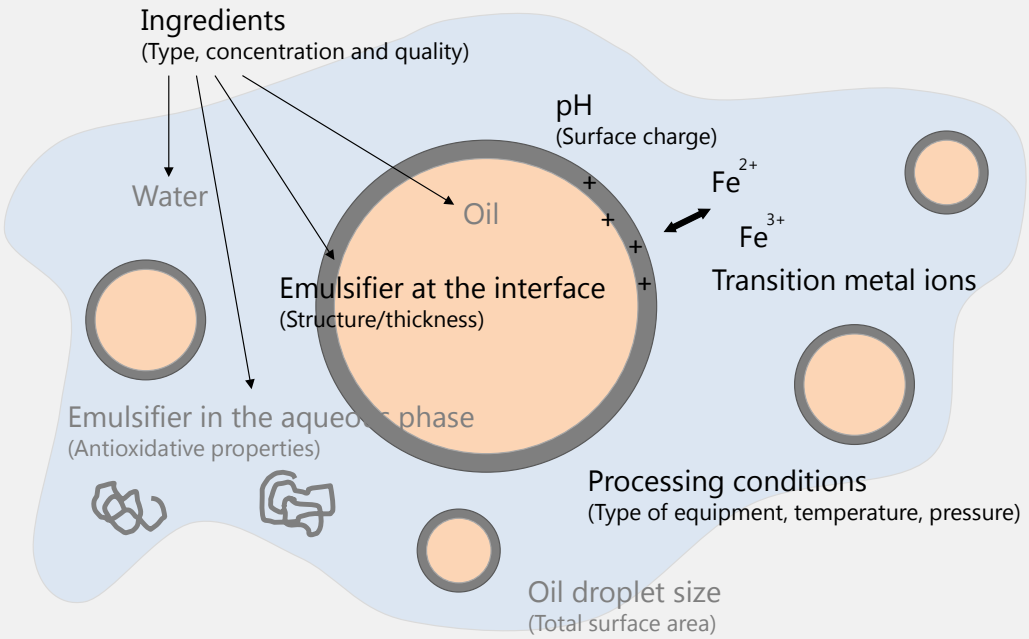
Some emulsifiers were used individually, whereas others were used in combinations as stated in Table 5. The aqueous phase in all simple emulsions was 10 mM sodium acetate imidazole buffer, which is widely used for these types of emulsions (Djordejevic et al., 2004; Faraji et al., 2004; Haahr and Jacobsen, 2008; Hu et al., 2003a Hu et al., 2003b). Dependent on the polarity of the emulsifiers, they were either dispersed in the oil or the aqueous phase prior to homogenization.

4.1.2 EMULSION PREPARATION AND EXPERIMENTAL DESIGN

The emulsions were produced by the use of various equipments. Two mechanical systems were used, namely a simple blade mixer (Paper I, II), and a hand held high speed (HS) mixer (for primary homogenization; Paper III-VI, Appendix II). The simple blade mixer was a Stephan mixer with a vacuum system for reducing pressure and a double sided bowl for cooling. The high speed mixers used were either an Ultra turrax (IKA T25, Janke & Kunkel IKA-Labortechnik, Staufen, Germany) or an Ystral mixer (Ballrechten-Dottingen, Germany). Three high pressure homogenizers were used for secondary homogenization, a M110L microfluidizer (MIC) (Microfluidics, Newton, MA, USA) and two two-stage valve homogenizers (VH), namely a lab scale Panda 2K (GEA, NiroSoavi, Parma, Italy) homogenizer and a pilot scale Rannie homogenizer (APV, Albertslund, Denmark) (Paper III-VI, Appendix II). The microfluidizer and the two-stage valve homogenizers differed in the design of their interaction chambers and thereby the means of droplet disruption (elaborated upon in section 2.3). The

homogenization conditions used were optimized for the purpose of each experiment. The experimental designs for studies of simple emulsion systems are summarized in Table 5:

Table 5. Overview of the experimental approach in the studies of simple oil-in-water emulsions.



	Oil %	Emulsifier type and %		pH	Iron	Homogenization
Paper I	70	CAS WPI MPL20 MPL75 LEC	2.8 or 1.4†	4.5 or 7.0	-	Blade mixer; 1200 rpm, 450 sec
Paper II	70	CAS WPI MPL20 MPL75 LEC	2.8 or 1.4†	4.5 or 7.0	+	Blade mixer; 1200 rpm, 450 sec
Paper III	10	CAS WPI	1.0	7.0	+	P: HS mixer (Ystral); 16000 rpm, 180 sec S: VH (Panda); 80/8 MPa, 3-4 pass or MIC; 69 MPa, 3 pass
Paper IV	10	WPIα WP WPI	1.0	4.0 or 7.0	+	P: HS mixer (Ystral); 16000 rpm, 180 sec S: MIC; 69 MPa, 3 pass
Paper V	10	WP LgCAS	1.0	7.0	-	P: HS mixer (Ystral); 16000 rpm, 180 sec S: VH (Rannie); 5/0.5 or 22.5/2.5 MPa, 3 pass
Paper VI	5	CAS WPI MPL20 MPL75 LEC	0.2 or 0.75	3.0 or 7.0	+ or -	P: HS mixer (Ultra-turrax); 13500 rpm, 180 sec S: VH (Panda) 80/8 MPa, 2 pass

†Only emulsions prepared with CAS

Please refer to Table 4 for interpretation of emulsifier code names

P: Primary homogenization; S: Secondary homogenization; HS: Hand held high speed mixer; VH: Two-stage valve homogenizer; MIC: Microfluidizer

4.2 CASE STUDIES - MILK AND CREAM CHEESE

Two dairy products were selected for comparison of the effect of adding fish oil as either neat oil or by the use of a delivery emulsion. Milk (~1.5% fat) was selected as a low fat product (Paper VII), and cream cheese (~16.5 % fat) as a medium fat product (Paper VIII). Milk and cream cheese are both considered emulsions themselves, and are rich in proteins that can potentially have antioxidative effects. Hence these products were considered good food systems for the incorporation of fish oil. As mentioned in section 3.3 fish oil enriched milk has been extensively studied by our research group. This knowledge makes it possible to compare different delivery emulsions in the present thesis. Cream cheese is less studied, but useful for investigating the applicability of 70% emulsions as delivery emulsions.

Fish oil enriched milk was prepared with 0.5 % (w/w) fish oil corresponding to a substitution of 1/3 of the original fat. In cream cheese, 1.3% (w/w) fish oil was added to the product without substitution. The choice of adding the fish oil to cream cheese without substitution of the original fat was mainly done out of practical reasons. However, as the fat content of the original cream cheese product normally varies in the range 15-18.7%, the addition of 1.3% fish oil was still within the range of normal variation. With the addition of 0.5 % fish oil to the milk, a serving size of 250 mL would provide 250 mg EPA and DHA in total, corresponding to the recommended daily intake (given by EFSA). The addition of 1.3% fish oil to the cream cheese would provide 82 mg EPA and DHA in a serving size of 30 g.

The delivery emulsions used for fish oil addition to milk were prepared with 10% fish oil and 1% of either CAS, WP or Lg as emulsifier. Emulsions were prepared using a hand held mixer for primary homogenization and the microfluidizer for secondary homogenization (similarly to the conditions stated in Table 5 for emulsions prepared for Paper IV). The oil concentration, type of emulsifiers and homogenization conditions were selected from the investigations of simple emulsions in Paper III-V. The delivery emulsions added to cream cheese were prepared with 70% fish oil and either 2.8% CAS, WPI or MPL20. These emulsions were prepared using a Stephan mixer (similarly to the conditions stated in Table 5 for emulsions prepared for Paper I). The choice of delivery emulsions for cream cheese was limited to the 70% emulsions, as all the other emulsions investigated had too high a water content to be suitable for addition to this type of food product. The emulsifiers were selected from the investigations of simple emulsions in Paper I, II. All delivery emulsions were prepared with water as the aqueous phase. The fish oil enriched dairy products were both compared to a reference product without fish oil.

4.3 STORAGE CONDITIONS AND ANALYSES

Simple emulsions were stored in closed blue cap bottles with 1-2 bottles representing each sample time point. An equal amount of emulsion was stored in each bottle, giving a similar headspace in all samples. Samples were stored at room temperature without exposure to light. All emulsions with 10% fish oil were stored for 14 days, and in most of them iron was added for accelerating lipid oxidation (as indicated in Table 5). Emulsions with 5 and 70% fish oil were stored for 7 days when additionally iron was added and for 42 days when only endogenous iron was present.

Fish oil enriched food products were stored in refrigerators to mimic real-life storage, without exposure to light. Milk was stored for 11 days at 2°C and cream cheese for 20 weeks at 5°C.

4.3.1 EVALUATION OF PHYSICAL PARAMETERS INFLUENCING LIPID OXIDATION

As described in Chapter 3 various physical parameters can influence the resulting lipid oxidation. However, as they are all influenced by the type of emulsifier used and furthermore the conditions under which the

emulsions are produced, they are very difficult to control. In some studies efforts were put into obtaining similar droplet sizes or viscosities of emulsions in order to rule out these parameters when considering lipid oxidation. In other studies measurements of these parameters have been made and discussed in relation to the resulting lipid oxidation.

As mentioned in Chapter 3, the antioxidative potential of milk proteins is suggested to relate to either their presence at the interfacial layer or in the aqueous phase, thus, it was found relevant to determine the partitioning of proteins between these two phases. The method commonly used for determining this is based on a centrifugation of the emulsions followed by a protein determination in the aqueous phase/the cream phase, and a separation of proteins by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page). This procedure was also employed in some of the studies described in the present thesis (Paper III-V, Appendix II). However, it should be kept in mind, that the centrifugation process itself could influence the partitioning of the proteins between the aqueous phase and the cream layer, and thus affect the results. Furthermore, the quantification of protein spots on gels from SDS-page carries a high uncertainty. Hence, in the present thesis, the protein determinations were only used to qualitatively compare emulsions, and not for actual quantifications.

In some of the studies, samples were subjected to confocal microscopy to image the partitioning of proteins and lipids in the emulsion (Paper I, VIII). In addition, cryo-scanning electron microscopy (cryo-SEM) imaging and cryo-transmission electron microscopy (cryo-TEM) imaging are reported in Paper I and III, respectively. Microscopy imaging was not a part of the present PhD work, but conducted by another PhD student. Hence, no further details on these methods will be given here. However, it should be mentioned that the microscopy imaging of emulsions is ongoing with the aim of developing methods for visualizing the interfacial layer in emulsions. If methods can be developed, this visualization can be used to support our hypotheses about the relations between lipid oxidation and structural changes of the interfacial layer in emulsions.

4.3.2 EVALUATION OF OXIDATIVE STABILITY DURING STORAGE

In both simple emulsion systems and fish oil enriched food products, the evaluation of lipid oxidation was based on a combination of measurements of lipid hydroperoxides (by PV) and volatile secondary oxidation products. Samples were taken several times during storage and frozen at -40°C until analyses. In the fish oil enriched foods, the fatty acid compositions and concentrations of tocopherols were furthermore determined at the beginning and the end of the storage experiment. In the experiment on cream cheese, chemical analyses were supplemented by sensory analysis. The sensory analysis was conducted by a trained panel. As our research group has previously studied the impact of volatiles on the sensory perception of fish oil enriched milk (Venkateshwarlu et al., 2004a; Venkateshwarlu et al., 2004b), and as sensory analyses are extremely time consuming, these were not prioritized for the milk samples in the present PhD work.

Volatile secondary oxidation products were determined by the use of gas chromatography-mass spectrometry (GC-MS), and volatiles were sampled either by the use of solid phase microextraction (SPME) (Paper I-IV) or dynamic headspace (DHS) (Paper V-VIII). SPME is a newer method for sampling volatiles that has been used in several studies in recent years (Beltran et al., 2005; Iglesias et al., 2007; Kataoka et al., 2000). In the beginning of the PhD work efforts were therefore put into optimizing a method for the use of SPME to sample volatile oxidation products in emulsions. However, as SPME was used during the first studies, it became obvious that it had some unforeseen drawbacks, such as batch dependent variations in the response of the fibers and a concentration dependent saturation of the fiber. In the middle of the project we therefore changed the

sampling method to DHS as we have several years of experience using this method. In addition, DHS has been shown to be a valuable method for the analysis of lipid oxidation in an early stage (Olsen et al., 2006).

Many studies in the literature, only report one or two volatiles (most often propanal or hexanal) as markers of lipid autoxidation of n-3 or n-6 fatty acids. However, as mentioned in the section 3.1.1, fishy and rancid off-flavours stem from a combination of volatile oxidation products. Thus, in the present PhD work, a range of volatiles were determined in each study. The choice of volatiles were made from a comparison of chromatograms obtained on samples from the first and last sampling day and knowledge acquired from the literature on which volatiles that are related to lipid oxidation. These volatiles were stated in Table 2.

4.3.3 STATISTICAL ANALYSES

In all experiments univariate statistical methods were employed. These methods included one-way and/or two-way analysis of variance, followed by a multiple comparison test of the samples to determine significant differences between individual samples or sampling time points. In addition to these univariate methods, principal component analysis was also performed when it was found relevant. This multivariate tool was used to give an overview of the variation in the samples.

Chapter 5:
Results and Discussion

In this chapter, the experimental findings reported in Paper I-VIII and Appendix I-III will be presented and discussed in connection to the hypotheses stated in Chapter 1 and relevant literature. The sample code names stated refer to the emulsifier or combination of emulsifiers used, as listed in Table 4 (p.26).

The chapter is divided in three sections, according to the overall focus areas stated in Chapter 4, namely the effect on lipid oxidation of

- factors related to the choice of emulsifier with specific emphasis on milk proteins (Section 5.1)
- factors related to the homogenization equipment and conditions (Section 5.2)
- the addition of fish oil to food products by the use of delivery emulsions (Section 5.3)

5.1 EMULSIFIER EFFECTS ON AUTOXIDATION IN EMULSIONS

To facilitate the interpretation of the observed effects of emulsifiers on oxidation in emulsions, emulsifiers were characterized by PV, oil content and compositions of proteins or phospholipids (Table 6). The data are partly based on analyses in our lab and the Laboratory of Food Technology and Engineering at Ghent University, and partly based on data sheets and communication with the manufacturers.

Table 6. Characterization of the emulsifiers used in Papers I-VIII and Appendix II.

Milk protein emulsifier	CAS	WPI	WPIα	Lg
PV [meq peroxides/kg oil]	2.3 ± 1.5[i]	4.3 ± 0.4[i]	---	---
Oil content [%]	0.8 ± 0.1[i]	0.3 ± 0.1[i]	~2[ii]	---
Protein content [%]	93.5[ii]	92[ii]	88-94[ii]	92.5[ii]
Protein composition [%]				
α-lactalbumin	tr[i]	22-24[ii]	~60[ii]	7.3[ii]
β-lactoglobulin	tr[i]	48-52[ii]	20-25[ii]	76.4[ii]
Caseins (α_{s1}, α_{s2}, β, κ)	Major part of total protein[i]	tr[i]	tr[i]	tr[i]
Phospholipid emulsifier	**MPL20**	**MPL75**	**LEC**	**EggPC**
PV [meq peroxides/kg oil]	1.5 ± 0.3[i]	21.9 ± 1.0[i]	1.3 ± 0.0[i]	---
Oil content [%]	24.1 ± 0.7[i]	82.6 ± 0.9[i]	85.8 ± 0.6[i]	---
Protein content [%]	53.8[ii]	3.1[ii]	---	---
Phospholipid content [%]	22.6[ii]	76.0[ii]	> 56[ii]	> 98[ii]
Phospholipid composition [%]	[iii]	[iii]	[iii]	[ii]
PC	27.70 ± 0.05	21.03 ± 0.16	29.70 ± 0.40	> 98
PE	25.55 ± 0.14	13.08 ± 0.02	15.31 ± 0.06	< 0.1
PI	8.80 ± 0.06	6.62 ± 0.04	28.50 ± 0.10	---
PS	8.60 ± 0.05	6.59 ± 0.10	3.48 ± 0.03	---
SM	20.59 ± 0.13	38.64 ± 0.11	0.00 ±0.00	< 1.0

Two batches of CAS, WPI and MPL20 was used in the studies. The stated data were obtained on the batch of these emulsifiers used in Paper I, II, VI and appendix I, III.

[i]Determined in our laboratory

[ii]Data provided by the manufacturer, either reported on data sheets or through personal communication

[iii]Determined by the Laboratory of Food Technology and Engineering, Department of Food Safety and Quality, Ghent University, Belgium. Only the main phospholipids are given.

---: No data available; tr: trace amounts detected from protein analysis using SDS-page in our lab

The PV was determined as an indicator of the oxidative status of the emulsifier. Among the emulsifiers measured PV ranged from 1.3 meq peroxides/kg oil in LEC to 21.9 meq peroxides/kg oil in MPL75. The oil contents of LEC and MPL75 were both above 80% whereas it was approximately 24% in MPL20, approximately 2% in WPIα, and below 1% in CAS and WPI. Analyses of the fatty acid composition of the oil in the phospholipid based emulsifiers showed that MPL75 contained about 60% saturated fatty acids, and MPL20 contained approximately equal amounts of saturated and unsaturated fatty acids. LEC, on the other hand, contained about 80% unsaturated fatty acids. The protein emulsifiers were reported to vary in their contents of α-lac and β-lg, with almost the opposite concentration of the two in WPI and WPIα. Lg was purified to contain as much as 76.4% β-lg and only 7.3% α-lac. The composition of casein components in CAS was stated by the manufacturer to correspond to the ratio in bovine milk (see Table 1).

To investigate the influence of different emulsifiers on lipid oxidation in emulsions, CAS, WPI, MPL20, MPL75 and LEC were all tested in 5% and 70% fish o/w emulsions under various conditions (Paper I, II, VI). Furthermore, 10% o/w emulsions were prepared with WPI and WPIα to investigate the influence of different ratios of α-lac and β-lg on oxidation (Paper IV), and with combinations of CAS and EggPC to investigate the combined effect of proteins and phospholipids (Appendix II). Overall, the results showed that both the type, concentration and composition of the emulsifiers used affected lipid oxidation in emulsions. In addition, their effects were greatly influenced by the pH at which the emulsions were produced and also by the presence of iron during storage. These results will be elaborated upon in the following sections.

5.1.1 THE TYPE OF MILK PROTEIN OR MILK PROTEIN COMPONENT

Irrespective of oil concentration, pH or iron addition, CAS generally increased the oxidative stability of emulsions compared to WPI in our studies as illustrated by PV in Figure 11 (Paper I, II and VI). Only in 5% emulsions prepared with 0.75% protein at pH 7 the opposite was observed. The better oxidative stability of CAS than of WPI is in accordance with previous studies on the oxidative stability of emulsions prepared with these milk proteins (Djordjevic et al., 2004; Hu et al., 2003b; Ries et al., 2010). Both these earlier studies and our own studies ascribed the better oxidative stability of CAS emulsions to the metal chelating activity of phosphorylated serine residues in CAS, as shown by Faraji et al. (2004) and Villiere et al. (2005). Faraji et al. (2004) reported specifically that CAS would bind 5.3-fold more iron than WPI at pH 7, when iron was kept soluble by the formation of a nitrilotriacetic acid-iron complex. The contradictory results obtained in our study of 5% emulsions with 0.75% protein at pH 7, might be related to a possible lower importance of the metal chelating effect of CAS in the aqueous phase if iron precipitates at the oil droplet surface as suggested by Mancuso et al. (1999).

Besides the differences in the amino acid compositions of caseins and whey proteins, the two proteins also have significantly different conformations, which may affect the exposure of individual amino acid residues with potential antioxidative properties. Whey proteins are globular with organized secondary and tertiary structures (Ng-Kwai-Hang, 2003). Hence, the lower oxidative stability of WPI emulsions could be caused by a lower accessibility of the reactive amino acid groups as they are hidden within the core of the highly structured protein (Elias et al., 2005). In contrast, the much more flexible CAS with no tertiary structures is likely to expose its reactive amino acid residues to a greater extent than WPI and thereby exert a better antioxidative effect.

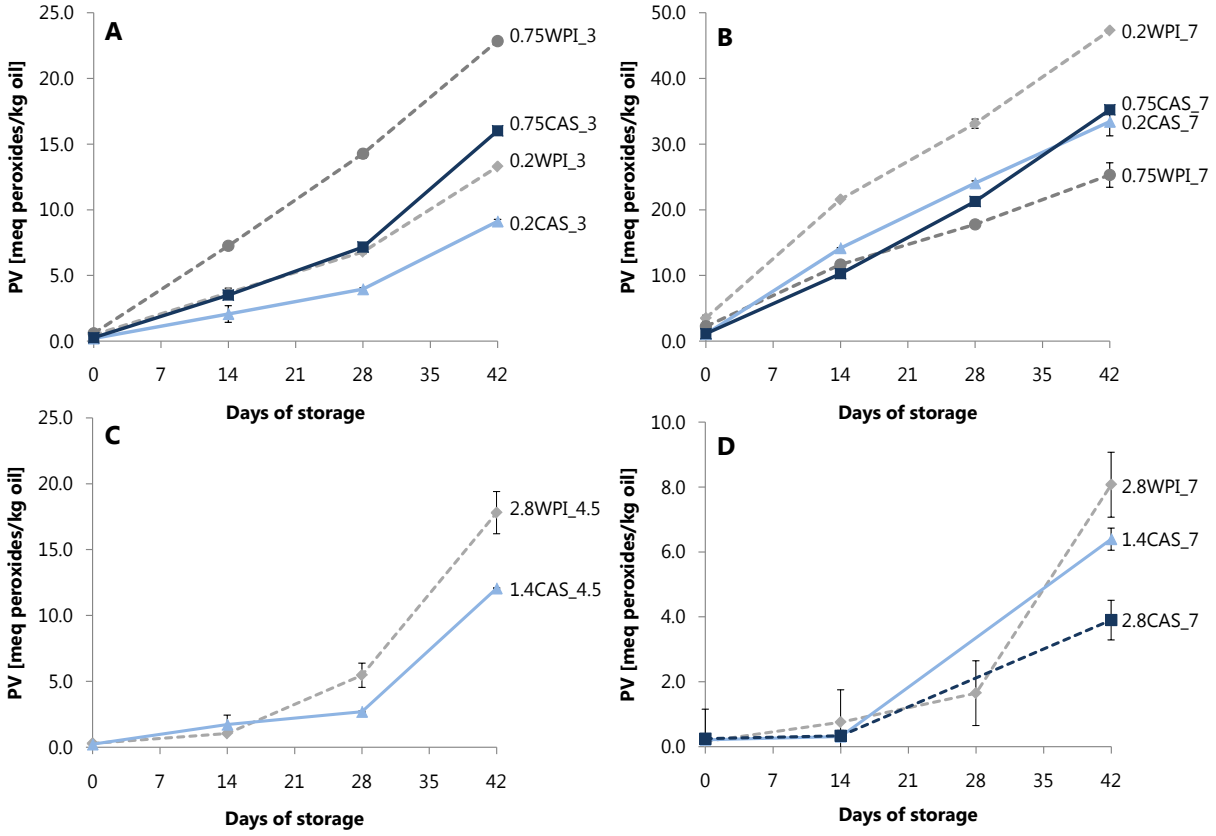


Figure 11. Peroxide values in 5% oil-in-water emulsions (Paper VI) at pH 3 (A) or pH 7 (B), and 70% oil-in-water emulsions (Paper I) at pH 4.5 (C) or pH 7 (D) during storage for 42 days at room temperature (19-22°C) without iron addition. Please note that the sample code names in this graph differ from the sample code names given in the papers. Sample code prefixes refer to concentration of emulsifier, 0.2 or 0.75% in 5% emulsions and 1.4 or 2.8% in 70% emulsions. Samples code postfixes refer to the pH of the emulsions. Data points represent means (n=2) ± standard deviations. Some error bars are within the data points.

The differences in the flexibility of the molecules were suggested by Hunt and Dalgleish (1994a) to affect the proteins abilities to cover the oil-water interface in emulsions. The authors showed that a lower surface concentration (mg/m^2) of CAS than of WPI was needed to obtain full coverage of the oil droplets in emulsions by protein (Hunt and Dalgleish, 1994a). This could explain the smaller droplets obtained in 70% emulsions with CAS (D3,2 = 8.69 µm) than with WPI (20.86 µm) at pH 7 in our studies (Paper I, II). In 5% emulsions prepared at the same pH and with a similar protein to oil ratio (1:25), the difference was less pronounced, as the average mean droplet sizes at day 1 were 0.17 µm and 0.23 µm in the CAS and WPI emulsion, respectively (Paper VI). However, this difference increased during storage, and already after 7 days, the WPI emulsion had a mean oil droplet size twice as large as the CAS emulsion. Increased protein coverage of the interfacial layer in emulsions with CAS is proposed to have contributed to the increased oxidative stability of these emulsions by providing a better protection against pro-oxidants present in the aqueous phase.

The coverage of the interface by whey proteins has also been shown to depend on the type of whey protein component used and the pH of the emulsion (Shimizu et al., 1981; Shimizu et al., 1985). However, in our study on 10% fish o/w emulsions prepared with different ratios of WPI and WPIα at pH 4 and pH 7 (Paper IV), only slight differences in mean oil droplet sizes were obtained. Nevertheless, differences in the oxidative stability of these emulsions at pH 7 and in the presence of iron were observed (refer to Table 4 for concentrations of α-lac and β-lg in WPI and WPIα). The emulsion prepared with WPIα, and thereby the highest concentration of α-lac,

had a significantly higher PV already at day 0 (10.0 meq peroxides/kg oil) as compared to the WPI emulsion (3.0 meq peroxides/kg oil) and also higher than the emulsion prepared with WP (4.0 meq peroxides/kg oil). Despite this, the overall increase in PV during 14 days of storage in the WPI emulsion was higher than in the WPIα emulsion (ΔPV = 14.3 and 9.9 meq peroxides/kg oil, respectively). In addition, the increases in concentrations of volatile oxidation products were also highest in the WPI emulsion. The overall conclusion was therefore that during the production of emulsions at pH 7, it was beneficial to use an emulsifier with a higher concentration of β-lg (WPI), whereas during storage, α-lac had a protective effect on the emulsion (WPIα).

It is known that many factors can influence the unfolding of whey protein components during emulsion formation, and we therefore suggest, that it is the unfolding and thereby exposure of different antioxidative amino acid components that differ between α-lac and β-lg under production and during storage of the emulsions. Further studies on purified α-lac and β-lg could be valuable together with a visualization of the interfacial layers by advanced microscopy in order to fully understand the obtained results.

Interestingly, no difference was observed between emulsions with WPI, WPIα or WP when prepared at pH 4 (Paper IV). This was in contrast to a previous study on the differences between the oxidative stability of 5% salmon o/w emulsions stabilized by 0.2% WPI, α-lac or β-lg at pH 3 (Hu et al., 2003a). In this study, the oxidative stability decreased in the order β-lg > α-lac ≥ WPI. Unfortunately, no other lipid oxidation studies are available on emulsions prepared with individual whey protein components at low pH for comparison. However, the different results obtained by Hu et al. (2003a) in comparison to those obtained in our study (Paper IV) could be caused by the difference in the protein to oil ratios or the difference in homogenization conditions used in the two studies. Thus, Hu et al. (2003a) prepared 5% salmon o/w emulsions stabilized by 0.2% protein (a protein to oil ratio of 1:25), whereas the emulsions in our study of whey protein components were prepared with 10% oil and 1% protein (a protein to oil ratio of 1:10).

For comparison of emulsions with similar protein to oil ratios we therefore turn to our 5% emulsions (Paper VI) prepared with 0.2% WPI. In these emulsions mean oil droplet sizes were observed to increase during storage, and we speculated that the increase in droplet sizes were due to the fact that a full coverage of the oil-water interface with protein was not obtained. Similarly, it is possible that the interface in the emulsions prepared by Hu et al. (2003a) were not fully covered by protein in contrast to the emulsions in Paper IV with a higher protein to oil ratio. Moreover, Hu et al. (2003a) prepared emulsions by homogenization on a two-stage valve homogenizer, whereas we used a microfluidizer for preparing emulsions for the study in Paper IV. In Paper III, we showed that for WPI emulsions the use of a two-stage valve homogenizer instead of a microfluidizer increased lipid oxidation due to a lower concentration of adsorbed protein. Hence, both the protein to oil ration, and the emulsification by the use of a two-stage valve homogenizer could have decreased the coverage of the interface by proteins and thereby increased the differences observed by Hu et al. (2003a) between individual whey protein components. It should, however, also be noted that Hu et al. (2003a) stored their emulsions with whey protein components at 37˚C, even though they observed much less oxidation at 4 and 20˚C in WPI emulsions.

The combined effects of the type of milk protein used as emulsifier and the homogenization equipment and conditions on lipid oxidation in emulsions will be elaborated upon in section 5.2.

5.1.2 THE CONCENTRATION OF MILK PROTEIN AS EMULSIFIER

A high concentration of milk protein as emulsifier is expected to increase the oxidative stability of an emulsion due to either the creation of a thick interfacial layer or due to potential antioxidative effects of proteins in the aqueous phase (Ries et al., 2010). A study was therefore set up in accordance with the study by Fang and Dalgleish (1993b) where emulsions were produced with almost similar droplet sizes, and different thicknesses of the interfacial layer, as described in Appendix I. It was hypothesized, that depending on the concentration, CAS would either stretch over the surface (Figure 12, A) or protrude out into the aqueous phase (Figure 12, B), whereby the thickness of the interfacial layer would change. In addition, it was hypothesized that the manner in which CAS was added would affect its structure at the interface.

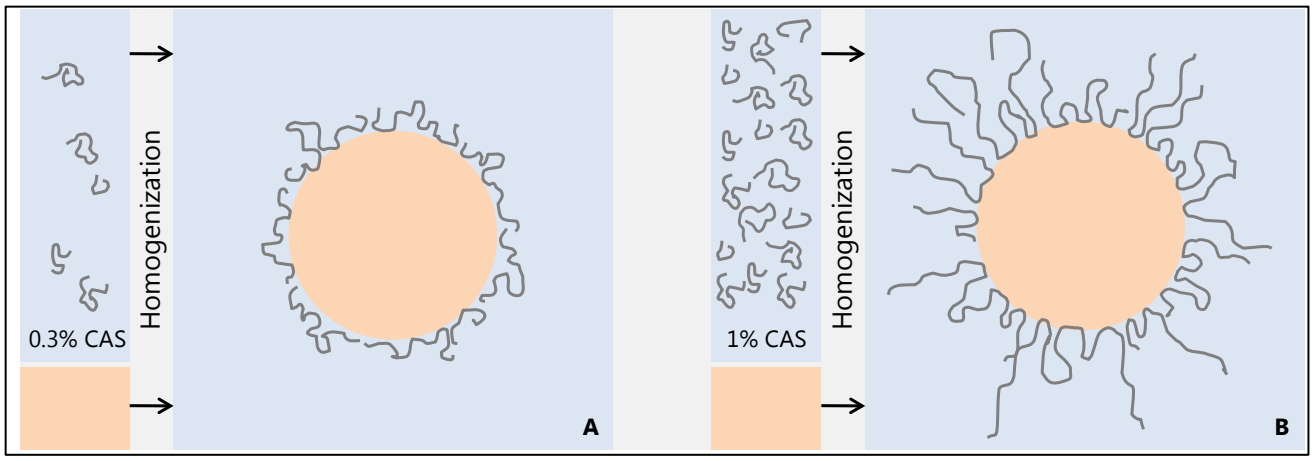


Figure 12. Schematic illustration of the hypothesized change in the thickness of the interfacial layer depending on the protein concentration. At low CAS concentration (A) the protein was expected to stretch over the interface, whereas at high concentration the protein was expected to protrude out in the aqueous phase (B).

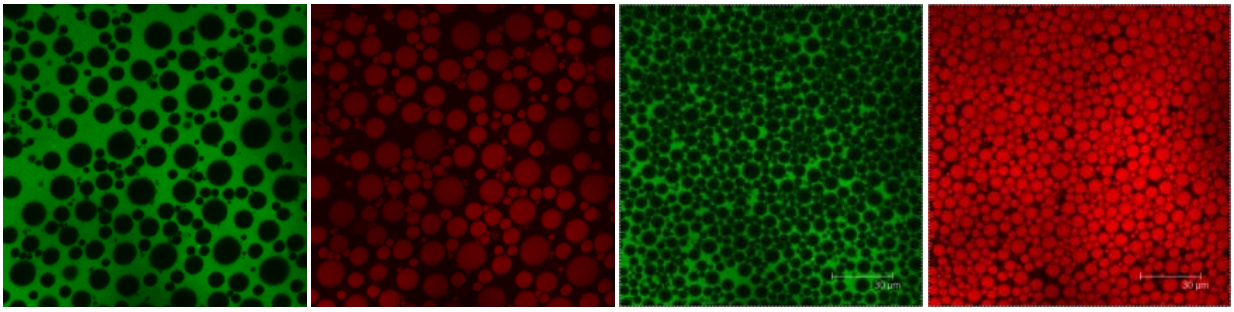
Several pre-experiments were carried out, and emulsions were prepared with CAS concentrations of 0.3, 0.5, 1.0 and 2.0%, with as similar mean oil droplet sizes as possible. After production, the emulsions were washed according to the procedure described by Faraji et al. (2004) in order to remove excess protein from the aqueous phase. However, the harsh centrifugation conditions during this removal disrupted the emulsions (as shown in Appendix I), and despite a huge number of trials and modifications to the procedure, it was not possible to repeat the emulsion production satisfactorily. Hence, the study of lipid oxidation in these emulsions could not be carried out. It was therefore not possible to study the influence of the thickness of the interfacial layers of CAS emulsions by this approach.

Other lipid oxidation studies were carried out in the present PhD work where the concentration of emulsifier was investigated. In Paper I, 70% emulsions were prepared with either 1.4% CAS or 2.8%, corresponding to emulsifier to oil ratios of 1:50 and 1:25. When emulsions were prepared without iron and stored for 42 days a better oxidative stability was observed from increasing the concentration of CAS.

For these emulsions, it was observed that the mean oil droplet size was approximately twice as large in the emulsion with 1.4% CAS ($D_{3,2}$ ≈ 21 µm) as in the emulsion with 2.8% CAS ($D_{3,2}$ ≈ 9 µm). This finding was in agreement with previous findings for 20% o/w emulsions, where it was shown that droplet size decreased up to an emulsifier to oil ratio of 1:20 (Fang and Dalgleish, 1993b). However, it was contradictory to the hypothesis about smaller droplets giving rise to more oxidation. Thus, other factors were assumed to influence lipid oxidation more than droplet size as will be discussed in the following.

When comparing emulsifier to oil ratios in the study by Fang and Dalgleish (1993b) with emulsifier to oil ratios in our study on 70% emulsions, it is likely, that the oil droplets in the emulsion with 2.8% protein are fully covered, whereas it could be speculated whether a full coverage of the interface by proteins was obtained in emulsions with 1.4% protein. The possible lack of full protein coverage of the interface in emulsions with 1.4% CAS could explain the lower oxidative stability during storage for 42 days. However, the coverage was still sufficient for no droplet coalescence to occur. The very different droplet sizes obtained in 20% and 70% emulsions also makes a direct comparison between the results obtained impossible. It could be argued that it would be valuable to calculate the coverage from the concentration of proteins and the oil droplet size distributions. However, the flexibility of CAS and thereby the ability of CAS to stretch or compact at the interface makes it too speculative to calculate the coverage of the interface by CAS from the present results.

The combination of a possible lack of full protein coverage at the interface and no droplet coalescence in the 70% emulsions supports the hypothesis of an effect of the thickness of the interfacial layer on lipid oxidation. Hence, in an emulsion where lipid droplets are found in such close proximity as shown on confocal micrographs in Figure 13, the diffusion of lipid hydroperoxides may increase as a result of a thinner interfacial layer/a lower coverage of the interface by proteins.



1.4% CAS 2.8% CAS

Figure 13. Confocal microscopy images of the two protein stabilized emulsions prepared in the study described in Paper I. Both emulsions are 70% oil-in-water emulsions prepared at pH 7. They have been emulsified with either 1.4% CAS or 2.8% CAS. The staining for proteins is green, and the staining for lipids is red.

Finally, the better oxidative stability of 70% o/w emulsions with a higher concentration of CAS in Paper I could be caused by a higher concentration of protein in the aqueous phase with metal chelating properties (Berton et al., 2011; Faraji et al., 2004; Ries et al., 2010). Confocal micrographs of the 70% emulsions confirmed the presence of proteins in the aqueous phase (stained green in Figure 13). Nevertheless, as droplet sizes were also differing, a quantification of these proteins in the aqueous phase would be needed to confirm whether more protein was actually present in the aqueous phase when emulsions were prepared with 2.8% instead of 1.4% CAS.

In contrast to the results obtained on 70% o/w emulsions, results on 5% o/w emulsions prepared with 0.2 and 0.75% CAS showed a slightly negative effect of increasing emulsifier concentration when emulsions were stored for 42 days and no additional iron was added (Paper VI). This effect was independent of pH, and results for WPI stabilized emulsions showed the same. This difference in lipid oxidation between 5% and 70% emulsions prepared with different concentrations of protein was interesting as it was unexpected. Hence, these results call for further studies on the differences in protein partitioning between the interface and the aqueous phase of the two types of emulsions, and furthermore investigations of the influence of the homogenization equipment used for their preparation.

To investigate the influence of iron and the metal chelating effect of CAS, emulsions similar to the ones described in Paper I were prepared. Iron was then added to these emulsions and they were stored for 7 days (Paper II). In this study, no difference was observed between emulsions with different concentrations of CAS. This could either be the result of a shorter storage period in this study than the study described in Paper I (and thereby less oxidation in general), or the earlier mentioned metal chelating properties of CAS. The similar oxidation levels in the emulsions prepared for Paper II with different concentrations of CAS could indicate that all metal ions were chelated even at the low CAS concentration. This suggestion was supported by a study on the oxidative stability of sodium caseinate stabilized emulsions (Villiere et al., 2005). In this study, the iron binding capacity of casein was investigated within a concentration range covering the range used in our 70% emulsions. Villiere et al. (2005) showed that when 0.7-5.2 mg/L iron was added to a solution of 1 g/L sodium caseinate (pH 6.5), nearly all the metal was bound by the proteins. Hence, this could explain the lack of difference between the emulsions with different CAS concentrations prepared for Paper II.

In 5% o/w emulsions where iron was added prior to storage (Paper VI), an increase in the concentration of CAS increased the oxidative stability when emulsions were prepared at pH 7. The effect was especially pronounced when considering volatiles data. At pH 3, the results were less clear, as no effect was observed on PV and most of the volatiles quantified. An increase in the concentration of WPI from 0.2 to 0.75% in these 5% emulsions clearly decreased PV and despite a lack of consensus among the volatiles quantified most volatiles supported results from PV. The better oxidative stability of 5% emulsions with a high emulsifier concentration was suggested to be caused by an increased metal chelation of proteins in the aqueous phase. This was supported by a calculation of the iron to casein ratio in the 5% emulsions with 0.2% CAS, which exceeds the ratio reported by Villiere et al. (2005) for the binding of iron by CAS. On the other hand, in 5% emulsions with 0.75% CAS the iron to CAS ratio was lower, and all iron was expected to be bound.

5.1.3 THE EFFECT OF SURFACE CHARGE AND PH IN EMULSIONS WITH MILK PROTEINS

The influence of pH and surface charge of emulsions were investigated in both 5% emulsions (Paper VI), 10% emulsions (Paper IV) and 70% emulsions (Paper I, II). Interestingly, a difference was observed depending on oil concentration. In 5% and 10% emulsions lipid oxidation was increased at neutral pH as compared to low pH (pH 3 and pH 4, respectively for the two studies). In contrast, emulsions with 70% fish oil were in general more oxidized at low pH (pH 4.5) than at neutral pH.

The observations for 5% and 10% emulsions were in agreement with several other studies, where lipid oxidation was reduced at low pH compared to neutral pH (Berton et al., 2011; Donnelly et al., 1998; Hu et al., 2003a; Kellerby et al., 2006). In some of these studies, the better oxidative stability of emulsions prepared at low pH was related to the positive surface charge of the oil droplets, and thus a potential repulsion of transition metal ions present in the aqueous phase. The finding that the pH effect was opposite in 70% emulsions compared to 5% and 10% emulsions suggests that the repulsion of transition metal ions is less important in emulsions with a high oil concentration. Emulsions with low oil concentrations in general had smaller droplets than the oil-rich 70% o/w emulsions and therefore needed more protein for a full coverage of the oil droplet interface. Hence, less protein is assumed to be present in the aqueous phase of emulsions with a low oil content. With more protein in the aqueous phase a better metal chelation could exist. Thereby, the influence of the surface charge would be less in emulsions with a high oil concentration than in emulsions with a low concentration. Protein determinations in the aqueous phase of the 70% o/w emulsions are, however, needed to confirm this hypothesis. In addition it would be valuable to be able to visualize or measure the iron partitioning between the different phases of the various emulsion.

In 10% o/w emulsions, the pH dependent adsorption of whey protein components was investigated in relation to lipid oxidation (Paper IV). In this study, we observed that all emulsions had a higher concentration of β-lg in the aqueous phase at pH 4 than at neutral pH. As lipid oxidation was also decreased at pH 4, this could imply that not only the surface charge, but also the presence of β-lg in the aqueous phase was preferential to obtain an oxidatively stable emulsion. Antioxidative effects of β-lg in the aqueous phase of Brij-stabilized emulsions was shown by Elias et al. (2005), and suggested to mainly depend on a radical scavenging effect of cysteine and tryptophan residues. However, the same authors later reported that β-lg may both possess radical scavenging activity and have metal chelating properties when present in the aqueous phase of Brij-stabilized emulsions (Elias et al., 2007). These observations support the observations in the present study on the importance of β-lg in the aqueous phase.

5.1.4 THE USE OF PHOSPHOLIPIDS ALONE OR IN COMBINATION WITH MILK PROTEINS

Despite the antioxidative properties generally associated with milk proteins in the literature, some potential drawbacks in using these emulsifiers also exist. Milk proteins (and especially casein) tend to precipitate at pH values around their isoelectric point, whereby the emulsion may become physically instable as described in section 2.5. Therefore, phospholipids were in the present thesis investigated as alternative food grade emulsifiers, either alone or in combination with milk proteins.

Soy lecithin (LEC) was used alone as emulsifier in both 5% and 70% emulsions (Paper I, II, VI). In 70% emulsions at pH 4.5, LEC was observed to increase or provide a similar oxidative stability as WPI. However, at pH 7 LEC was shown to decrease the oxidative stability as compared to WPI and CAS. These differences might be linked to the thickness of the interfacial layer provided by the different emulsifiers. Milk proteins are in general known to provide thick interfacial layers, but at low pH the structure of adsorbed whey proteins, and especially of β-lg, has been shown to change, with an increase in its unordered structure (Fang and Dalgleish, 1997). Hence, this might also change its protective effect at the interfacial layer. The latter is in accordance with the fact that WPI stabilized 70% o/w emulsions was observed to be more stable towards oxidation at high pH than at low (Paper I, II).

In 5% emulsions LEC samples were in general observed to oxidize more than all samples with milk proteins independently of emulsifier concentration, pH or iron addition. The difference between emulsions prepared with 5% or 70% fish oil and LEC is speculated to be caused by the presence of emulsifier in the aqueous phase. The oil-rich emulsions had larger emulsion droplets and thereby possibly a higher amount of phospholipids in excess in the aqueous phase than the emulsions with low oil concentration, which have a larger total oil droplet surface area. Hence, phospholipids in the aqueous phase of 70 % o/w emulsions could have created micelles that have previously been shown to inhibit lipid oxidation (Nuchi et al., 2002). Nuchi et al. (2002) suggested that surfactant micelles can remove lipid hydroperoxides from the oil droplets and thereby prevent radicals resulting from hydroperoxide breakdown from attacking unsaturated lipids in the emulsion droplets and thus inhibit the propagation of lipid autoxidation.

Previous studies on the combination of casein and egg-yolk lecithin suggested that a thicker interfacial layer and thereby a better emulsifier coverage of the oil droplets could be obtained by this combination (Dalgleish, 1996; Fang and Dalgleish, 1993a). Hence, in the present PhD work two emulsifiers with a combination of milk proteins and milk phospholipids (MPL20 and MPL75) were investigated as emulsifiers in 5% and 70% emulsions (Paper I, II, VI). Unfortunately, the quality of MPL75 was very poor (PV = 21.9 meq peroxides/kg oil) and this might have obscured the actual effect of using a combination of protein and phospholipid in emulsions prepared with this emulsifier. Hence, all emulsions prepared with MPL75 were much more oxidized

than emulsions prepared with other emulsifiers (CAS, WPI, MPL20 or LEC) (Paper I, II, VI). An interesting observation was, however, done from cryo-SEM images of 70% emulsions with MPL75. In comparison to emulsions with CAS or MPL20, the surface of the oil droplets in the MPL75 emulsion had a more rough structure (Figure 14).

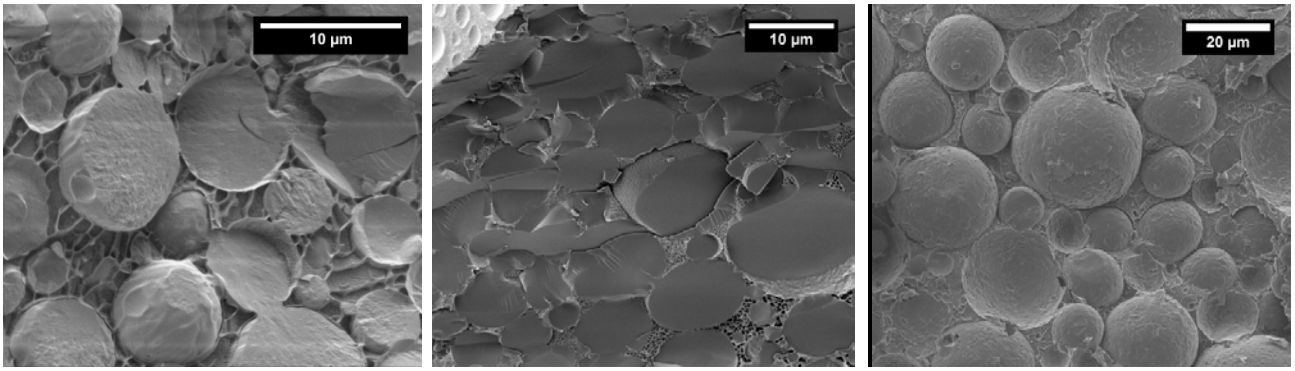


Figure 14. Cryo-scanning electron microscopy images of 70% oil-in-water emulsions prepared with 2.8% CAS (left), 2.8% MPL20 (middle) or 2.8% MPL75 (right). The surface morphology of the emulsion with MPL75 was different from the other two, with a more rough appearance, and a different fracture plane.

In addition, the fracture plane was different, as the MPL75 sample tended to break along the interfacial layer, instead of through the droplets as CAS and MPL20 emulsions did. Hence, these micrographs indicated that the surface structure of an emulsifier with a high phospholipid content was different from an emulsifier with protein or a lower phospholipid content. Better results were obtained with MPL20 as emulsifier than with MPL75. MPL20 was shown to provide a similar or better oxidative stability than WPI when 70% o/w emulsions were prepared without iron addition (Paper I) (Illustrated by PV data in Figure 15).

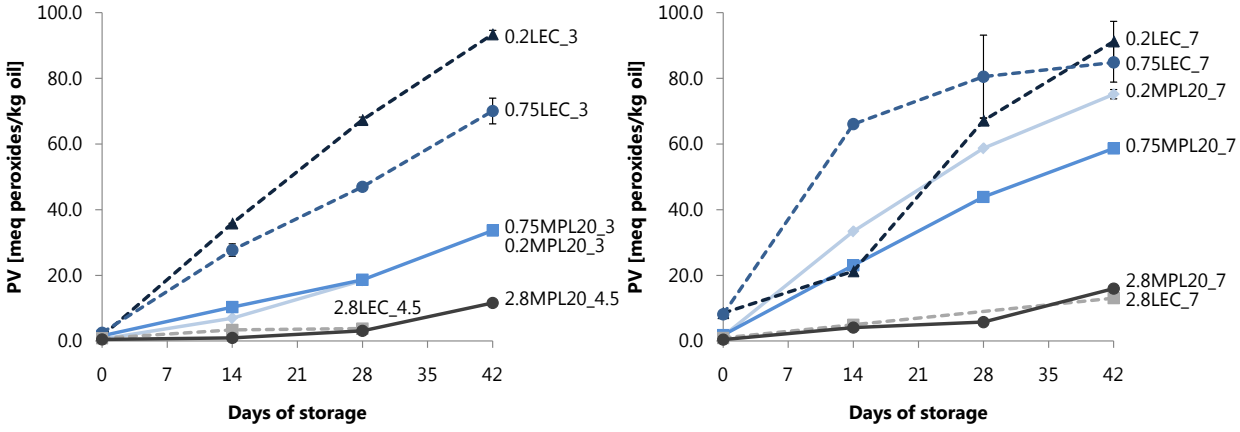
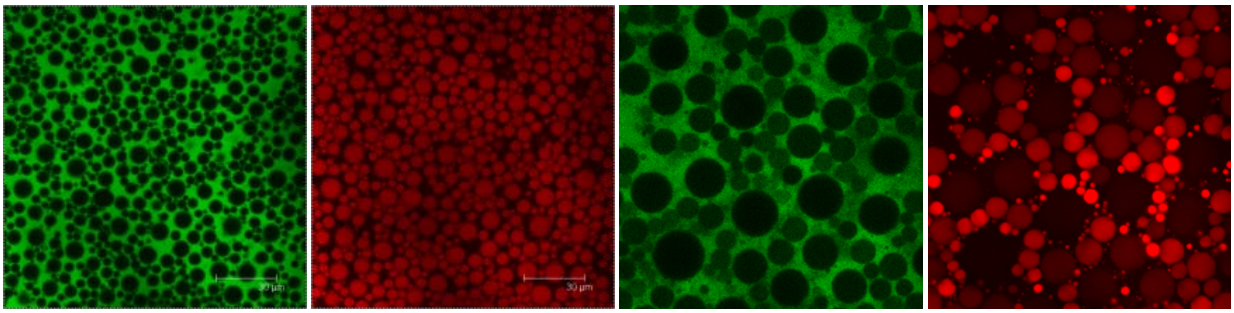


Figure 15. Peroxide values in emulsions prepared with LEC (broken lines) or MPL20 (full lines) during storage for 42 days at room temperature (19-22°C) without iron addition. Blue and grey lines are 5% (Paper VI) and 70% (Paper I) oil-in-water emulsions, respectively. Please note that the sample code names in this graph differ from the sample code names given in the papers. Sample code prefixes refer to concentration of emulsifier, 0.2 or 0.75% in 5% emulsions and 2.8% in 70% emulsions. Samples code postfixes refer to the pH of the emulsions. Data points represent means (n=2) ± standard deviations. Some error bars are within the data points.

When iron was added, the results were not as clear (Paper II). Nevertheless, in all 70% emulsions prepared, MPL20 provided better oxidative stability than LEC (Paper I, II), and a similar trend was observed for 5% emulsions (Paper VI).

Even though, the knowledge available on antioxidative effects of individual phospholipid components is very limited, some general considerations can be made on the differences in the compositions of phospholipids in the two emulsifiers LEC and MPL20. The main difference between the two is that MPL20 contains PC and PE as its main phospholipid constituents, whereas LEC contains mainly PI and PC (Table 6). PE has been suggested to work in synergy with α-tocopherol whereas PI has been suggested to inactivate transition metal ions (Bandarra et al., 1999; Pokorný, 1987). Thus, the presence of PE could enhance the antioxidative effect of MPL20 by acting in synergy with tocopherols naturally present in the fish oil (Table 3). However, relative to the total concentration of phospholipids the content of PE was actually slightly higher in LEC. In addition, the presence of PI would have been expected to increase the oxidative stability of LEC emulsions when iron was added, but this was not the case. Hence, other factors than the individual phospholipid constituents have contributed to the differences observed for LEC and MPL20 emulsions.

Other components in the emulsifiers might have influenced oxidation. In the data sheet for MPL20 it is reported that it contains 53.8% milk proteins (Table 6). These milk proteins might have exhibited antioxidative effects as well (Faraji et al., 2004; Hu et al., 2003b; Ries et al., 2010), and therefore increased the oxidative stability of MPL20 emulsions. The zeta potentials confirmed that proteins were present at the interface, whereas confocal microscopy imaging showed that proteins were also present in the aqueous phase (Figure 16). Thus, the proteins at the interface could have increased the thickness of the interfacial layer as suggested by Hunt and Dalgleish (1994) and/or led to a repulsion of transition metal ions at low pH (Hu et al., 2003a). Moreover, the proteins in the aqueous phase could have had a metal chelating effect (Faraji et al., 2004).



2.8% MPL20 2.8% MPL75

Figure 16. Confocal microscopy images of the two phospholipid/protein stabilized emulsions prepared in the study described in Paper I. Both emulsions are 70% emulsions prepared at pH 7. They have been emulsified with either 2.8% MPL20 or 2.8% MPL75. The staining for proteins is green, and the staining for lipids is red.

To further investigate the effect of using combinations of milk proteins and phospholipids, 10% o/w emulsions were prepared with a fixed concentration of EggPC and varying concentrations of CAS (Appendix II). Lipid oxidation was accelerated by iron addition in this study. From the results obtained, it was evident that at low CAS concentration (0.3%), the addition of EggPC increased both the physical and oxidative stability of the emulsion. However, when CAS was present in a concentration of 0.5% or 1.0% the addition of EggPC either did not change or decreased the oxidative stability of the emulsions (Figure 17A).

When 0.3% CAS was used as emulsifier (without addition of EggPC) the mean oil droplet size increased during 14 days of storage to approximately the double (Figure 17B). The addition of EggPC reduced this increase. As

the protein concentration in the aqueous phase of the two emulsions did not differ significantly, a combination of CAS and EggPC must have been present at the interfacial layer. Whether the increase in the oxidative stability from using a combination of CAS and EggPC was a result of decreased oil droplet coalescence in the emulsion or a change in the thickness of the interfacial layer cannot be concluded from the present results. Interestingly, emulsions with 0.5% or 1.0% CAS as the sole emulsifier were more or similarly stable towards oxidation as the corresponding emulsions with EggPC added. In both cases the protein concentration in the aqueous phase was higher in the emulsions with EggPC added whereas no change in droplet sizes was observed. Hence, it was suggested that part of CAS at the interface was substituted by EggPC in both cases as also suggested by Fang and Dalgleish (1993a). However, in contrast to our hypothesis, this possible protein-phospholipid complex at the interface did not improve the oxidative stability. The difference in the effect of EggPC on the oxidative stability of emulsions with 0.5% and 1.0% CAS was speculated to be related to the higher total concentration of CAS in the aqueous phase in the latter. This high concentration of CAS in the aqueous phase could compensate for the lower protection of the interfacial layer shown to occur when CAS was substituted by EggPC in the 0.5% CAS emulsion.

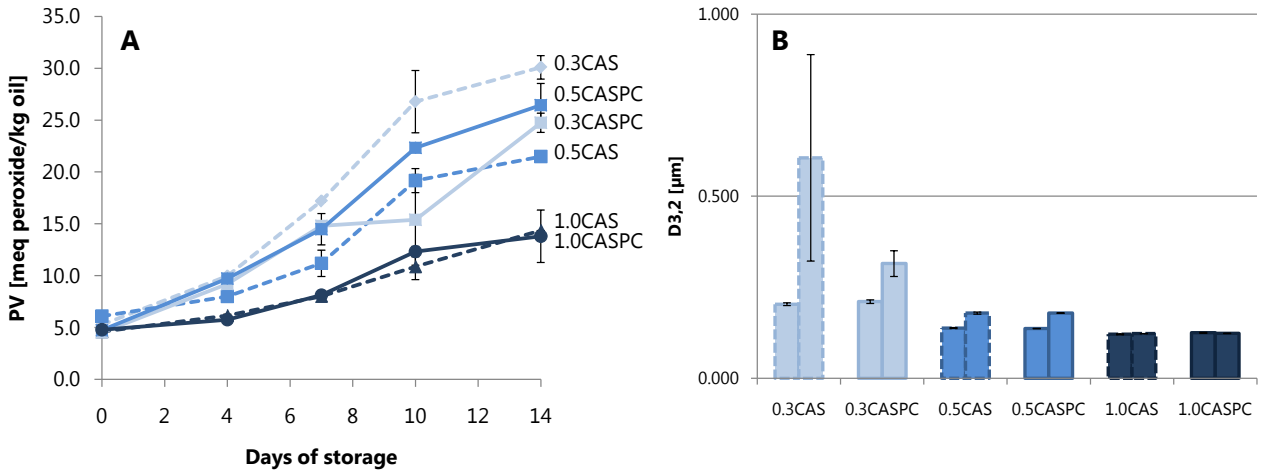


Figure 17. Peroxide values (A) and mean oil droplet sizes, $D_{3,2}$ (B), in emulsions prepared with different concentrations of CAS (0.3, 0.5 or 1%), or combinations of CAS and EggPC, with EggPC in a fixed concentration (0.5%) and CAS in different concentrations (0.3, 0.5 or 1%). Data points for both peroxide values and oil droplet sizes represent means (n=2) ± standard deviations. Some error bars are within the data points.

The overall conclusion from studies of combinations of milk proteins and phospholipids was therefore that only under some specific conditions (70% emulsions at low pH or 10% emulsions with a deficit concentration of protein) the combination of milk proteins and phospholipids was preferential.

5.1.5 THE EFFECT OF OIL DROPLET SIZE

In almost all the studies carried out in this PhD work, oil droplet sizes were shown to differ depending on emulsifier type, concentration or emulsification conditions. In studies on the influence of emulsifier type and concentration (Paper I, II, VI), these parameters were generally shown to influence lipid oxidation more than the actual droplet size. Furthermore, the study on the use of different equipments (Paper III) where droplet size distributions were optimized to be similar, a difference in oxidation was still observed between samples prepared with WPI on the two equipments. Hence, the majority of the studies in this PhD work implied that other factors influencing the macrostructure of the emulsion were more important than the oil droplet size for

the resulting lipid oxidation. In one case however, namely when emulsions with WP were prepared at different pressures (Paper V), a smaller oil droplet size seemed to increase lipid oxidation.

To investigate whether the oil droplet size itself could influence lipid oxidation, a membrane homogenizer was purchased with the aim of producing o/w emulsions with monomodal oil droplet size distributions and different mean oil droplet sizes. A number of experiments were carried out with this equipment, but whereas emulsions with surfactants such as Tween20 could be produced with monomodal droplet size distributions, this was not possible for emulsions prepared with WPI (Appendix III). Hence, it was not possible to perform a lipid oxidation study on the influence of oil droplet size in WPI emulsions by this approach.

5.1.6 LIPID OXIDATION IN EMULSIONS VERSUS IN NEAT FISH OIL

In the two studies on 70% emulsions, lipid oxidation in emulsions was also compared to lipid oxidation in neat oil stored under similar conditions (Paper I, II). Our results on emulsions prepared with pure milk proteins (WPI or CAS) described in Paper I showed that these emulsions oxidized less or similar to neat oil. Hence, the interfacial barrier created by the milk proteins between the oil phase and the transition metal ions in the aqueous phase was expected to protect the lipids from oxidizing.

However, when emulsions were added iron and stored for 7 days (independent of the emulsifier used), or when emulsions were prepared with phospholipids and stored for 42 days without iron addition, emulsions oxidized more than neat oil. This indicated that upon iron addition or when phospholipids were used, the protective role of the emulsifier at the interface was overruled by other factors. This could be the physical stress that the lipids were put through during emulsion production. Even though precautions were taken to minimize the risk of initiating lipid oxidation during emulsion production (limited oxygen availability and cooling), the emulsions did undergo harsher production conditions as compared to the neat oil that was poured directly into glasses for storage. This suggestion was confirmed by results for the initial PV, which was much lower in the neat oil than in the emulsions. The fact that emulsions prepared with milk proteins were less or similarly oxidized as neat oil when iron was not added (Paper I) but more oxidized when iron was added (Paper II) might therefore be related to this presence of significant levels of lipid hydroperoxides in emulsions already after production. These lipid hydroperoxides can react with the iron added, and readily increase oxidation in the emulsion samples compared to neat oil.

5.2 THE EFFECT OF HOMOGENIZATION EQUIPMENT AND CONDITIONS

Different homogenization equipments differ in their emulsification principle as described in section 2.3. Furthermore, Lee et al. (2007b) suggested that the type of homogenization equipment used could influence the structural properties of proteins. On this background, the present PhD work set out to investigate the influence of the type of homogenization equipment used on lipid oxidation in emulsions. The aim was to compare emulsions prepared on two types of high pressure homogenizers and a membrane homogenizer. Unfortunately, it was not possible to produce milk protein emulsions with sufficiently small oil droplets on the membrane homogenizer that could enable a comparison to emulsions produced by high pressure homogenization (Appendix III). Hence, the oxidative stability of emulsions was compared solely using two types of high pressure homogenizers (Paper III).

In milk, high pressure homogenization conditions such as temperature and pressure has previously been shown to influence lipid oxidation through the partitioning of protein components between the interface and the aqueous phase (Let et al., 2007b; Sørensen et al., 2007). These factors were further investigated for emulsions in this PhD work.

5.2.1 THE EFFECT OF HOMOGENIZATION EQUIPMENT

To investigate the influence of the type of high pressure homogenizer on the oxidative stability of 10% fish o/w emulsions, emulsions were prepared on a two-stage valve homogenizer and on a microfluidizer (Paper III). Emulsions were optimized to have similar oil droplet size distributions (Figure 18).

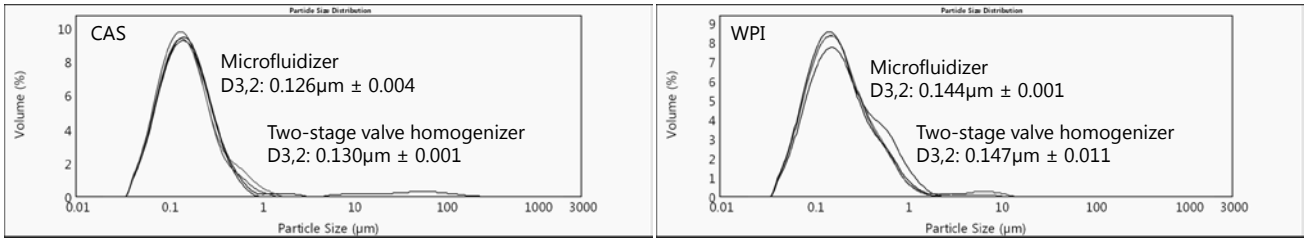


Figure 18. Oil droplet size distributions and mean oil droplet sizes ($D_{3,2}$) in 10% oil-in-water emulsions prepared with CAS or WPI on a microfluidizer or a two-stage valve homogenizer (Paper III). Homognization conditions were optimized to obtain similar droplet size distributions for each protein prepared on the two different equipments.

Results showed that the type of high pressure homogenizer used influenced lipid oxidation when emulsions were prepared with WPI, but not when prepared with CAS. In WPI emulsions the oxidative stability was increased when prepared on a microfluidizer. Based on results from a previous study on milk homogenized on two different equipments (Dalgleish et al., 1996), the difference observed in the present study was hypothesized to be caused by a homogenizer dependent difference in the adsorption behaviour of whey protein components. This was confirmed by the findings that when WPI emulsions were prepared on a microfluidizer, less protein was present in the aqueous phase, and thereby more protein was present at the interface (4.96 mg/mL and 2.86 mg/mL in the aqueous phase of WPI emulsions prepared on a valve-homogenizer and a microfluidizer, respectively). Thus, a thicker or denser interfacial layer was obtained when the emulsion was prepared on a microfluidizer.

A preferential adsorption of individual protein components (α-lac and β-lg) has been shown to exist and depend upon various conditions, such as the total protein concentration (Fang and Dalgleish, 1997; Fang and Dalgleish, 1998; Ye, 2008) and pH (Fang and Dalgleish, 1997; Hunt and Dalgleish, 1994b; Yamauchi et al., 1980). Moreover, Lee et al. (2007b) compared their own results to results obtained in the above-mentioned studies and suggested that the choice of homogenization equipment could have had an influence on the structural differences of the interfacial proteins observed in the other studies. Combined with differences in the antioxidative properties of β-lg and α-lac (Allen and Wrieden, 1982; Hu et al., 2003a), it seems likely that the preferential adsorption of one whey protein component over the other could influence lipid oxidation in WPI emulsions prepared on different homogenization equipments. From SDS-page analysis on the aqueous phases of similar emulsions as those prepared with WPI in Paper III, we confirmed differences in the protein compositions as a result of the homogenization equipment used for their production (microfluidizer vs two-stage valve homogenizer). However, the results were not clear, and further studies are needed to clarify the exact influence of the homogenization equipment used for the partitioning of protein components between the aqueous phase and the interface in emulsions.

Similar to the whey protein components, a competition for the adsorption of different casein components (α_{s1}-α_{s2}-, β- and κ-casein) to the interface has been shown to exist (Dickinson et al., 1988; Srinivasan et al., 1999; Sun and Gunasekaran, 2009; Ye, 2008). In addition, different antioxidative properties of these components have been shown (Cervato et al., 1999). However, no difference was observed in the oxidative stability of CAS emulsions prepared on the two equipments in our study. Hence, a possible difference in the adsorption of

casein components to the interface due to the homogenization equipment used was suggested to be less important than the fact that metal chelating CAS was present in the aqueous phase (as discussed in section 5.1.1).

5.2.2 THE EFFECT OF HOMOGENIZATION PRESSURE AND TEMPERATURE

To further study the influence of homogenization conditions in a high pressure homogenizer, emulsions were produced with a combination of whey proteins, WP (WPI and WPIα, 1:1) or a combination of CAS and β-lg in the ratio corresponding to milk, LgCAS (9:1) (Paper V). These emulsions were either pre-heated to 72°C prior to homogenization, or homogenized at room temperature (approximately 22°C), and furthermore either homogenized at 5 or 22.5 MPa. Results demonstrated an emulsifier dependent difference in the effect of pressure on lipid oxidation, whereas no clear effect of temperature was observed.

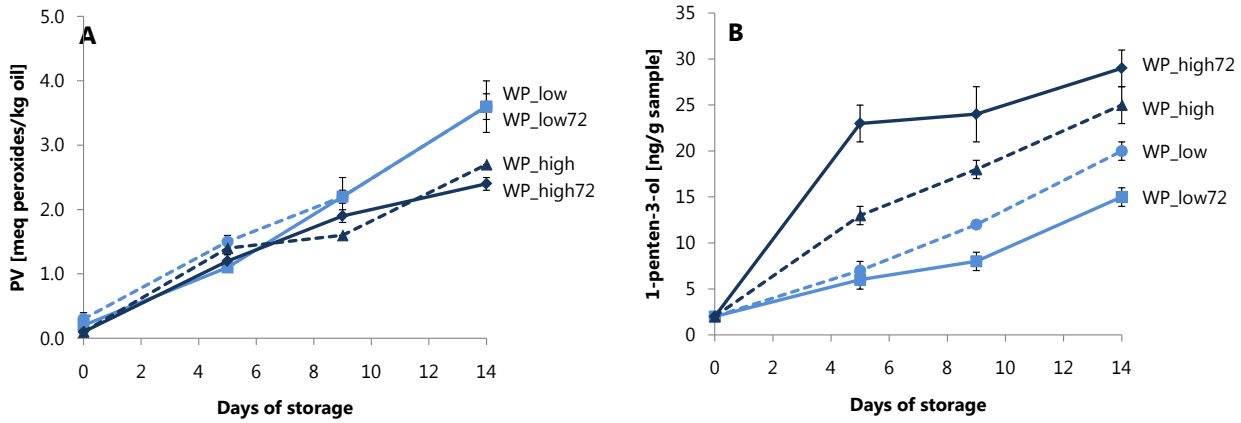


Figure 19. Peroxide values (A) and concentrations of 1-penten-3-ol (B) in 10% oil-in-water emulsion samples prepared with 1% WP (0.5% WPI + 0.5% WPIα). Sample code postfixes refer to homogenization conditions. Low: Prepared at low pressure, 5 MPa; High: Prepared at high pressure, 22.5 MPa; 72: Prepared at 72°C. Storage for 2 weeks at room temperature (~20°C). Data points for PV represent means (n=2) ± standard deviations, and for 1-penten-3-ol means (n=3) ± standard deviations. Some error bars are within the data points.

In emulsions with a combination of whey proteins (WP) as emulsifier, an increase in pressure led to a decrease in PV, but an increase in volatiles formation (Figure 19). A low PV and a high concentration of volatiles could be the result of a fast degradation of lipid hydroperoxides in these emulsions. This degradation could stem from an increase in the exposure of lipid hydroperoxides towards transition metal ions in the aqueous phase when droplet sizes were reduced due to increased homogenization pressure. A similar link between oil droplet size and increased lipid oxidation has previously been suggested in other studies (Jacobsen et al., 2000; Kargar et al., 2011; Lethuaut et al., 2002). Results from studies on the influence of droplet size on lipid oxidation are, however, unclear and most often other factors are concluded to influence lipid oxidation more than the actual droplet size (Azuma et al., 2009; Gohtani et al., 1999; Hu et al., 2003b; Sørensen et al., 2007), as also discussed in section 5.1.4. Hence, the droplet size might not be the sole explanation for the results obtained in the study described in Paper V.

Besides the differences in oil droplet size, the protein composition in the aqueous phase was also slightly different when WP emulsions were prepared at different pressures. At high pressure, less β-lg was present in the aqueous phase than at low pressure. Hence, it was speculated that the antioxidative activity of individual whey protein components was different at the interface and in the aqueous phase, and specifically that the antioxidative activity of β-lg was higher when present in the aqueous phase than at the interface. As previously

mentioned, structural changes have been observed upon adsorption of β-lg to an interface (Fang and Dalgleish, 1997), which could potentially change the accessibility of amino acid residues with antioxidative properties. The possible better antioxidative effect of β-lg than of α-lac in the aqueous phase was supported by our study on individual whey protein components (Paper IV). To fully explain the results on WP emulsions produced at different homogenization pressures, more studies are needed on the unfolding of whey proteins under different conditions.

The emulsions produced with a combination of CAS and Lg (ratio as in milk) demonstrated the opposite effect of an increase in pressure (Paper V). In these emulsions, a higher pressure led to both lower PV and lower concentrations of volatile secondary oxidation products (Figure 20). This was in accordance with observations in milk (Let et al., 2007b). Thus, despite a decrease in oil droplet size and an increased total surface area of the oil droplets, lipid oxidation was decreased when emulsions were produced at high pressure in both the milk study and the present study on LgCAS emulsions.

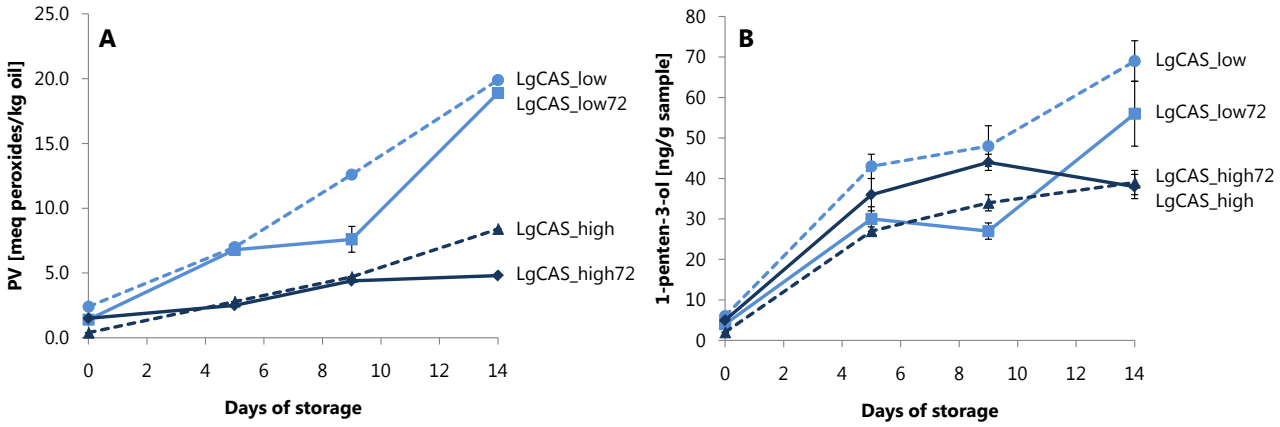


Figure 20. Peroxide values (A) and concentrations of 1-penten-3-ol (B) in 10% oil-in-water emulsion samples prepared with 1% LgCAS (0.1% Lg + 0.9% CAS). Sample code postfixes refer to homogenization conditions. Low: Prepared at low pressure, 5 MPa; High: Prepared at high pressure, 22.5 MPa; 72: Prepared at 72°C. Storage for 2 weeks at room temperature (~20°C). Data points for PV represent means (n=2) ± standard deviations, and for 1-penten-3-ol means (n=3) ± standard deviations. Some error bars are within the data points.

In milk, it was suggested that an optimal partitioning of proteins between the interface and the aqueous phase was responsible for the higher oxidative stability when emulsions were produced at a high pressure than at a low pressure (Let et al., 2007b; Sørensen et al., 2007). Similar results on the partitioning of proteins were obtained in our study (Paper V), where the concentration of caseins was higher in the aqueous phase when emulsions were produced at high pressure, and similarly the concentration of β-lg was lower. The presence of caseins in the aqueous phase has previously been shown to provide a good antioxidative effect by effectively chelating transition metal ions both in emulsions (Faraji et al., 2004) and algal oil enriched milk (Gallaher et al., 2005).

Combining the results from the studies of WP and LgCAS emulsions it is suggested that when CAS is present (as in LgCAS) it is most beneficial to have this protein in the aqueous phase and β-lg at the interface, whereas when CAS is not present (as in WP), it is more beneficial to have β-lg in the aqueous phase and α-lac at the interface.

The lack of a temperature effect for any of the emulsifier combinations in Paper V was surprising, as results previously obtained on milk reported a better oxidative stability upon pasteurization (72°C) prior to

homogenization due to unfolding of β-lg (Let et al., 2007b). In addition, Kiokias et al. (2007) showed a reduction in conjugated diene formation when 30% sunflower o/w emulsions were stabilized by heat-treated whey protein concentrate instead of native whey protein concentrate. These authors reported structural changes in the whey protein between 60°C to 80°C, with an optimum at 80°C whereafter no beneficial effect of further heating was observed. It was expected that the whey proteins had all their reduced sulfhydryls in the reactive form at 80°C.

In contrast to these studies on adsorbed whey proteins in milk and emulsions (Kiokias et al., 2007; Let et al., 2007b; Sørensen et al., 2007), Elias et al. (2007) reported that in order to decrease lipid hydroperoxides and TBARS formation, β-lg should be pre-heated to 95°C. Preheating to 70°C did not have any effect as compared to native β-lg. It should however be noted that in the latter study the pre-heated β-lg was not adsorbed at the interface during emulsification, instead the native or pre-heated β-lg was added to the aqueous phase of Brij-stabilized 5% menhaden o/w emulsions after emulsification. However, Elias et al. (2007) also demonstrated that despite the observation that heating to 70°C did not have any effect, the exposure of cysteine and thereby sulfhydryl residues were highest at 70°C. In addition, they showed that the ability to scavenge free radicals was better for β-lg pre-heated to 70°C than for native β-lg (Elias et al., 2007). Hence, the lack of clear results on heat treatment in Paper V calls for further oxidation studies on the partitioning of protein components as a result of heat treatment over a wider range of temperatures.

5.3 OXIDATIVE STABILITY OF FISH OIL-ENRICHED FOODS WITH DELIVERY EMULSIONS

Based on the results obtained in the studies described in Paper I-V and Appendix II, emulsions were selected for the study of lipid oxidation in fish oil enriched milk (Paper VII) and fish oil enriched cream cheese (Paper VIII). Milk was prepared with either neat oil or 10% fish o/w emulsions with WP, CAS or Lg, whereas cream cheese was prepared with either neat oil or 70% fish o/w emulsions with WPI, CAS or MPL20, as elaborated in section 4.2. In general, a higher degree of lipid oxidation was observed in both food products when fish oil was added, as compared to the reference samples.

5.3.1 FISH OIL ENRICHED MILK

In milk, both PV data (Figure 21A) and results for volatile secondary oxidation products (represented by 1-penten-3-ol in Figure 21B) revealed that the oxidative stability was better when fish oil was added as neat oil instead of as a delivery emulsion (Paper VII). In addition, no clear differences were observed between the three milks added different delivery emulsions. The lower oxidative stability of the milks added delivery emulsions compared to the milk added neat oil was in contrast to the overall conclusions from a previous study on the addition of neat fish oil and pre-emulsified fish oil to milk (Let et al., 2007a). In this study, a higher PV was observed when a 50% fish o/w emulsion prepared with 1.5% whey protein was added to milk than when neat oil was added. However, lower concentrations of volatiles and a reduced rancid/old odour and flavour was observed with the addition of a fish o/w emulsion, and the authors based their general conclusions on the latter data.

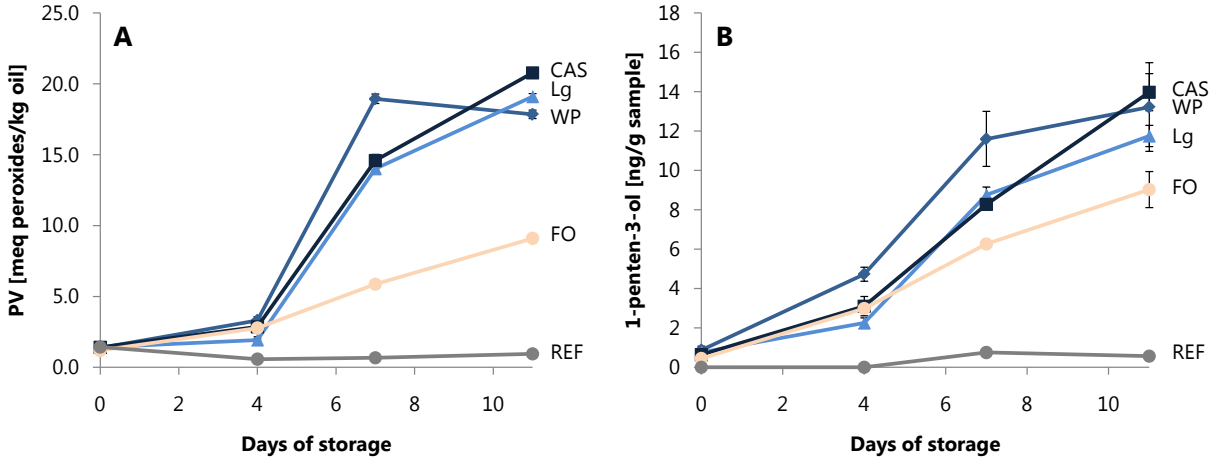


Figure 21. Peroxide values (A) and concentrations of 1-penten-3-ol (B) in milk samples added fish oil as neat oil (FO) or in a delivery emulsion (CAS, WP and Lg) compared to a reference milk sample without fish oil (REF). Storage for 11 days at ~2°C. Data points for PV represent means (n=2) ± standard deviations, and for 1-penten-3-ol means (n=3) ± standard deviations. Some error bars are within the data points.

The divergence in the overall results on the use of delivery emulsions in our study and the study by Let et al. (2007a) could be caused by the differences in the delivery emulsions used. Let et al. (2007a) used a 50% fish o/w emulsion, whereas we used a 10% fish o/w emulsion. As a result of the pressures used for preparing the delivery emulsions in the two studies, the mean oil droplet size was much smaller in the emulsions in our study than in the delivery emulsion prepared by Let et al. (2007a). However, as the homogenization of the milk added neat oil was done at a similar pressure in the two studies (22.5 MPa), this resulted in the opposite relation between oil droplet sizes when prepared with neat oil or delivery emulsions in the two studies. Hence, Let et al. (2007a) had smaller oil droplets in the milk prepared with neat oil than the milk prepared with delivery emulsions, whereas we had the smallest droplets in our milks added delivery emulsions.

In this case oil droplet size might therefore have had an influence, with the smaller oil droplets oxidizing more than the larger droplets in both studies, resulting in a lower oxidative stability of the milk added neat oil in the study by Let et al. (2007a), and a lower oxidative stability of the milk added delivery emulsions in our study. As already mentioned, the emulsifier used for preparing the delivery emulsions in our study (WP, Lg or CAS) did not influence the resulting lipid oxidation in milk (Paper VII). This observation was surprising, since differences between the oxidative stability of emulsions prepared with whey proteins and caseins were observed in other studies in this PhD work (Paper I-III, VI). This could indicate that the differences in the protective effect of the various protein components in the aqueous phase observed in the emulsions was diminished in the milk.

From this comparison of the use of different delivery emulsions, it was concluded that to improve the oxidative stability of fish oil enriched milk, a pre-emulsified fish oil with a high oil concentration and larger droplets should be used. In addition, the production conditions used when incorporating the delivery emulsion could be improved, e.g. by production in an oxygen-free environment.

5.3.2 FISH OIL ENRICHED CREAM CHEESE

The only studies published on fish oil enriched cream cheese so far investigated solely the sensory quality (Kolanowski and Weißbrodt, 2007). The study carried out in this PhD work is therefore the first lipid oxidation study on fish oil enriched cream cheese in which chemical measurements, sensory evaluation and microscopy imaging have been conducted in combination (Paper VIII). Results showed that cream cheese with fish oil

added as delivery emulsions prepared with CAS or WPI oxidized the most during 20 weeks of storage, whereas cream cheese with fish oil added as neat oil or in a delivery emulsion prepared with MPL20 oxidized the least, at least during the initial part of the storage period (Figure 22). Hence, in this type of system the effect of using a delivery emulsion was dependent upon the emulsifier by which it was prepared, in contrast to the results for fish oil enriched milk (Paper VII).

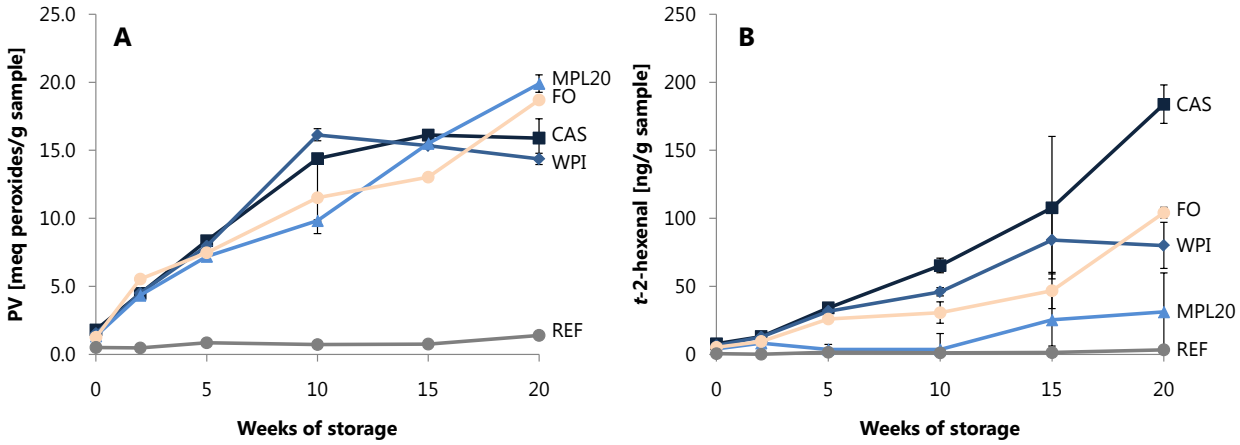


Figure 22. Peroxide values (A) and concentrations of *t*-2-hexenal (B) in cream cheeses with 1.3% fish oil added as neat oil (FO) or in a delivery emulsion (CAS, WPI and MPL20) compared to a reference cream cheese without fish oil (REF). Storage for 20 weeks at ~5°C. Data points for PV represent means (n=2) ± standard deviations, and for *t*-2-hexenal means (n=3) ± standard deviations. Some error bars are within the data points.

Interestingly, differences in the macrostructures were observed in the cream cheese samples when imaged by confocal microscopy (Figure 23). The reference cream cheese without fish oil (REF) and the cream cheese with neat fish oil (FO) had relatively large unprotected oil droplets (stained red in Figure 23), whereas the three cream cheeses with fish oil added as a delivery emulsion (MPL20, WPI and CAS) had far fewer unprotected lipid droplets. Particularly MPL20 could be distinguished from the other samples as more of the lipid (including the milk lipid) was hidden within the protein structure (less visible red colour in the image).

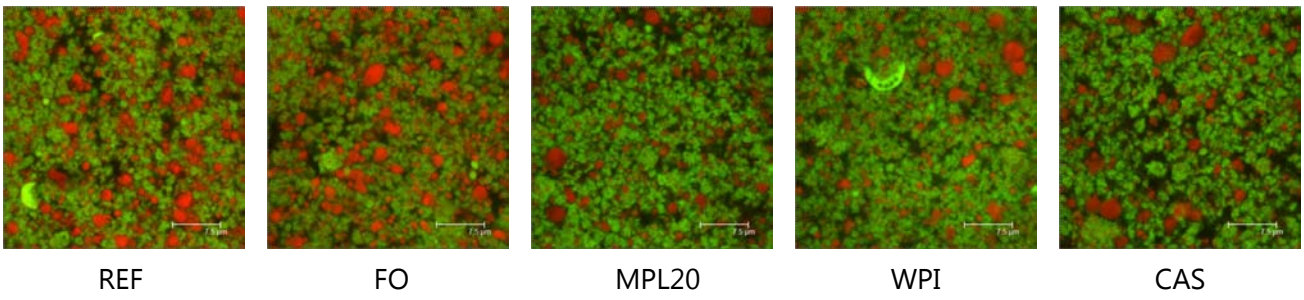


Figure 23. Confocal micrographs of the cream cheeses (Paper VIII), prepared without fish oil addition (REF), with fish oil added as neat oil (FO) or fish oil added in a 70% fish oil-in-water delivery emulsion emulsified by MPL20, WPI or CAS.

The differences in the macrostructure between the cream cheeses added delivery emulsions and the other two cream cheeses might be ascribed to the excess protein present in the aqueous phase of the 70% delivery emulsions. Hence, when added to the cream cheese, the emulsifier that was present in the aqueous phase in the delivery emulsion emulsifies milk fat present in the cream cheese. This results in fewer unprotected milk fat

droplets observed in the micrographs (Figure 23). The otherwise protective effect of CAS in the aqueous phase compared to WPI, as observed in 70% emulsions (Paper I and II), is therefore lacking in the cream cheeses. Hence, as discussed for the milk, this implies that the emulsifier at the interface is not as important as the protein present in the aqueous phase, and in this system an emulsion droplet covered by CAS is not better protected than an emulsion droplet covered by WPI.

After the production of these cream cheeses, we were informed by the manufacturer of the MPL20 emulsifier that antioxidants are added during its production. Hence, the better oxidative stability of the cream cheese with the MPL20 delivery emulsion compared to the WPI and CAS delivery emulsions could be a result of this. However, the macrostructure is also different in the cream cheese with the MPL20 delivery emulsion. Hence, the increased oxidative stability compared to the other two cream cheeses with delivery emulsion could also be ascribed to a better protection of the lipids in the protein structure.

Overall, the conclusion from the study of fish oil enriched cream cheese was that the approach of using a delivery emulsion did not improve the oxidative stability of the product as compared to adding neat oil. To be able to add fish oil to this type of product in the future, more studies on the oil-rich delivery emulsions should be conducted or other approaches for protecting the fish oil should be considered, e.g. the addition of antioxidants.

Chapter 6:
Conclusions and Perspectives

6.1 CONCLUSIONS

Overall, this PhD work has shown that factors related to both the choice of emulsifier, homogenization equipment and emulsification conditions influence the oxidative stability of simple fish o/w emulsions. These factors include the oil concentration, the type of milk protein or phospholipid used as emulsifier, the pH, the addition of iron, preheating of the protein prior to homogenization, the equipment used for homogenization and the pressure applied during high pressure homogenization. Most importantly, lipid oxidation in simple fish o/w emulsions was shown to depend on combinations of these factors, and not of any of these factors alone.

It was shown that despite an attempt to optimize the above-mentioned factors for creating oxidatively stable fish o/w delivery emulsions, this was not enough to ensure a protection of the fish oil when the delivery emulsions were added to either milk or cream cheese.

Regarding the choice of emulsifier, it was initially hypothesized that differences in the antioxidative properties of different milk proteins would influence lipid oxidation. This hypothesis was confirmed, and generally CAS was observed to increase the oxidative stability of emulsions more than WPI. Thus, in general CAS was shown to provide a good oxidative stability of simple o/w emulsions independent of oil concentration, pH, the presence of transition metal ions or the choice of homogenization equipment used for emulsion production. The antioxidative effect of CAS was suggested to be ascribed mainly to its contents of amino acid residues with metal chelating properties, and specifically its presence in the aqueous phase. It could not be confirmed whether the thickness of the interfacial layer influenced the oxidative stability of CAS emulsions. Despite the protective effect of CAS in simple o/w emulsions compared to other emulsifiers, CAS provided a similar (in milk) or a lower (in cream cheese) protective effect when used as emulsifier in a delivery emulsion for the addition of fish oil to these food products. Hence, the protective effect of CAS in the aqueous phase, as shown in simple o/w emulsions was suggested to be lacking in these real food systems.

In contrast to emulsions prepared with CAS, the oxidative stability of simple o/w emulsions prepared with the structured WPI emulsifiers were highly dependent on both the ratio between individual protein components (α-lac and β-lg), pH, the homogenization equipment used, and the homogenization conditions applied. Conditions that could be related to an unfolding of whey proteins and thereby an exposure of amino acid residues with antioxidative properties were shown to improve the oxidative stability. In addition, the partitioning of individual whey protein components between the aqueous phase and the interfacial layer was shown to be of importance. It was suggested, that β-lg in the aqueous phase increased the oxidative stability compared to α-lac. More studies are needed to fully understand the role of the partitioning of whey protein components and their unfolding in relation to lipid oxidation in simple o/w emulsions.

We hypothesized that a positive oil droplet surface charge would lead to a repulsion of transition metal ions and thereby decrease lipid oxidation as compared to a negative oil droplet surface charge. This was confirmed in 5% and 10% emulsions but not in 70% emulsions. In the latter, the results indicated that the presence of a high concentration of proteins with a metal chelating effect in the aqueous phase was more important than the repulsion of transition metal ions.

The use of soy lecithin (LEC) or combinations of milk phospholipids and milk proteins (MPL20 or MPL75) were shown not to improve the oxidative stability of simple o/w emulsions compared to the use of CAS alone. Nevertheless, under certain conditions LEC and MPL20 emulsions were more stable towards oxidation than WPI emulsions, namely at low pH in 70% emulsions. This was possibly due to pH dependent structural changes in the emulsifiers that affected their protective effects at the interfacial layer differently. Moreover, the interfacial layer of CAS deficient 10% o/w emulsions was improved by the addition of EggPC as hypothesized. The creation of protein-phospholipid complexes at the interface of emulsions with a sufficient CAS concentration was, as opposed to our hypothesis, not shown to improve the oxidative stability. Instead, a similar or a lower oxidative stability was observed in these emulsions with both CAS and EggPC, compared to emulsions with CAS alone. At a sufficient CAS concentration it was therefore shown preferential to have this emulsifier at the interface instead of having EggPC. Furthermore, it was concluded that it required a good quality of the phospholipid emulsifier to obtain a beneficial effect compared to the use of milk proteins alone.

As hypothesized, the type of high pressure homogenization equipment and the conditions applied influenced lipid oxidation in milk protein stabilised emulsions by affecting the total protein adsorption and the partitioning of protein components between the interfacial layer and the aqueous phase. However, the equipment and homogenization conditions affected milk proteins differently. Whereas lipid oxidation in CAS emulsions was shown to be unaffected by the type of homogenization equipment used, WPI emulsions oxidized more when prepared on a two-stage valve homogenizer than on a microfluidizer. Moreover, an increase in the pressure applied increased lipid oxidation in WP emulsions, but decreased lipid oxidation in LgCAS emulsions. The differences between the influence of pressure on WP and LgCAS emulsions was suggested to be related to the partitioning of protein components between the aqueous phase and the interfacial layer. Hence, when CAS was present (in LgCAS) it was most beneficial to have this protein in the aqueous phase and β-lg at the interface, whereas when CAS was not present (in WP), it was more beneficial to have β-lg in the aqueous phase and α-lac at the interface. The effect of increasing the temperature to unfold whey proteins was not clear from the present study.

WPI stabilized emulsions could not be prepared with monomodal droplet size distributions by membrane homogenization. The relation between oil droplet size and oxidative stability of WPI emulsions could therefore not be established by the planned approach. From measurements of oil droplet sizes throughout the studies in this PhD work, it was concluded that in most cases other factors than the actual droplet size was more important for lipid oxidation, such as the composition of proteins at the interfacial layer, or the partitioning of proteins between the aqueous phase and the interfacial layer.

In the case studies of fish oil enriched dairy products, it was hypothesized, that the addition of oxidatively stable delivery emulsions would be advantageous as compared to addition of neat oil. However, in both cases this hypothesis was shown to be too simple, as the matrix to which the delivery emulsion was added played a significant role.

In milk, a better oxidative stability was not obtained by the use of a 10% fish o/w delivery emulsion compared to addition of neat oil. This was explained by an increased oxidation due to smaller oil droplets in milks with delivery emulsions as compared to milk added neat oil. Furthermore, the type of emulsifier (CAS, WP or Lg) used for the production of the delivery emulsion had no significant effect under these conditions. In contrast to milk, the oxidative stability of cream cheese was shown to be influenced by the type of emulsifier used for preparation of the delivery emulsion (CAS, WPI or MPL20). Despite an overall oxidatively instable product, the cream cheese with fish oil added as a delivery emulsion prepared with MPL20, and the cream cheese with neat

fish oil oxidized less than the cream cheeses with fish oil added as delivery emulsions prepared with WPI or CAS. These differences were linked to differences in the macrostructure of the cream cheeses, and the lack of antioxidative effects of CAS and WPI in the aqueous phase compared to when these emulsifiers were used in simple o/w emulsions. The approach used in the present PhD work with an optimization of delivery emulsions in general, prior to their addition to different food products should possibly be reconsidered. Hence, to obtain oxidatively stable fish oil enriched food products, delivery emulsions should be individually optimized in relation to the specific food matrix to which it is added.

6.2 PERSPECTIVES

The studies included in this PhD thesis have touched upon research areas where previous publications are scarce or non-existing. This thesis includes the first two papers published on lipid oxidation in simple o/w emulsions prepared with as high oil concentration as 70% and results on these emulsions used as delivery emulsions in cream cheese. Despite the lack of a protective effect of using delivery emulsions in both milk and cream cheese, this approach cannot be turned down from the present results. Instead the results call for an individual optimization of the type of delivery emulsion used in each food product specifically.

In milk, we suggest that it would be beneficial to use a delivery system with a high oil concentration and large oil droplets. As emulsifier for the delivery emulsion, it would be valuable to investigate whether a casein product that has properties similar to the original casein in milk could be advantageous over caseinate. As the macroctructure of the cream cheese prepared with MPL20 was different from the other cheeses, this could imply that the approach of using combinations of milk proteins and milk phospholipids in delivery emulsions for cream cheese would be advantageous. Hence, this should be further explored. The observation that the type of emulsifier used in the delivery emulsions may not be as important for the oxidative stability as the type of emulsifier used in simple o/w emulsions should, however, also be further investigated. Hence, to investigate solely the effect of the emulsifier at the interface of the fish oil droplets, the delivery emulsions should be separated in a cream phase and an aqueous phase, and then only the cream phase should be added to the food product.

Further studies are, however, also needed to optimize the production conditions for the 70% delivery emulsions. Hence, it would probably be beneficial to explore the use of other emulsification equipments, such as a colloid mill, and to gain knowledge on the protein composition at the interface and in the aqueous phase as affected by conditions such as pH and emulsifier concentration in these emulsions. Furthermore, it would be valuable to determine the metal ion contents of both milk and cream cheese, and especially the partitioning of these within the food product. Finally, a protection of the oil during incorporating of the delivery emulsion in the food product could be considered, e.g. by production in an oxygen-free environment.

If none of these approaches to improving the delivery emulsions result in more oxidatively stable fish oil enriched dairy products, the use of antioxidants should be evaluated. A possible synergy could exist if peptides with antioxidative properties could be used for both emulsification and antioxidant protection.

This thesis also includes the first paper investigating the influence of the type of high pressure homogenization equipment used on lipid oxidation in oil-in-water emulsions. Hence, the knowledge obtained in this study should be used in the future when different studies on e.g. the effects of emulsifier type and pH are compared, but where emulsions have been produced on different equipments. In addition, it could be valuable for the food industry to consider their use of different high pressure homogenization systems, when

food emulsions are prepared with whey proteins or other emulsifiers that could possibly be equally sensitive towards the kind of treatment.

In this PhD work results on the oxidative stability of simple o/w emulsions have been combined with studies of protein partitioning to elucidate the effects of pH and homogenization conditions. Valuable information has been obtained by this approach. However, it would be beneficial to improve the method used for determining the protein partitioning for future use. The method used in the present project includes two centrifugation steps where the oil droplets surrounded by emulsifier are separated from the aqueous phase. Hence, it is a destructive method, and it would be valuable to be able to determine the protein partitioning directly on the emulsion, or to develop a less destructive method for separating the oil droplets from the aqueous phase, e.g. a filtration.

The inclusion of phospholipids in the present PhD work did not provide a convincingly good alternative to milk proteins. However, under certain conditions combinations of milk proteins and milk phospholipids had beneficial effects. To date, results published on the antioxidative effects of individual phospholipids are extremely scarce, and most often obtained under conditions where oxidation is accelerated by e.g. heating. To fully understand the effect of using phospholipids, lipid oxidation studies should be carried out where the specific effects of individual phospholipids are evaluated both when phospholipids are present alone and when they are present in combination with milk proteins.

As discussed in this PhD thesis, the influence of oil droplet size on lipid oxidation is contentious, because differences in oil droplet size most often exist because other factors, that could influence lipid oxidation themselves, are varied. To investigate the influence of oil droplet size on lipid oxidation in emulsions in general, the results we obtained from preparing emulsions by the use of a membrane homogenizer should be followed up. Hence, emulsions should be produced by this equipment with an emulsifier such as Tween20 to obtain monomodal droplet size distributions with different mean droplet sizes. To better understand the influence of oil droplet size on lipid oxidation in milk protein stabilized emulsions, the use of microchannel emulsification should be exploited. In a microchannel emulsification system monomodal droplet size distributions could possibly be obtained since the membrane material might be better suited for proteins and less fouling would occur.

Finally, microscopy imaging has been explored for the visualization of differences in the structure of emulsions, and fish oil enriched food products. Confocal microscopy provided some valuable information about the macrostructure. However, as both the influence of the structure and thickness of the interfacial layer are still under debate, it would be highly relevant to develop more advanced methods for microscopy imaging and visualization of the interfacial layer in emulsions. This could also increase our understanding of the differences between using milk proteins alone or in combination with phospholipids. Likewise, advanced microscopy could potentially be used for investigating the partitioning of transition metal ions and proteins between the aqueous phase and the interfacial layer. The development of such advanced microscopic methods is ongoing in a PhD study related to the same research project as the present PhD work.

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Appendices and Papers

APPENDIX I

Emulsions with Different Thicknesses of the Interfacial Layers

APPENDIX II

Combinations of Caseins and Phospholipids

APPENDIX III

Emulsions Prepared by Membrane Homogenization with Monomodal Oil Droplet Size Distributions

PAPER I

Horn AF, Nielsen NS, Andersen U, Søgaard LH, Horsewell A & Jacobsen C (2011) Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH. *European Journal of Lipid Science and Technology 113:1243-1257*

PAPER II

Horn AF, Nielsen NS & Jacobsen C (2012) Iron-mediated lipid oxidation in 70% fish oil-in-water emulsions: effect of emulsifier type and pH. *International Journal of Food Science and Technology 47:1097-1108*

PAPER III

Horn AF, Nielsen NS, Jensen LS, Horsewell A & Jacobsen C (2012) The choice of homogenisation equipment affects lipid oxidation in emulsions. *Food Chemistry 134:803-810*

PAPER IV

Horn AF, Wulff T, Nielsen NS & Jacobsen C (2012) Effect of α-lactalbumin and β-lactoglobulin on the oxidative stability of 10% fish oil-in-water emulsions depends on pH. *Food Chemistry (submitted).*

PAPER V

Horn AF, Barouh N, Nielsen NS, Baron CP & Jacobsen C (2012) Homogenization pressure and temperature affect protein partitioning and oxidative stability of emulsions. *Journal of the American Oil Chemist´s Society (corrected and resubmitted).*

PAPER VI

Nielsen NS, **Horn AF** & Jacobsen C (2012) Effect of emulsifier type, pH and iron on oxidative stability of 5% fish oil-in-water emulsions. *European Journal of Lipid Science and Technology (submitted).*

PAPER VII

Horn AF, Nielsen NS & Jacobsen C - Lipid oxidation in milk enriched with neat fish oil or pre-emulsified fish oil. *Draft intended for Food Chemistry.*

PAPER VIII

Horn AF, Green-Petersen D, Nielsen NS, Andersen U, Hyldig G, Jensen LHS, Horsewell A & Jacobsen C (2012) Addition of fish oil to cream cheese affects lipid oxidation, sensory stability and microstructure. *Agriculture 2:359-375.*

Appendix I:
Emulsions with Different Thicknesses of the Interfacial Layers

BACKGROUND AND HYPOTHESIS

In emulsions, the interface between the oil and the aqueous phase is the place of contact between lipids and prooxidative components (McClements and Decker, 2000). Hence, lipid oxidation is to a great extent considered an interfacial phenomenon. Factors related to the interface are therefore most often considered important for the resulting lipid oxidation, e.g. the surface charge or the thickness of the interfacial layer. A positive surface charge may lead to a repulsion of prooxidative transition metal ions present in the aqueous phase (Donnelly et al., 1998; Hu et al., 2003; Kellerby et al., 2006), whereas a thick interfacial layer may serve as a physical barrier between lipids and prooxidants. However, no studies are available that have investigated lipid oxidation in emulsions as a result of the thickness of the interfacial layer.

Dalgleish and co-workers have in several studies looked into changes in the conformation and thickness of the interfacial layer when changing the concentration of different milk proteins, or milk protein components (Dalgleish, 1993; Fang and Dalgleish, 1993; Hunt and Dalgleish, 1994). In a study on 20% soy oil-in-water emulsions with caseins obtained from skim milk (0.2-2.0%), it was shown that at casein concentrations above 0.5%, the oil droplet size was almost independent of the protein concentration. Hence, $D_{3,2}$ ranged from 340 to 310 nm when the concentration of casein was increased from 0.7 to 2.0% (Fang and Dalgleish, 1993). At concentrations of 0.7% casein or less, all of the protein present was adsorbed to the surfaces of the oil droplets. Thus, at casein concentrations of 1% or above appreciable quantities of non-adsorbed casein was found in solution. These observations were used to suggest that casein has different conformations depending on the concentration used. At the lowest casein concentration the interfacial coverage by protein was less than 1 mg m^{-2}, and the emulsions were not stable. Right above this value, the emulsions became stable, however, the caseins were spread to their maximum extent and gave an interfacial layer thickness of about 5 nm (Figure 1A).

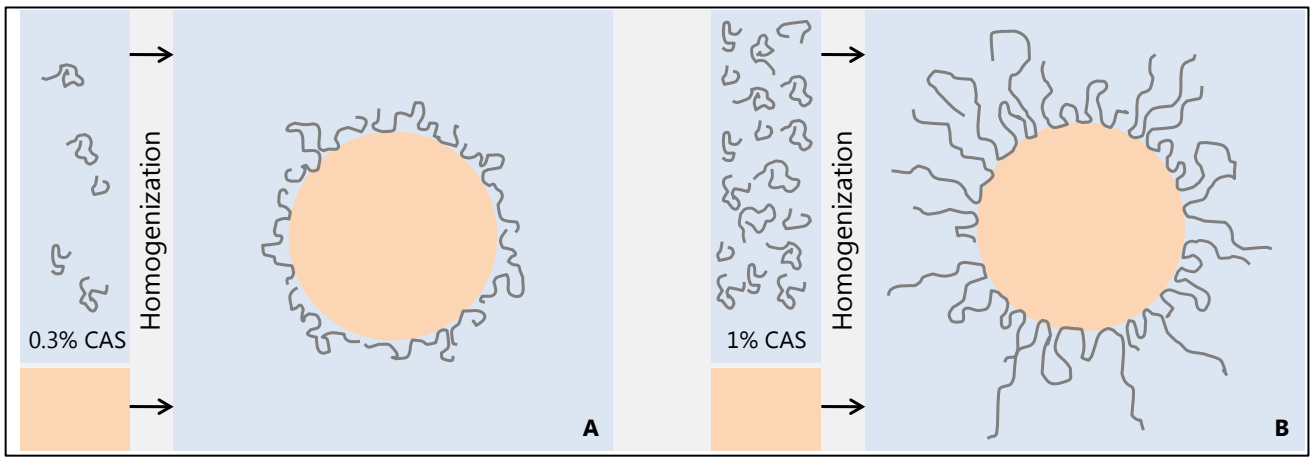

Figure 1. A schematic illustration of the differences in the thickness of the interfacial layers between an oil droplet in an oil-in-water emulsion prepared with a low (A) and a high (B) concentration of CAS.

When the surface coverage by proteins was increased to above 1.5 mg m^{-2}, the otherwise stretched casein molecules tended to compact, giving space for additional casein molecules at the surface, and thereby they protruded out into the aqueous phase creating a thicker interfacial layer of about 10 nm (Figure 1B) (Fang and

Dalgleish, 1993). In the same study, the authors investigated the surface coverage by proteins as a consequence of adding casein solution to already formed, but casein-deficient emulsions. It was discussed whether this treatment resulted in a different structure, or whether the adsorption pattern was similar to the one suggested when a sufficient amount of casein was added before homogenization. The differences are schematically illustrated in Figure 2.

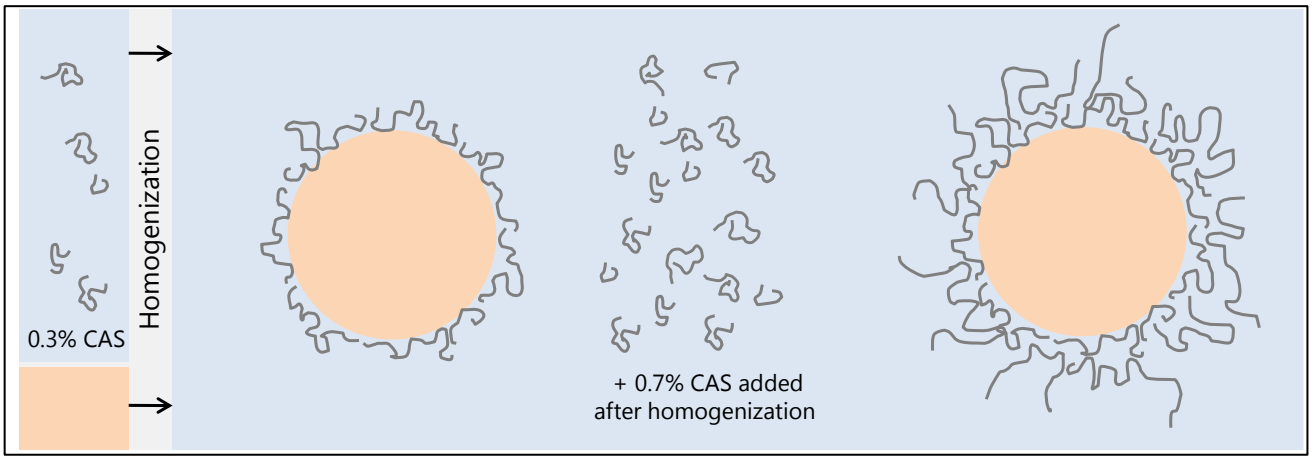

Figure 2. A schematic illustration of the structural differences at the interfacial layer, when CAS is adsorbed during homogenization and added after homogenization to a CAS-deficient oil droplet.

On this background, it was hypothesized that the oxidative stability of CAS stabilized emulsions would depend on the concentration of CAS used for emulsification, and thereby the thickness of the interfacial layer. In addition, that a possible difference in the structure of the interfacial layer (introduced by the manner in which CAS was added to the emulsion) would affect lipid oxidation.

AIM

The aim of the study was to prepare emulsions with similar oil droplet sizes according to the method description by Fang and Dalgleish (1993) with varying concentrations of CAS (0.3, 0.5, 1.0 and 2.0%), and thus varying thicknesses of the interfacial layer. A second aim was to prepare emulsions with a low concentration of CAS (0.3%) and then add more CAS (0.7%) after homogenization to possibly obtain varying protein structures at the interface. Afterwards, to remove the proteins from the aqueous phase in these emulsions by the procedure described by Faraji et al. (2004) to avoid that their antioxidative effects in the aqueous phase would affect the results. Finally, to conduct a storage experiment with these emulsions to determine their oxidative stability, as a result of the thickness and structures of the interfacial layers.

MATERIALS

The materials used were

- Commercial cod liver oil provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway).
- Sodium caseinate (Miprodan®30) donated by Arla Foods Ingredients amba (Viby J, Denmark).
- All other chemicals and solvents used were of analytical grade.

EMULSION PREPARATION

Emulsions were prepared from 20% (absolute w/w) fish oil, and 20 mM imidazole buffer (pH 7) with CAS in concentrations of 0.3, 0.5, 1.0 or 2.0% (absolute w/w). CAS was dissolved in the buffer overnight at approximately 5°C. Prior to homogenization, a premix was prepared by adding the fish oil slowly to the aqueous phase (buffer and protein) during mixing with a hand-held mixer (Tissue tearor, model 985370-395, Biospec Products Inc., Bartlesville, USA). Secondary homogenization was done using a microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75µm).

Even though the method description by Fang and Dalgleish (1993) was followed as precisely as possible, emulsions could not be prepared with similar mean oil droplet sizes. Hence, a range of experiments were carried out to optimize the method. Both the duration of the premixing and the pressure and number of passes on the microfluidizer were varied in order to prepare stable emulsions with 0.3, 0.5, 1.0 and 2.0% CAS, and mean droplet sizes as similar as possible. Droplet size distributions were determined on a Mastersizer (Model MSS, 1998, Serial 33544/394, Malvern Instruments, Ltd., Worcestershire, UK). The emulsion was suspended directly in recirculating buffer (20 mM imidazole).

The emulsions were subjected to a washing procedure as described by Faraji et al. (2004). However, as shown in Figure 3, the oil droplet size distributions became bimodal and the emulsion could not be restored after the washing procedure.

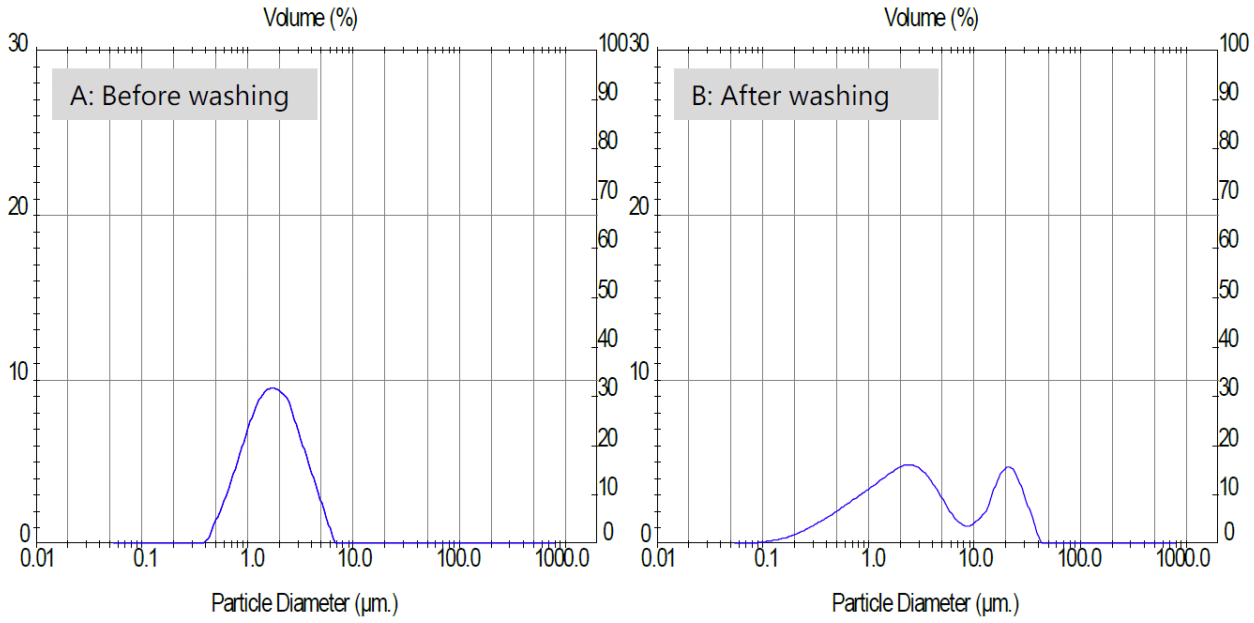

Figure 3. Oil droplet size distributions in an oil-in-water emulsion prepared with 20% fish oil and 0.3% CAS before (A) and after (B) washing.

Several attempts to perform the procedure under less harsh conditions (lower speed and shorter duration of the centrifugation) did not result in emulsions that were suitable for the aim of this study.

Appendix I

THICKNESS OF THE INTERFACIAL LAYER

CONCLUSION AND PERSPECTIVES

Unfortunately, it was not possible to carry out the storage experiment that we aimed for due to instability of the prepared emulsions after the washing procedure. In addition, greater deviations in the mean droplet sizes were obtained for emulsions with different CAS concentrations than those reported by Fang and Dalgleish (1993). Hence, it could not be verified that we had obtained different thicknesses of the interfacial layers.

Knowledge is still lacking on the influence of the thickness of the interfacial layer and the resulting lipid oxidation in emulsions. However, for the moment we do not have any suggestions to another kind of approach that could be used for looking into this matter. A few suggestions to developing and/or improving the presented method, however, do exist. Firstly, the use of casein extracted directly from milk instead of using a sodium salt of casein could maybe improve the stability of the emulsions. Secondly, a less harsh filtration step could possibly replace the centrifugation in the procedure for removing proteins from the aqueous phase.

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Appendix II:
Combinations of Caseins and Phospholipids

BACKGROUND AND HYPOTHESIS

The interface between the oil and the aqueous phase in emulsions is the place of contact between lipids and prooxidative components (McClements and Decker, 2000). Hence, it is hypothesized, that the creation of a thick interfacial layer, which could serve as a physical barrier between the oil and the aqueous phase, would protect the lipids against oxidation.

A change in the thickness and structure of the interfacial layer was suggested by Fang and Dalgleish (1993) when a combination of caseins and lecithins were used as emulsifier as compared to using solely casein. The addition of lecithin was found to enhance the physical stability of emulsions when casein concentration was low (< 0.3%) in 20% emulsions. This was suggested to be due to the creation of protein-phospholipid complexes, and thereby a better coverage of the oil droplet surface by emulsifier. The phospholipids were expected to supplement the caseins at the interface, whereby the caseins could protrude more into the aqueous phase and stretch less over the surface (Fang and Dalgleish, 1993). However, when protein concentrations were ≥ 0.4%, a displacement of protein at the interface rather than a supplementation occured (Courthaudon et al., 1991; Dickinson and Iveson, 1993; Fang and Dalgleish, 1993).

On this background, it was hypothesized, that a change in the thickness and structure of the interfacial layer due to the use of a combination of casein and phospholipids would improve the oxidative stability of the emulsion compared to the use of casein alone.

AIM

The aim of the study was to compare the oxidative stability of emulsions prepared with 0.3, 0.5 or 1.0% CAS to emulsions prepared with similar concentrations of CAS and then additionally 0.5% EggPC in a storage experiment.

MATERIALS

The materials used were

- Commercial cod liver oil provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway).
- CAS, Sodium caseinate (Miprodan® 30) donated by Arla Foods Ingredients amba (Viby J, Denmark).
- EggPC, Phosphatidylcholine from eggs was purchased from Larodan (Larodan Fine Chemicals AB, Malmö, Sweden).
- All other chemicals and solvents used were of analytical grade.

EMULSION PREPARATION

Six emulsions were prepared with 10% w/w fish oil and emulsifier concentrations as stated in Table 1. The buffer used was a 10 mM sodium acetate imidazole buffer (pH 7.0), and CAS was dispersed in the buffer overnight at approximately 5°C. EggPC was dispersed in the oil phase approximately one hour prior to homogenization. Primary homogenization was done by adding the fish oil (with or without EggPC) slowly to the buffer during mixing at 16,000rpm (Ystral mixer, Ballrechten-Dottingen, Germany). The fish oil was added during the first minute of mixing, and the total mixing time was 3 minutes. Secondary homogenization was

done on a microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75µm). Emulsions were homogenized at a pressure of 10,000 psi (69 MPa), running 3 passes. Emulsions were added 100 µM $FeSO_4$ to accelerate lipid oxidation and 0.05% sodium azide to prevent microbial growth.

Table 1. The experimental design with sample code names.

	0.3CAS	0.5CAS	1.0CAS	0.3CASPC	0.5CASPC	1.0CASPC
Concentration of CAS [%]	0.3	0.5	1.0	0.3	0.5	1.0
Concentration of EggPC [%]	0.5	0.5	0.5	0.5	0.5	0.5

Emulsions were stored in 100mL Bluecap bottles at room temperature (20°C ± 0.2) in the dark for 14 days. Samples were taken at day 0, 4, 7, 10 and 14 for lipid oxidation measurements. Measurements of pH, viscosity, and droplet size distributions were carried out at day 1 and 14, whereas zeta potential was determined at day 4. Samples for protein content in the aqueous phase were taken at day 0, and stored at 5°C until centrifugation at day 7.

CHARACTERIZATION OF THE EMULSIONS

PH, VISCOSITY, ZETA POTENTIAL AND DROPLET SIZE

The pH values of emulsions were measured at room temperature directly in the sample during stirring (pH meter, 827 pH Lab, Methrom Nordic ApS, Glostrup, Denmark).

Viscosities of the emulsions (15 mL) were measured using a stress controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden) equipped with a CC25 standard bob cup system in a temperature vessel. Measurements were done at 20°C over a shear stress range from 0.0125 to 1.64Pa. Viscosities are given as the average viscosity of the linear part of the plot of shear stress vs viscosity expressed in mPa·s. Viscosities were measured twice on each emulsion.

Zeta potentials were measured in a Zetasizer (Zetasizer Nano ZS, Malvern instruments Ltd., Worcestershire, UK) with a DTS-1060C cell. Before analysis, the emulsions were diluted 2:1000 in 10mM sodium acetate imidazole buffer (pH 7). Samples were analyzed with 100 runs and measurements were done in triplicate.

Droplets size distributions were measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). Emulsion droplets were diluted in recirculating water (3000rpm), reaching an obscuration of 13-18%. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively.

PROTEINS IN THE AQUEOUS PHASE

Samples (approximately 35g) were centrifuged at 45,000g for 50 min at 10°C (Sorvall RC-6 PLUS, Thermo Fisher Scientific, Osterode, Germany). The lower aqueous phase was extracted with a syringe and needle, before centrifuging once more at 70,000g for 60 min at 15°C (Beckman ultracentrifuge L8-60M, Fullerton, CA, USA). Once more the lower aqueous phase was extracted and then filtered (Minisart NML 0.20µm filter, Sartorius, Hannover, Germany). The protein content in the aqueous phase was determined with a BCA Protein Assay Reagent Kit (Pierce, Thermo Scientific, Rockford, IL, USA). Prior to analysis samples were diluted 1:9 in 10 mM sodium acetate imidazole buffer (pH 7). Measurements were performed in duplicate.

MEASUREMENTS OF LIPID OXIDATION

PEROXIDE VALUES

A lipid extract was prepared from each emulsion according to the method described by Bligh and Dyer (1959) using 10g emulsion and a reduced amount of solvent (30.0ml methanol and chloroform, 1:1). Peroxide values were subsequently determined on this lipid extract by colorimetric determination of iron thiocyanate at 500nm as described by Shantha and Decker (1994).

VOLATILE SECONDARY OXIDATION PRODUCTS

Approximately 4 g of emulsion, 30 mg internal standard (4-methyl-1-pentanol, 30 µg/g water) and 2 mL antifoam (Synperonic 800 µL/L water) was weighted out in a 100 mL purge bottle. The bottle was heated in a water bath at 45°C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile secondary oxidation products were trapped on Tenax GR tubes. The volatiles were desorbed again by heat (200°C) in an Automatic Thermal Desorber (ATD-400, Perkin Elmer, Norwalk, CN), cryofocused on a cold trap (-30°C), released again (220°C), and led to a gas chromatograph (HP 5890IIA, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m x 0.25 mm x 1.0 µm; J&W Scientific, CA, USA). The oven program had an initial temperature of 45°C for 5 min, increasing with 1.5°C/min until 55°C, with 2.5°C/min until 90°C, and with 12.0°C/min until 220°C, where the temperature was kept for 4 min. The individual compounds were analyzed by mass-spectrometry (HP 5972 mass-selective detector, Agilent Technologies, Palo Alto, CA, USA; Electron ionisation mode, 70 eV; mass to charge ratios between 30 and 250). From a comparison of chromatograms from non-oxidised and oxidised samples, the following volatiles were selected for quantification: butanal, pentanal, 2-penten-1-ol, 1-penten-3-ol, 1-penten-3-one, hexanal, 2,4-hexadienal, 2-hexenal, heptanal, 4-heptenal, 2,4-heptadienal and 2,6-nonadienal. In the chromatograms two peaks were identified as 2,4-heptadienal. From previous studies of these two peaks (not published) it is anticipated that they represent the two isomers *t,c*-2,4-heptadienal and *t,t*-2,4-heptadienal. Calibration curves were made from dissolving the compounds in 96% ethanol and diluting to concentrations of approximately 0.01, 0.025, 0.05, 0.1, 0.25 and 0.5mg/g. These solutions were injected (1µL) directly on the Tenax GR tubes (in triplicate) using a small syringe (Hamilton syringe 7105N, Bonaduz, Switzerland). Ethanol was subsequently removed by nitrogen (purge flow 50mL/min, 5 min). The samples for calibration curves were run similarly to the emulsion samples on the GC-MS.

STATISTICS

All data (except pH and volatiles) were analysed by one or two-way analysis of variance with Bonferronis multiple comparison test as post test (GraphPad Prism, version 4.03, GraphPad Software Inc). All references to significant differences ($p < 0.05$) between samples or between sampling times, are based on this statistical analysis of data.

RESULTS AND DISCUSSION

The pH values of the emulsions ranged from 7.1-7.3 as stated in Table 2. At low CAS concentrations (0.3 and 0.5%) a less negative zeta potential was observed in emulsions with EggPC added. Emulsions with 1.0% CAS (1.0CAS and 1.0CASPC) did not differ (Table 2). Emulsions with the highest concentrations of CAS (1.0CAS and 1.0CASPC) had significantly higher viscosities than emulsions with 0.3 or 0.5% CAS (Table 2). However, emulsions with similar concentrations of CAS with or without EggPC did not differ significantly. The higher viscosities in 1.0CAS and 1.0CASPC compared to the other samples were most likely due to the higher concentration of protein in the aqueous phase, as shown in Table 2.

Table 2. Physico-chemical data for 10% oil-in-water emulsions prepared with different emulsifiers. Refer to Table 1 for sample code names.

	pH	Viscosity [mPa·s]	Zeta potential [mV]	Protein in the aqueous phase [mg/mL]
0.3CAS	7.3	2.98 ± 0.04 ^{a}	-40.8 ± 1.7 ^{a}	0.41 ± 0.06 ^{a}
0.5CAS	7.1	3.00 ± 0.02 ^{a}	-39.6 ± 0.7 ^{ab}	1.03 ± 0.04 ^{b}
1.0CAS	7.2	3.27 ± 0.02 ^{bc}	-41.3 ± 1.6 ^{a}	1.78 ± 0.00 ^{d}
0.3CASPC	7.2	3.09 ± 0.08 ^{ab}	-35.8 ± 0.5 ^{b}	0.68 ± 0.04 ^{a}
0.5CASPC	7.1	3.14 ± 0.02 ^{ab}	-29.9 ± 0.8 ^{c}	1.35 ± 0.01 ^{c}
1.0CASPC	7.1	3.35 ± 0.05 ^{c}	-38.7 ± 2.2 ^{ab}	2.14 ± 0.12 ^{e}

Results for pH and viscosity are only reported from measurements at day 1. pH was not changing during storage, and only the viscosity of 0.3CAS increased significantly between day 1 and 14. Letters refer to significant differences between samples ($P<0.05$).

An increase in the concentration of CAS significantly increased the concentration of protein in the aqueous phase both in emulsions with and without EggPC. A comparison of samples with similar concentrations of CAS showed that at a CAS concentration of 0.3% addition of EggPC did not change the protein concentration in the aqueous phase, whereas the concentration of protein increased significantly upon addition of EggPC to emulsions with 0.5 or 1.0% CAS (Table 2). At the lowest concentration of CAS (0.3%) the mean oil droplet size increased during the 14 days of storage (Figure 1). Hence, a total coverage of the interface by proteins had most likely not been obtained.

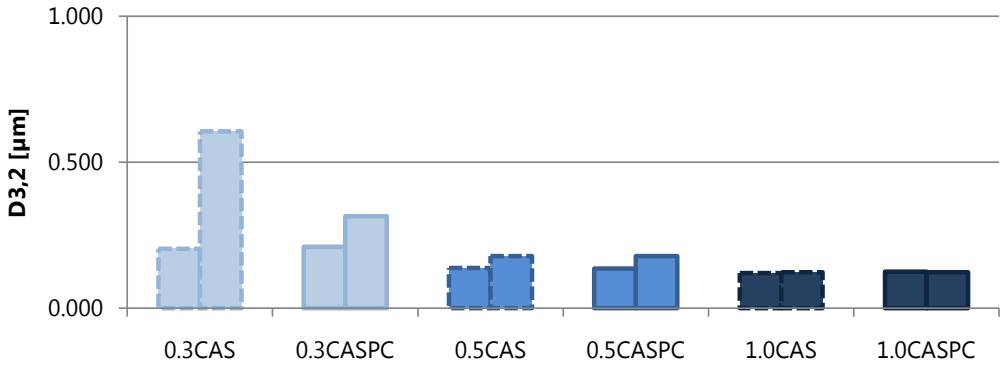

Figure 1. Mean oil droplet sizes ($D_{3,2}$) in 10% oil-in-water emulsions measured at day 1 (left bars for each emulsion) and day 14 (right bars for each emulsion). Refer to Table 1 for sample code names.

The addition of EggPC reduced this increase. However, as the protein concentration in the aqueous phase of the two emulsions did not differ significantly (Table 2), a combination of CAS and EggPC must have been present at the interfacial layer. Whether the increase observed in the oxidative stability (illustrated by PV and the development in concentrations of 1-penten-3-ol in Figure 2) from using a combination of CAS and EggPC was a result of an overall improved physical stability of the emulsion or a change in the thickness of the interfacial layer cannot be concluded from the present results.

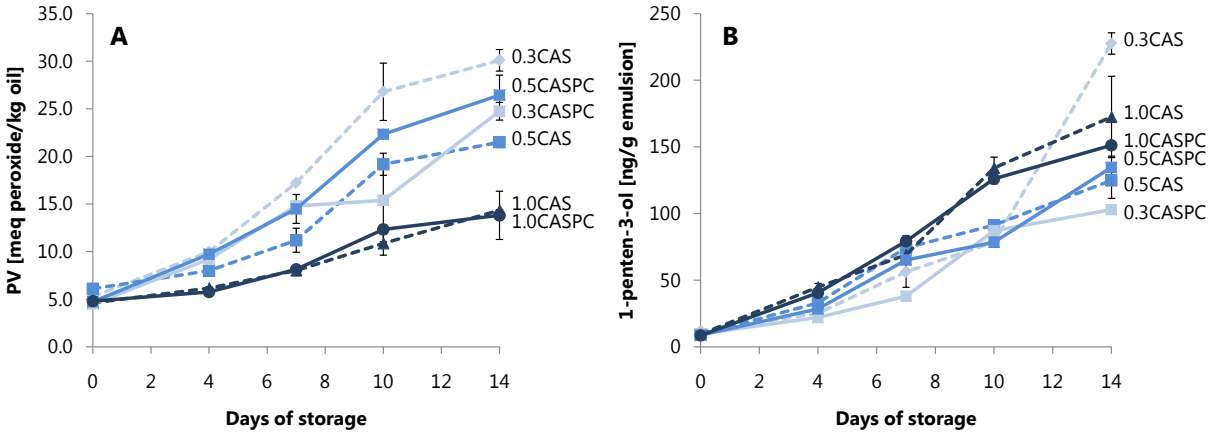

Figure 2. Peroxide values (A) and concentrations of 1-penten-3-ol (B) in oil-in-water emulsions during storage for 14 days. Refer to Table 1 for sample code names.

Interestingly and in contrast to our hypothesis, emulsions with a concentration of 0.5% or 1.0% CAS as the sole emulsifier were more or similarly stable towards oxidation as the corresponding emulsions with EggPC added (Figure 2). In both cases the protein concentration in the aqueous phase was higher in the emulsions with EggPC added (Table 2) whereas no change in droplet sizes was observed (Figure 1). Hence, it is suggested that part of CAS at the interface was substituted by EggPC in both cases. This was in accordance with the suggestion by Fang and Dalgleish (1993). However, in contrast to our hypothesis, a possible protein-phospholipid complex did not increase the oxidative stability in these emulsions. The difference in the effect of EggCAS on the oxidative stability of emulsions with 0.5% CAS and 1.0% is speculated to be related to the antioxidative potential of the higher total concentration of CAS in the aqueous phase in the latter (Faraji et al., 2004;Ries et al., 2010). This high concentration of CAS in the aqueous phase could compensate for the lower protection of the interfacial layer shown to occur when CAS was substituted by EggPC in the 0.5% CAS emulsion.

CONCLUSION AND PERSPECTIVES

The use of a combination of CAS and EggPC was only preferential when the concentration of CAS alone was insufficient for a full coverage of the interface by proteins. At higher concentrations of CAS, a similar or lower oxidative stability was obtained when a combination of CAS and EggPC was used, as opposed to using CAS alone.

The focus in this study was on the physical change in the thickness of the interfacial layer by using a combination of casein and egg-phosphatidylcholine. In the future, it could be interesting to further evaluate the antioxidative properties of other phospholipids, such as phosphatidylinositol or phosphatidyl-ethanolamine, both alone, and in combinations with milk proteins as emulsifiers in emulsions, as these most likely display different antioxidative properties.

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Appendix III:
Emulsions Prepared by Membrane Homogenization with Monomodal Oil Droplet Size Distributions

BACKGROUND AND HYPOTHESIS

In emulsions, the contact between lipids and prooxidants occur at the interface between the oil and the aqueous phase (McClements and Decker, 2000). Thus, lipid oxidation is to a great extent considered an interfacial phenomenon. Emulsions with large oil droplets have a smaller total surface area exposed to prooxidants in the aqueous phase than emulsions with smaller droplets. Hence, it is hypothesized that emulsions with larger oil droplets are less susceptible to lipid oxidation than emulsions with smaller droplets (McClements and Decker, 2000). However, conclusions on the relation between oil droplet size and lipid oxidation are not clear. This is at least partly due to the fact that obtaining different droplet sizes often requires that other parameters are varied. These parameters might influence lipid oxidation themselves, as e.g. the type and concentration of emulsifier used or the homogenization conditions.

In both mayonnaise and BSA oil-in-water emulsions, lipid oxidation was observed to progress faster in smaller droplets than in larger ones in the initial part of the storage period, whereas no dependence of droplet size on lipid oxidation was observed in the later part of the storage period (Jacobsen et al., 2000; Lethuaut et al., 2002). In accordance with the above-mentioned, a study on emulsions prepared with caseinate or Tween20 showed that an increase in the oil volume fraction and a concomitant increase in the oil droplet size resulted in a better oxidative stability of the emulsions (Kargar et al., 2011). The authors explained these results by the smaller total surface area exposed to iron in the aqueous phase, when droplet size increased. In contrast, other studies have shown no correlation between oil droplet size and lipid oxidation (Gohtani et al., 1999; Hu et al., 2003; Sun and Gunasekaran, 2009).

Most of the studies mentioned here obtained different oil droplet sizes by varying the emulsifier type or concentration, the oil volume fraction or the homogenization conditions. Hence, lipid oxidation might in these studies not only be influenced by oil droplet size, but rather by a combination of factors determining the macrostructure of the emulsion. To investigate the influence of oil droplets on lipid oxidation it would be beneficial to have as narrow droplet size distributions as possible and to decrease the overlap in these distributions between emulsions that are compared.

A membrane homogenizer was purchased for this project, for the purpose of preparing emulsions with monomodal droplet size distributions with varying mean oil droplet sizes. This should be done by using membranes with different pore sizes (Nakashima et al., 1991b).

AIM

The aim of the study was to prepare 5% oil-in-water emulsions by membrane homogenization. These emulsions should be prepared with different monomodal droplet size distributions using protein as the emulsifier. Subsequently, a storage experiment should be conducted to determine the oxidative stability of the emulsions in dependence of the oil droplet size.

Appendix III

OIL DROPLET SIZE - MEMBRANE HOMOGENIZATION

The materials used were

- Commercial rapeseed oil bought locally.
- Commercial cod liver oil provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway).
- Whey protein isolate (Lacprodan®DI-9224) donated by Arla Foods Ingredients amba (Viby J, Denmark).
- Tween®20, Calbiochem, Merck KGaA, Darmstadt, Germany.
- All other chemicals and solvents used were of analytical grade.

EMULSION PREPARATION AND MEASUREMENTS OF DROPLET SIZE DISTRIBUTIONS

Emulsions were prepared by a membrane homogenizer (High speed minikit, SPG Technology CO., LTD., Miyazaki, Japan) equipped with a SPG membrane module (Diameter 10mm and length 125mm). The membranes used were all hydrophilic with pore sizes of 0.1, 1.1 and 10.1µm. The pressure used was varied depending on the emulsifier and the membrane pore size. More than 30 experiments were conducted and more than 100 different emulsions were prepared, with the aim of preparing appropriate emulsions for the aim of this study. A range of different emulsifiers or emulsifier combinations with varying properties were tested, namely whey protein isolate (WPI), Tween20, soy lecithin (LEC), Tween80, milk phospholipid PL20, sodium dodecyl sulphate, polyglycerol ester (PGE) and monoglycerid (MG). In this appendix only the work on WPI and Tween20 emulsions will be reported.

Droplet size distributions were determined in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) by laser diffraction. The emulsion was suspended directly in recirculating buffer (10mM sodium acetate imidazole; 3000rpm, 12-14% obscuration). The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively.

RESULTS ON WPI EMULSIONS

A summary of the experimental approaches to obtain oil-in-water emulsions with monomodal droplet size distributions with WPI is given in Table 1. Where nothing else is stated the emulsions were prepared with 5% rapeseed oil at pH 7, and by the use of a 1.1µm membrane.

Firstly, two different membranes were used for comparison. However, emulsions could not be obtained with the 10.1µm membrane. Hence, it was decided to continue the experiments with the 1.1µm membrane.

Table 1. Summary of selected experiments on the preparation of WPI emulsions by membrane homogenization.

Parameter investigated	Concentration of WPI used [%]	Conclusions
Influence of membrane pore size (1.1µm vs 10.1 µm)	0.2	It was not possible to prepare emulsions with the 10.1µm membrane. The emulsion prepared with the 1.1µm membrane were creaming shortly after production.
Concentration of WPI	0.2, 0.5, 1.0, 2.0, 3.0, 5.0	Emulsions were creaming, but no coalescence was observed during 2 days of storage. Emulsions had broad droplet size distributions as shown for selected emulsions in Figure 1.
Concentration of WPI at pH 3	0.2, 0.5, 1.0, 2.0, 3.0	Emulsions were creaming with a tendency to coalesce. Droplet size distributions in Figure 2.
Concentration of WPI with fish oil as the oil phase	0.025, 0.1, 0.5, 1.0, 2.0	Large oil droplets. Emulsions were creaming. Droplet size distributions are shown in Figure 3.
Heating of WPI prior to emulsification	0.5	No effect of heating
Continuously addition of WPI to the water phase during emulsification	0.75 to 2.0	No effect of a continuously addition of WPI to the recirculating water phase.

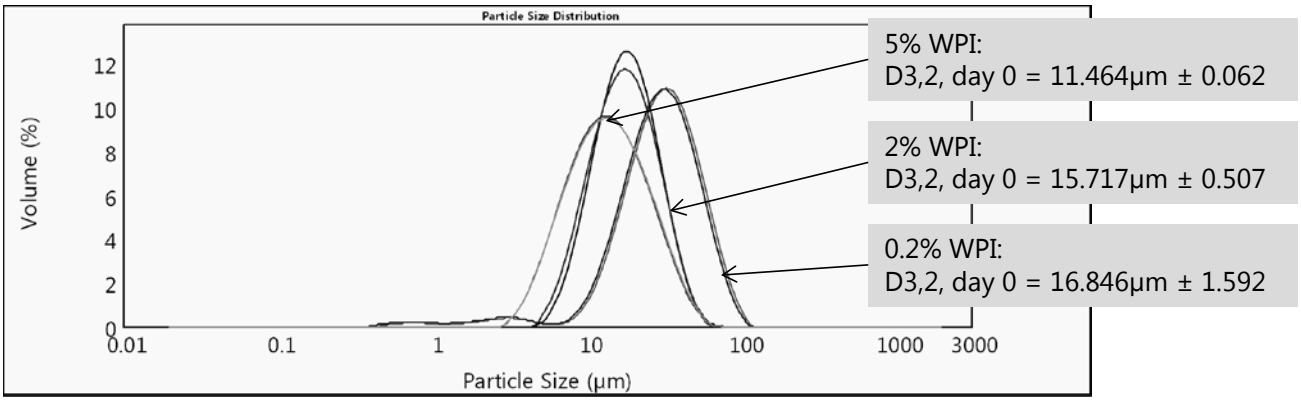

Figure 1. Oil droplet size distributions in oil-in-water emulsions prepared with 5% oil and 0.2, 2 or 5% WPI. Emulsions were prepared at pH 7 with a 1.1µm membrane. Mean oil droplet sizes decreased with increasing WPI concentration.

From the experiments on varying the concentration of WPI in the water phase, it was obvious that smaller mean droplet sizes could be obtained when the concentration of WPI was increased (Figure 1). However, emulsions had very broad droplet size distributions and were creaming shortly after production.

A decrease in pH increased the mean oil droplet size when emulsions were prepared with a low concentration of WPI (Figure 2). At high concentrations only slight differences were observed in mean droplet sizes compared to emulsions at pH 7, however the distributions were broader.

Emulsions prepared with fish oil instead of rapeseed oil did not give any better results (Figure 3). Actually the mean oil droplet sizes were larger as compared to the emulsions prepared with rapeseed oil at pH 7 (Figure 1).

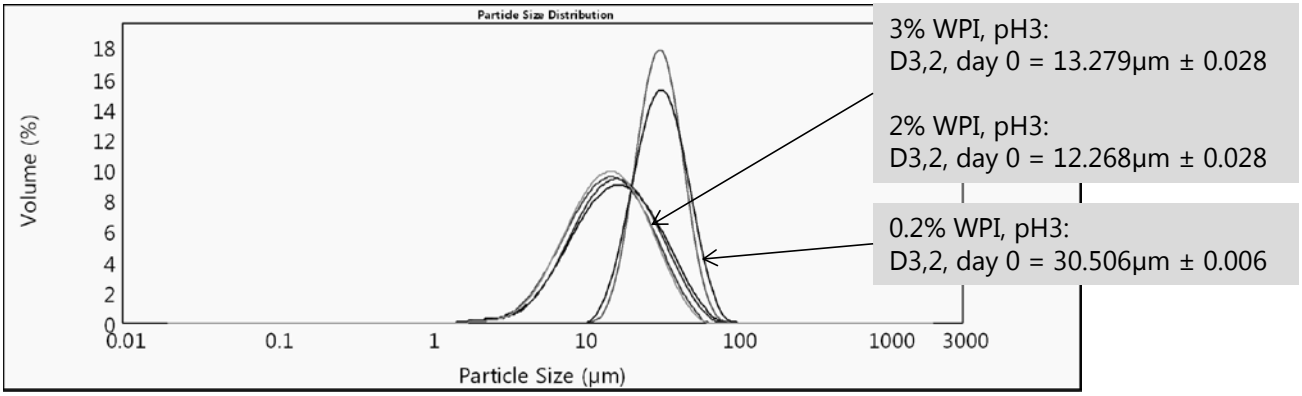

Figure 2. Oil droplet size distributions in oil-in-water emulsions prepared with 5% oil and 0.2, 2 or 3% WPI. Emulsions were prepared at pH 3 with a 1.1µm membrane. Mean oil droplet sizes decreased with increasing WPI concentration, but the distribution was broadened.

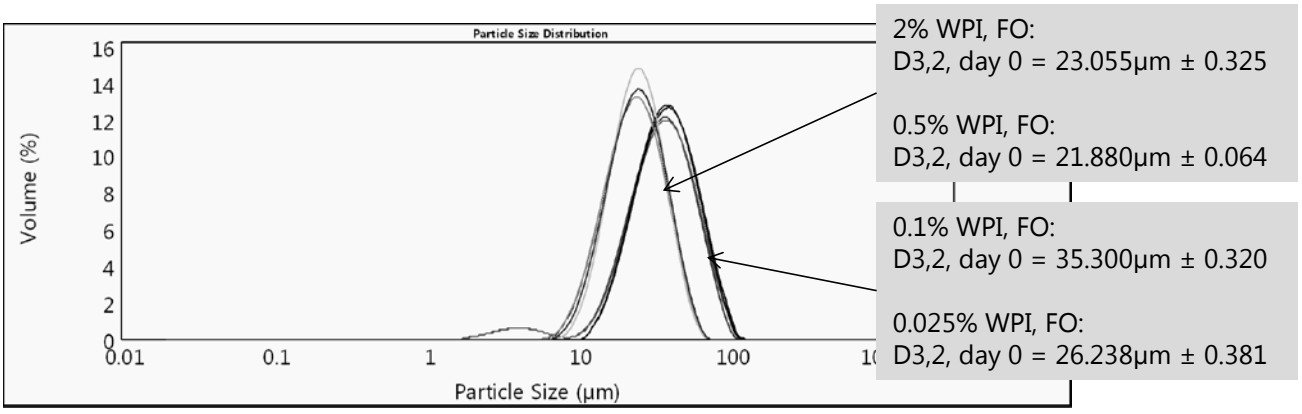

Figure 3. Oil droplet size distributions in oil-in-water emulsions prepared with 5% rapeseed oil and 0.025, 0.1, 0.5 or 23% WPI. Emulsions were prepared at pH 7 with a 1.1µm membrane.

To increase the emulsifying capacity of WPI the effect of heating and a slow addition of WPI to the circulating water phase were tried. However, this did not give any better results with regards to mean oil droplet size and more narrow distributions. Emulsions were also prepared with combinations of WPI and LEC, PGE or MG, but none of these emulsions gave satisfactory results either.

As illustrated in Figures 1-3, all WPI emulsions had much larger mean oil droplet sizes and broader distributions than what was expected from the description of the capabilities of the membrane homogenizer (Nakashima et al., 1991b), and studies by Nakashima et al. (1991a). These authors reported a linear relationship between pore size and oil droplet size, with oil droplets being 3.25 times larger than the pore size. Hence, the emulsions prepared with WPI could not be used for the aim of this study.

RESULTS ON TWEEN20 EMULSIONS

A series of experiments was therefore carried out with Tween20 as emulsifier. In contrast, to emulsions prepared with WPI these emulsions gave much narrower distributions and smaller mean oil droplet sizes. The experiments with Tween20 are summarized in Table 2. All emulsions were prepared at pH 7, and where nothing else is stated the emulsions were prepared with 5% oil and by the use of a 1.1µm membrane.

Table 2. Summary of selected experiments on the preparation of Tween20 emulsions by membrane homogenization. RO: Rapeseed oil; FO: Fish oil.

Parameter investigated	Concentration of Tween20 used [%]	Conclusions
FO vs RO oil	2.0%	No difference in the obtained droplet size distributions.
Concentration of Tween20 and membrane pore size (FO/RO)	Dependent on the pore size of the membrane 0.1µm and 0.5µm: 0.1, 0.5, 1.0, 2.0, 3.0 1.1µm: 0.025, 0.1, 0.5, 1.0, 2.0 10. 1µm: 0.025, 0.05, 0.1, 0.5, 1.0	It was not possible to prepare emulsions with the 0.1µm membrane. Emulsions prepared with the 0.5µm membrane were best at a Tween20 concentration of 1%. The concentration of Tween20 did not affect the oil droplet sizes in emulsions prepared with the 10.1µm membrane. Emulsions were creaming, but no coalescence occurred over 8-15 days of storage. Selected droplet size distributions are shown in Figure 4.
Concentration of the oil phase (2.5, 5, 10 or 20% FO), when the emulsifier to oil ratio was kept constant at 2:5.	Dependent on the oil concentration 1.0, 2.0, 4.0, 8.0	The oil and emulsifier concentrations did not influence the oil droplet size distributions, as long as the ratio between them was constant. Droplet size distributions are shown in Figure 5.

In contrast to emulsions prepared with WPI, emulsions prepared with rapeseed oil and fish oil was shown not to differ when prepared with Tween20 as emulsifier (Data not shown). As observed from droplet size distributions in Figure 4 much smaller mean oil droplet sizes and much narrower distributions were obtained with this emulsifier as compared to the emulsions prepared with WPI (Figure 1).

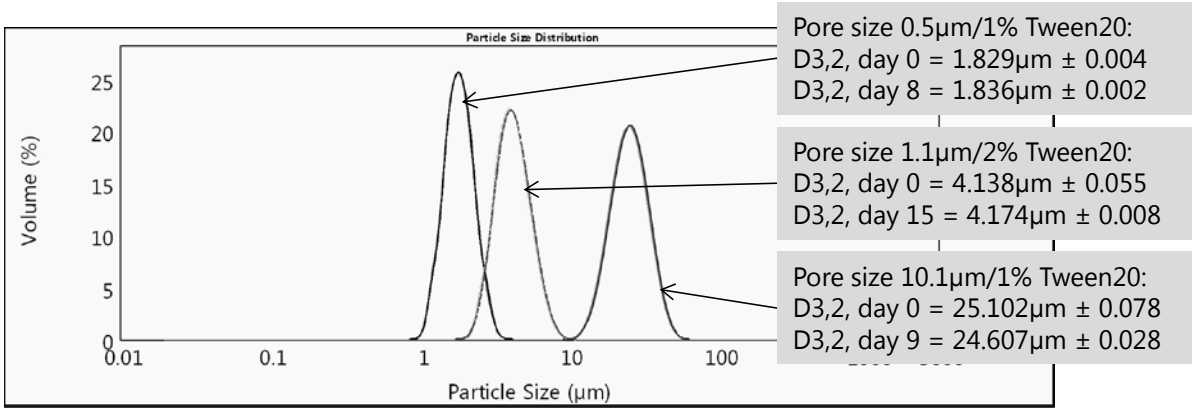

Figure 4. Oil droplet size distributions for emulsions prepared with 5% oil and 1 or 2% Tween 20. Emulsions were prepared at pH 7 with a 0.5, a 1.1 or a 10.1µm membrane. Oil droplet sizes did not change during storage.

It was also shown that the oil and emulsifier concentration could be varied, and similar oil droplet size distributions be obtained, as long as the protein to oil ratio was kept constant (Figure 5).

In general, results from the preparation of emulsions with Tween20 showed that emulsions could be obtained with very narrow droplet size distributions. Using the membrane with pore size of 0.5µm, 1.1µm or 10.1µm emulsions were prepared with mean oil droplet sizes of 1.83µm, 4.17µm or 21.10µm, respectively. Emulsions could not be produced with the membrane having a pore size of 0.1µm. All emulsions were creaming during storage, but no coalescence occurred for up to 15 days of storage.

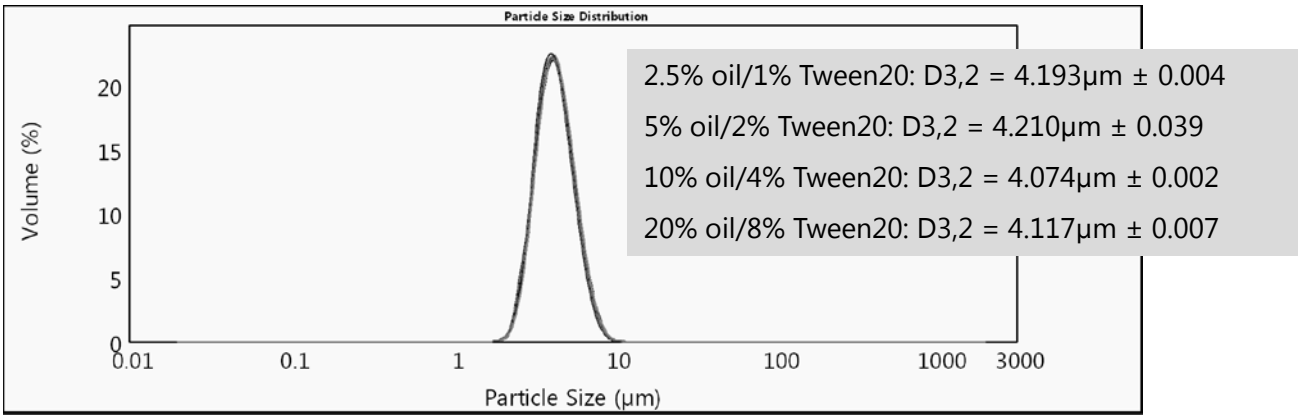

Figure 5. Oil droplet size distributions for emulsions prepared with 2.5, 5, 10 and 20% fish oil and correspondingly 1.0, 2.0, 4.0 or 8.0% Tween20. Emulsions were prepared at pH 7 with a 1.1µm membrane.

In Figure 6, oil droplet size distributions for emulsions prepared with Tween20 and WPI by the use of a membrane homogenizer is compared to emulsions prepared with Tween20 on two types of high pressure homogenizers, namely a two-stage valve homogenizer and a microfluidizer. Obviously, emulsions with Tween20 had much narrower oil droplet size distributions when prepared by the use of a membrane homogenizer than when prepared by the use of high pressure homogenizers. However, WPI emulsions could not be obtained with these narrow droplet sizes distributions. We anticipate that the emulsions could not be produced with WPI because the circulation flow of the aqueous phase in the membrane system was too high, and whey proteins require more time to get to the surface than what could be provided in this system.

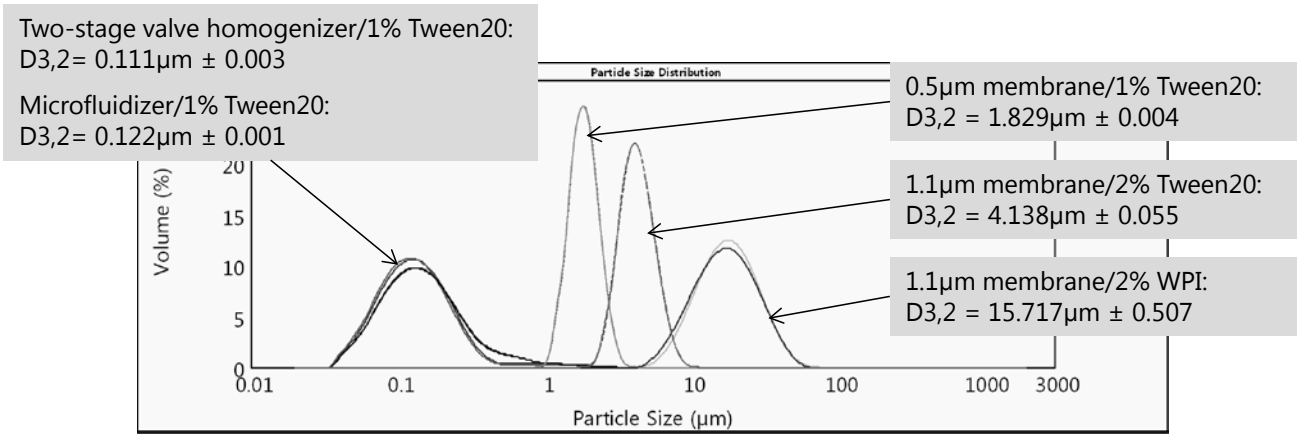

Figure 6. Oil droplet size distributions in emulsions prepared by a membrane homogenizer, a 2-stage valve homogenizer or a microfluidizer, with varying oil droplet sizes and width of their distributions. All emulsions were prepared with 5% oil.

CONCLUSION AND PERSPECTIVES

Unfortunately, it was not possible to prepare WPI stabilized emulsions with monomodal droplet size distributions for the aim of this study by the membrane homogenizer. However, emulsions with Tween20 were prepared with satisfying droplet size distributions, and studies of the influence of oil droplet size on lipid oxidation should be conducted by the use of this emulsifier in the future.

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PAPER I

Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH

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European Journal of Lipid Science and Technology 113:1243-1257

Research Article

Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH

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The objective of this study was to evaluate the protective effects of five different emulsifiers on lipid oxidation in 70% fish oil-in-water emulsions to be used as delivery systems for long chain polyunsaturated omega-3 fatty acids to foods. The emulsifiers were either phospholipid (PL) based or protein based. The PL-based emulsifiers were soy lecithin and two milk PL concentrates (with either 20 or 75% PL). The protein-based emulsifiers were whey protein isolate and sodium caseinate. Lipid oxidation was studied at two pH values (pH 4.5 and 7.0) and results were compared to lipid oxidation in neat fish oil. Lipid oxidation was followed by determination of peroxide values and volatile oxidation products. Emulsions were furthermore imaged by confocal and cryo-scanning electron microscopy. Results showed that emulsions prepared at high pH with proteins oxidized less than or equally to neat oil, whereas, all other emulsions oxidized more. In addition, there was a tendency toward a faster progression in lipid oxidation at low pH compared to high pH for emulsions prepared with protein-based emulsifiers. The opposite was observed for emulsions prepared with PL-based emulsifiers. Hence, at low pH PL-based emulsions may be more suitable as delivery systems than protein-based emulsions. Moreover, the quality of the PL-based emulsifiers seemed to affect lipid oxidation.

Practical applications: Results from the present study give an insight into the physical and oxidative stability of 70% fish oil-in-water emulsions prepared with whey protein isolate, sodium caseinate, milk phospholipids, or soy lecithin. The emulsions can be used as delivery systems for fish oil to foods. However, only emulsions prepared with proteins at high pH offered advantages with respect to better oxidative stability during storage compared to neat fish oil. Thus, when fish oil is added to a food product in a delivery emulsion, the type of emulsion used should be carefully considered.

Keywords: Delivery system / Lipid oxidation / n-3 PUFA / Phospholipids / Proteins

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1 Introduction

Despite the steadily growing body of evidence supporting health beneficial effects of long-chain polyunsaturated omega-3 fatty acids [1], a concomitant increase in fish intake has not occurred in the Western populations. Therefore, incorporation of marine oils into foods has gained an increased interest during the last decade. However, the highly unsaturated lipids present in fish oils are prone to lipid oxidation. The addition of these healthy lipids to food products is therefore limited by the development of unpleasant off-flavors. Hence, efficient strategies for protecting the fish oil, when

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Abbreviations: cryo-SEM, cryo-scanning electron microscopy; **MPL,** milk phospholipid; **PC,** phosphatidylcholine; **PE,** phosphatidylethanolamine; **PL,** phospholipid; **SFA,** saturated fatty acids; **CAS,** sodium caseinate; **SM,** sphingomyelin; **LEC,** soy lecithin; **WPI,** whey protein isolate

added to food products, are necessary in order to make fish oil enriched foods successful in the marketplace.

In an attempt to increase the oxidative stability of fish oil enriched food products, several studies have been carried out comparing the effect of adding the fish oil as neat oil or as a delivery emulsion prepared with milk proteins as the emulsifier. However, the success of using an emulsion as a delivery system has been shown to depend on the food system to which the fish oil is added. In fish oil enriched yoghurt and salad dressing the addition of neat oil gave the most oxidatively stable food product, whereas, in milk, cheese, and energy bars the most oxidatively stable food product was obtained when the fish oil was added as an emulsion [2–4]. Thus, a better understanding of lipid oxidation in the delivery emulsions themselves could be valuable in order to understand the differences observed between food systems.

A large number of studies have been performed on lipid oxidation in emulsions containing up to 40% oil and particularly on emulsions with 5–20% oil. However, oxidation studies on simple emulsions prepared with as much as 70% oil are to the author's knowledge non-existing. This is despite the obvious advantage of having as high an oil content and as low a water content as possible in the emulsion, when the purpose of its use is as a delivery system, particularly in food products with a low water content.

In both neat oil and emulsions the presence of oxygen and iron can promote lipid oxidation [5]. In emulsions it is therefore crucial to choose an emulsifier with appropriate physicochemical properties for providing both physical and oxidative stability to the emulsion.

In the food industry milk protein-based emulsifiers such as caseins and whey proteins are commonly used. In emulsions milk proteins can easily adsorb to the oil droplets, due to more or less hydrophobic regions in their amino acid structure. However, caseins and whey proteins create different thicknesses of the interfacial layer [6]. Protein-based emulsifiers may also increase the viscosity of the interfacial layer and the surrounding water phase, and thereby restrict the penetration and mobility of prooxidants into the oil [7]. In addition, the milk proteins are charged at pH values above and below their isoelectric point. Thus, at low pH where the surface charge of the proteins is positive a repulsion of the cationic transition metal ions is expected to take place [8]. Finally, the different amino acid residues in milk proteins have also been suggested to possess metal chelating effects [9] or free radical scavenging properties [10].

Compared to proteins, phospholipids (PL) will behave differently at the interfacial layer. PL consists of a hydrophilic head group and a lipophilic tail group, with the latter extending into the emulsion oil droplet. Excess PL that are not associated with the oil–water interface can also lead to the formation of micelles in the continuous phase [11]. Surfactant micelles have been hypothesized to influence lipid oxidation either by increasing the partitioning of lipid hydroperoxides out of emulsion droplets [12] or by influencing the physical location of prooxidant transition metal ions [13]

Besides the possible antioxidative properties of PL in micelles, PL at the interface might also possess antioxidant activity. However, the mechanisms by which PL act are still under debate, possibly because the antioxidant mechanisms differ between different PL classes [14–17]. Furthermore, oxidation studies have been carried out over a wide range of temperatures, which might partly explain the different results on their antioxidant properties. One mechanism that has been studied by several research groups is the ability of PL to work synergistically with tocopherols [14, 15, 18]. Some PL, such as phosphatidylethanolamine (PE), has also been proposed to possess antioxidant activity in themselves, by their ability to interact with free radicals [19]. Furthermore, the successful use of PL as antioxidants in oils or emulsions has been shown to depend on the fatty acid composition of both the oil and on the individual PL used [15, 17, 20].

In addition to the physicochemical properties of the individual emulsifiers, the oil droplet size in the emulsions is also expected to influence lipid oxidation. Thus, small droplets are hypothesized to oxidize more easily than larger ones, due to a larger total surface area, and thereby an increased contact area between prooxidative metal ions in the water phase and lipid hydroperoxides present at the oil–water interface [5]. Nevertheless, previous studies have shown varying results, with some studies confirming the hypothesis [21] and some showing that droplet size might not be as important as, e.g., the emulsifier composition at the interface [22, 23].

Based on this, the hypothesis was that PL-based and protein-based emulsifiers would behave differently at the interfacial layer, and thereby affect lipid oxidation differently. Furthermore, it was hypothesized, that pH, protein concentration, and the composition of the different PL would influence lipid oxidation in emulsions. The aim of this study was therefore to evaluate the protective effect of five different food grade emulsifiers against lipid oxidation in 70% fish oil-in-water emulsions and to compare lipid oxidation in the emulsions with that in neat oil. An additional aim was to study and visualize possible differences in the microstructure by applying confocal microscopy and scanning electron microscopy (cryo-SEM). The emulsifiers chosen were two milk proteins, sodium caseinate (CAS) and whey protein isolate (WPI) and three PL-based emulsifiers, a soy lecithin (LEC) and two milk phospholipids (MPL) containing approximately 20 and 75% of PL (MPL20 and MPL75, respectively). Since the food products used for fish oil enrichment vary over a wide pH range, the effect of pH was also studied, by preparing emulsions at both a low pH (4.5) corresponding to the pH of yoghurt and at a high pH (7.0), corresponding to the pH of milk. Furthermore, for CAS, the effect of different protein concentrations was studied.

2 Materials and methods

2.1 Materials

Commercial cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway) and stored at -40°C until use. The fatty acid content of the major fatty acids was (in %) as follows: 16:0 9.2, 16:1(n-7) 8.3, 18:1(n-9) 16.7, 18:2(n-6) 1.9, 21:1(n-9) 10.6, 20:5(n-3) 9.2, 22:1(n-11) 5.8, and 22:6(n-3) 12.4, as determined by the method described in Section 2.3. The initial PV and tocopherol content was <0.1 meq peroxides/kg oil and approximately 200 mg α-tocopherol/kg, respectively. CAS (Miprodan® 30), WPI (Lacprodan® DI-9224), and MPLs (Lacprodan® PL-20, and Lacprodan® PL-75 MPL concentrate) were kindly donated by Arla (Arla Foods Ingredients amba, Viby J, Denmark). On data sheets from Arla protein contents of 93.5% in CAS, 92% in WPI, 53.8% in MPL20, and 3.1% protein in MPL75 were reported. Furthermore, MPL20 was reported to contain 22.6% PL, and MPL75 to contain 76% PL, mainly sphingomyelin (SM), phosphatidylcholine (PC), and PE in both. LEC (SolecTM E-40-B) was donated by The Solae Company (Århus, Denmark). The data sheet from the Solae Company reported a PL content of <56% (as acetone insolubles). The PL were further characterized as described in Section 2.3. All other chemicals and solvents used were of analytical grade.

2.2 Preparation of emulsions and sampling

In general, the emulsions consisted of 70.0% fish oil, 2.8% emulsifier, and 27.2% 10 mM sodium acetate–imidazole buffer (refer to Table 1 for sample codes), corresponding to an emulsifier to oil ratio of 1:25. The only exceptions were two of the emulsions prepared with CAS, where only 1.4% emulsifier was used and consequently they contained more buffer (28.6%). Prior to emulsification, the emulsifiers were dissolved in either the oil or the water phase depending on their solubility. WPI, CAS, and MPL20 were dissolved in the buffer, whereas, MPL75 and LEC were dissolved in the fish oil. The pH of the buffer was adjusted with HCl or NaOH to reach pH values of 4.5 and 7.0 in the final emulsions. Emulsions were produced in 500 g batches in a Stephan Universal mixer (Stephan, UMC5, 1995, Hameln, Germany) equipped with an emulsification blade. During processing the mixer bowl was cooled with circulating water at 0°C. Firstly, the buffer (with or without the emulsifier) was mixed for 30 s under reduced pressure (approximately 40 kPa), and then the oil (with or without the emulsifier) was slowly added during mixing (3 min, 1200 rpm). The emulsions were mixed for additional 2 min × 2 min under reduced pressure, with a scrape-down of splashes from the walls of the mixing bowl in between. Sodium azide (0.05% w/v) was added to the emulsions to prevent microbial growth. Emulsions were stored in 100 mL blue cap bottles at around 19°C in the dark for 6 wk. Samples were taken at day 0, 14, 28, and 42 (except for samples LEC_high, 1.4CAS_high, and CAS_high which were not taken on day 28). The viscosity and pH were measured on day 1, and zeta potential was measured in all samples during the first 8 days of storage. Droplet sizes were measured on day 1 and 42. Where nothing else is mentioned results are given as averages of double determinations on the same sample. However, for WPI_high two emulsions were made, and therefore results are given as quadruple determinations (a double determination on each of the two emulsions).

2.3 Characterization of the ingredients

Fatty acid composition of the fish oil and the three PL-based emulsifiers was determined on the lipid extract (prepared as described in Section 2.5.1) or directly on the oil by fatty acid methylation [24], followed by separation through GC (HP5890A, Hewlett Packard, Palo Alto, CA, USA; Column: DBWAX, 10 m × 0.10 mm × 0.1 μm; J&W Scientific, CA, USA) [25]. The quality of all five emulsifiers and the fish oil was determined by PV, using the same procedure as described for the emulsions in Section 2.5.1. Furthermore, the PL classes of the three PL-based emulsifiers, MPL20, MPL75, and LEC were determined by the Laboratory of Food Technology and Engineering, Department of Food Safety and Quality, Ghent University, Belgium. This was done by using a HPLC method in combination with an evaporative light scattering detector. Prevail Silica 3U was used and the PL species were eluted with a gradient mobile phase of dichloromethane, methanol, and

Table 1. Emulsions for the experiment with sample codes

		Protein based emulsifiers			Phospholipid based emulsifiers		
Emulsifier		Sodium caseinate		Whey protein isolate	Soy lecithin	Milk phospholipid (20%)	Milk phospholipid (75%)
Emulsifier concentration		1.4%	2.8%	2.8%	2.8%	2.8%	2.8%
pH	4.5	1.4CAS_low		WPI_low	LEC_low	MPL20_low	MPL75_low
	7.0	1.4CAS_high	CAS_high	WPI_high	LEC_high	MPL20_high	MPL75_high

acetic acid/triethylamine buffer. Polar lipid standard solutions of milk and soy origins (Spectral Service GmbH, D-50996 Köln, Germany) were also injected for quantitative determination.

2.4 Characterization of emulsions

2.4.1 Droplet size

Droplet sizes were measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) using the method described by Let et al. [2]. The emulsions were pretreated by dissolving 1 g emulsion in 5 g buffer (10 mM NaH_2PO_4 and 5 mM SDS), mixing for 30 s and then sonicating for 15 min in a waterbath at 0°C. Droplets of the pretreated emulsions were diluted in recirculating water (3000 rpm), reaching an obscuration of 12–15%. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively.

2.4.2 Zeta potential and pH

The surface charge of the emulsion droplets were determined by the zeta potential with a Zetasizer Nano 2S (Malvern Instruments, Ltd.) at 25°C. Each sample was diluted in 10 mM sodium acetate–imidazole buffer (approximately 0.08 g sample in 10 g buffer) before measuring, and the zeta potential range was set to −100 to +50 mV. Results are given as averages of four or more consecutive measurements on the same sample.

For the determination of pH, emulsion and distilled water were mixed in a 1:1 ratio and pH was measured during stirring. No repetitions were made.

2.4.3 Viscosity measurements

The initial viscosities of the emulsions were measured using a Brookfield viscometer Model RV DV II (Brookfield Engineering Labs. Inc., Stoughton, MA, USA). Different RV spindles were used to adjust the measuring range, no 2 for LEC_low, no 4 for MPL emulsions at pH 4.5, and no 5 for all protein emulsions and MPL emulsions at pH 7.0. Measurements were done on 400 mL sample in a 600 mL beaker, at 100 rpm. During measurements the emulsion temperature was 20.4 ± 0.3°C.

2.4.4 Microscopy

Four emulsions (CAS_high, 1.4CAS_high, MPL20_high, and MPL75_high) were subjected to imaging by confocal microscopy and cryo-SEM. For confocal microscopy staining was done with Fluorescein isothiocyanate, for the proteins and Nile red for the oil. Microscopy was performed on a Leica TCS SP II (Leica Microsystems GmbH, Heidelberg, Germany) inverted vertically, at RT with a 100× oil immersion objective. To investigate the specificity of the staining, a control staining for PL was carried out. This confirmed that the green Fluorescein isothiocyanate staining was specific for proteins.

For cryo-SEM emulsions were put into copper rivets (Quorum Technologies Ltd, East Grinstead, East Sussex, UK) and plunged into slush nitrogen (−210°C). The frozen samples were transferred under vacuum to a preparation chamber (Quorum Polar Prep 2000 Cryo Transfer System, Newhaven, East Sussex, UK) where they were fractured with a cooled knife, subjected to sublimation at −95°C for 4 min, and sputter coated with platinum. Microscopy was performed on a FEI Quanta 200 FEG MKII (FEI, Eindhoven, The Netherlands) at 2 kV at −120°C and 1.66×10^{-4} Pa.

2.5 Measurements of lipid oxidation

2.5.1 Primary oxidation products—peroxide values

For determination of primary oxidation products, a lipid extract was prepared according to the method described by Bligh and Dyer [26] using 5–10 g emulsion or emulsifier sample for each extraction and a reduced amount of solvent (30.0 mL of methanol and chloroform, 1:1). PVs were subsequently measured on the lipid extracts or directly on the oil in the oil samples, by colorimetric determination of iron thiocyanate at 500 nm, as described by Shantha and Decker [27].

2.5.2 Secondary oxidation products—SPME GC-MS

Approximately 1 g of emulsion, 1 mL 100 mM Tris buffer, pH 8.5, and 0.5 g NaCl, were mixed on a whirly mixer for 30 s in a 10 mL vial. The sample was heated for 3 min to a temperature of 60°C, followed by extraction for 45 min while agitating the sample at 500 rpm. Extraction of headspace volatiles was done by the use of a 50/30 μm DVB/CAR/PDMS SPME fiber (Supelco, Bellafonte, PA, USA) installed on a CTC Combi Pal (CTC Analytics, Agilent Technologies, Waldbronn, Germany). Volatiles were desorbed in the injection port of a gas chromatograph (HP 6890 Series, Hewlett Packard; Column: DB-1701, 30 m × 0.25 mm × 1.0 μm; J&W Scientific) for 60 s at 220°C. The oven program had an initial temperature of 35°C for 3 min, increased with 3.0°C/min until 140°C, with 5.0°C/min until 170°C, and with 10.0°C/min until 240°C, where the temperature was kept steady for 8 min.

The individual compounds were analyzed by mass-spectrometry (HP 5973 inert mass-selective detector, Agilent Technologies, USA; Electron ionisation mode, 70eV, mass to charge ratio scan between 30 and 250). From a comparison of chromatograms from non-oxidized and oxidized samples the following volatiles were selected for quantification: pentanal (derived from oxidation of n-6 PUFA), heptanal

(derived from oxidation of n-9 MUFA), and 2,4-heptadienal (derived from oxidation of n-3 PUFA). In the chromatograms two peaks were identified as 2,4-heptadienal. From previous studies of these two peaks (not published) it is anticipated that they represent the two isomers, *t*,*c*-2,4-heptadienal and *t*,*t*-2,4-heptadienal. Calibration curves were made by dissolving the compounds in rapeseed oil followed by the addition of an amount corresponding to 0–1000 ng of the compounds to 1 g emulsion or oil. Since emulsions made with different emulsifiers retained the compounds differently, calibration curves were made for the oil, and for emulsions with MPL75, MPL20, LEC, and WPI individually. Thus, the latter was used for quantifying volatiles in all protein-based emulsions. Calibration curves were parallel shifted in order to obtain positive values. Thus, amounts of volatiles are not given as exact values and should therefore not be used for comparison to other studies. Measurements were made in triplicates on each sample.

2.6 Statistical analyses

Data were analyzed by one- or two-way ANOVA, the latter with Bonferronis multiple comparison test as post test (GraphPad Prism, version 4.03, GraphPad Software, Inc). All references to significant differences ($p < 0.05$) between samples or between sampling time points, are based on this statistical analysis of data.

3 Results

3.1 Characterization of emulsions and emulsifiers

3.1.1 The quality and composition of the emulsifiers

The PV was used to assess the quality of the emulsifiers, and results are listed in Table 2. PVs ranged from 1.3 meq peroxides/kg oil in LEC to 21.9 meq peroxides/kg oil in MPL75.

Table 2. Characterization of the emulsifiers

	WPI	CAS	MPL20	MPL75	LEC
PV (meq peroxides/kg oil)	4.3 ± 0.4	2.3 ± 1.5	1.5 ± 0.3	21.9 ± 1.0	1.3 ± 0.0
Oil content (%)	0.3 ± 0.1	0.8 ± 0.1	24.1 ± 0.7	82.6 ± 0.9	85.8 ± 0.6
Fatty acid composition (%)	–	–			
Total SFA			45.2 ± 0.5	60.6 ± 0.4	19.4 ± 0.1
14:0			6.1 ± 0.1	5.6 ± 0.0	0.1 ± 0.0
16:0			24.4 ± 0.1	29.3 ± 0.2	15.7 ± 0.1
18:0			13.1 ± 0.2	19.9 ± 0.0	3.2 ± 0.0
20:0			0.4 ± 0.0	3.3 ± 0.0	0.3 ± 0.0
Total MUFA			35.9 ± 0.1	26.7 ± 0.1	23.6 ± 0.0
18:1n-9			32.5 ± 0.2	23.2 ± 0.1	21.8 ± 0.0
Total PUFA			12.9 ± 0.4	4.0 ± 0.0	56.0 ± 0.0
18:2n-6			8.0 ± 0.5	1.7 ± 0.0	50.4 ± 0.1
18:3n-3			1.1 ± 0.0	0.1 ± 0.0	5.3 ± 0.0
Protein content (%)[a)]	92.0	93.5	53.8	3.1	–
PL content (%)[a)]	–	–	22.6	76.0	> 56
PL class composition (%)[b)]	–	–			
GluCer			2.00 ± 0.01	3.57 ± 0.01	–
LacCer			6.76 ± 0.02	10.47 ± 0.02	–
PC			27.70 ± 0.05	21.03 ± 0.16	29.70 ± 0.40
PE			25.55 ± 0.14	13.08 ± 0.02	15.31 ± 0.06
PI			8.80 ± 0.06	6.62 ± 0.04	28.50 ± 0.10
PS			8.60 ± 0.05	6.59 ± 0.10	3.48 ± 0.03
SM			20.59 ± 0.13	38.64 ± 0.11	–
PG					1.08 ± 0.02
PA					9.71 ± 0.09
LysoPC					12.23 ± 0.28

WPI, whey protein isolate; CAS, sodium caseinate; MPL20, milk phospholipid 20%; MPL75, milk phospholipid 75%; LEC, soy lecithin; SFA, saturated fatty acids; PL, phospholipids; GluCer, glucosylceramide; LacCer, lactosylceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PG, phosphatidylglycerol; PA, phosphatidic acid; LysoPC, lysophosphatidylcholine.
Individual fatty acids are given when they constitute more than 2.0%.
[a)] As reported on the data sheets provided by the manufacturers.
[b)] As determined by the Laboratory of Food Technology and Engineering, Department of Food Safety and Quality, Ghent University, Belgium.

The oil contents for LEC and MPL75 were both above 80%, whereas, it was approximately 24% for MPL20 and below 1% for each of the two protein-based emulsifiers (Table 2). Further analyses of the fatty acid composition in the PL-based emulsifiers showed that MPL75 contained about 60% saturated fatty acids (SFA), whereas, MPL20 contained approximately the same amount of saturated and unsaturated fatty acids. LEC on the other hand contained about 80% unsaturated fatty acids, of which around 63% was α-linoleic acid, 18:2n-6 (refer to Table 2 for more details). Analysis of PL classes showed that MPL75 contained mainly SM (38.64%) and PC (21.03%). However, the measured amount of SM was approximately 25% higher than that reported in the data sheet, and consequently the amount of mainly PE was measured to be lower. The main constituents of MPL20 were PC (27.70%), PE (25.55%), and SM (20.59%). In this case, the measured amounts of the different PL classes corresponded well with that reported in the data sheet. LEC contained mainly PC (29.70%) and phosphatidylinositol (PI; 28.50%; Table 2).

3.1.2 pH, viscosity, zeta potential, and mean droplet sizes

For emulsions aimed at a pH of 4.5 the actual pH was measured to be in the range from 3.8 to 4.7, whereas, for the emulsions aimed at a pH of 7.0, the actual pH was measured to be between 6.0 and 7.1 (Table 3). The difference between the actual pH and the intended pH was due to the influence of the addition of sodium azide.

The viscosities were determined on day 1 to compare the initial differences between emulsions. Viscosities ranged from 146 cP for LEC_low to 3650 cP for 1.4CAS_low (Table 3). The first one having a consistency like milk and the latter having a consistency like mayonnaise-based dressing. Viscosity data for LEC_high are missing. Initial viscosities were in the order: LEC_low^{a} ≤ MPL20_low^{a,b} ≤ MPL75_low^{b} < MPL75_high^{c} = 1.4CAS_high^{c} < MPL20_high^{d} = WPI_high^{d} < WPI_low^{e} < CAS_high^{f} < 1.4CAS_low^{g} (letters indicating significant differences on a 95% level). Hence, emulsions with proteins generally had higher viscosities than emulsions with PL.

The zeta potential was negative for all emulsions, except WPI_low and MPL20_low that had zeta potentials of 42.7 and 26.8 mV, respectively. For WPI_high and MPL20_high the zeta potential was approximately −30 mV, for 1.4CAS_high, CAS_high, and MPL75_low approximately −40 mV and for the two emulsions with LEC and MPL75_high the zeta potential was approximately −56 mV (Table 3). No data are available for 1.4CAS_low, but since it has been shown in previous studies that emulsions with CAS at a low pH have a positive surface charge [28], it is assumed, that this is also the case in the present study.

Generally the mean droplet sizes of the emulsions did not change significantly during storage (Table 3). However, emulsions prepared with different emulsifiers showed some differences in mean droplet sizes, but no clear effect of neither pH nor emulsifier type (protein vs. PL) was observed. At day 1 the droplet sizes were in the order MPL20_high^{a} = CAS_high^{a} ≤ WPI_low^{a,b} = LEC_high^{a,b} = MPL75_high^{a,b} = 1.4CAS_low^{a,b} ≤ MPL20_low^{b} = WPI_high^{b} = 1.4CAS_high^{b} < MPL75_low^{c} = LEC_low^{c}.

3.1.3 Microscopy

Both from the confocal micrographs (Fig. 1A) and the cryo-SEM micrographs (Fig. 1B) it was observed, that oil droplets were uniformly dispersed in the emulsions. From the staining in the confocal micrographs a clear separation was observed between lipid in the interior of the droplets (red color) and protein in the surrounding water phase (green color). For all emulsions excess protein was dispersed in the water phase.

Table 3. Physico-chemical data for the emulsions

Emulsion	pH	Viscosity (cp)	Zeta potential (mV)	D[3,2], day 1 (μm)	D[3,2], day 42[a)] (μm)
WPI_low	3.8	2566 ± 8	42.7 ± 2.8	12.09 ± 0.07	14.83 ± 3.00^{ns}
WPI_high	7.1	1955 ± 121	−30.7 ± 0.6	20.86 ± 0.38	18.75 ± 2.39^{ns}
1.4CAS_low	4.2	3650 ± 139	–	19.23 ± 3.41	14.74 ± 1.46^{ns}
1.4CAS_high	7.0	1230 ± 3	−40.0 ± 1.1	21.40 ± 0.05	21.73 ± 0.28^{ns}
CAS_high	7.1	3075 ± 16	−39.8 ± 0.7	8.69 ± 0.10	8.99 ± 0.02^{ns}
MPL20_low	4.1	459 ± 7	26.8 ± 2.0	20.39 ± 6.82	27.66 ± 5.10^{ns}
MPL20_high	7.0	1834 ± 37	−31.6 ± 0.2	8.19 ± 0.05	10.13 ± 0.36^{ns}
MPL75_low	4.5	479 ± 10	−41.9 ± 4.0	43.76 ± 3.28	31.37 ± 7.79***
MPL75_high	6.0	1008 ± 17	−56.6 ± 0.5	16.25 ± 0.42	13.80 ± 2.49^{ns}
LEC_low	4.7	146 ± 4	−55.6 ± 3.1	50.81 ± 0.60	–
LEC_high	6.5	–	−55.8 ± 3.3	13.91 ± 0.87	9.06 ± 3.66^{ns}

Please refer to Table 1 for sample codes.

[a)] Significant differences refer to the difference between day 1 and 42 (ns: not significantly different; ***: significantly different, $p < 0.001$).

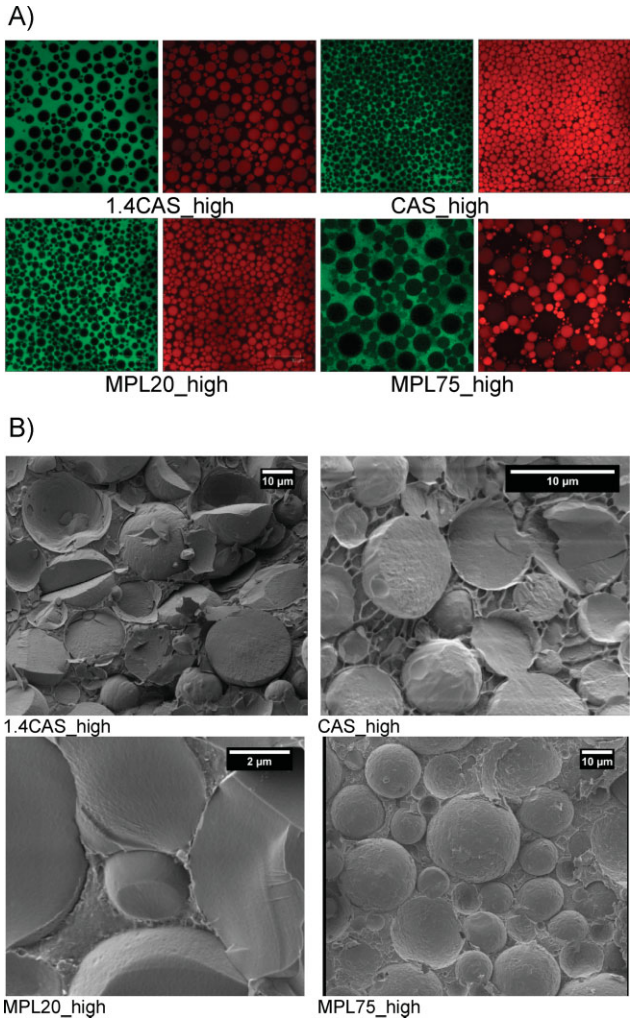

Figure 1. (A) Confocal micrographs of MPL20_high, MPL75_high, 1.4CAS_high, and CAS_high stained for lipids (red) and protein (green). Micrographs are 150 µm × 150µm. The staining shows that there were no lipids in the water phase and no protein in the lipid phase. (B) Cryo-SEM micrographs of 1.4CAS_high, CAS_high, MPL20_high, and MPL75_high. The surface morphology was different in MPL75_high with a more rough appearance and seemingly several layers of emulsifier (black arrows).

Confocal imaging (Fig. 1A), furthermore, visualized that droplet sizes were smaller in MPL20_high and CAS_high than in the other two emulsions, and from the micrographs droplet sizes were estimated to be ~500 nm–15 µm in 1.4CAS_high and ~500 nm–9 µm in CAS_high. In MPL75_high some fairly large droplets (up to 25 µm) were observed surrounded by droplets estimated to be within almost the same size range as the droplets in MPL20_high (~500 nm–10 µm).

From the cryo-SEM micrographs (Fig. 1B) a distinct difference in surface structure was observed for the emulsion with MPL75, compared to the other emulsions. The emulsions CAS_high, 1.4CAS_high, and MPL20_high displayed smooth droplet surfaces with only the imprint from the ice crystals creating structure. In contrast MPL75_high had a more rough appearance with bumps and holes on the surface of the oil droplets and it seemed that there were several layers of emulsifier covering the droplets (see black arrows). We also observed that almost all droplets in CAS_high, 1.4CAS_high, and MPL20_high emulsions were broken across after freeze-fracture while the droplets in MPL75_high emulsions were almost always intact. In the cryo-SEM micrographs a mesh-like structure with small holes was furthermore observed, possibly caused by evaporation of ice crystals when the samples were subjected to sublimation.

3.2 Lipid oxidation in neat oil

The initial PV of the neat oil was <0.1 meq peroxides/kg oil increasing to 7.4 meq peroxides/kg oil at day 42, with the main increase in the last part of the storage period (Figs. 2 and 4). Contents of pentanal, heptanal, and *t*,*t*-2,4-heptadienal did not change significantly during storage, whereas, *t*,*c*-2,4-heptadienal increased significantly from day 0 to 42 (data not shown).

3.3 Lipid oxidation in emulsions prepared with proteins

3.3.1 Peroxide values

PV in all emulsions prepared with proteins, except the emulsion with caseins at pH 7.0 (CAS_high), increased significantly during storage (Fig. 2). The three emulsions prepared with CAS had an initial PV of 0.2 meq peroxides/kg oil. At pH 7.0 the PV increased to 3.9 in the emulsion with the high casein concentration (CAS_high), and to 6.4 in the emulsion with the low casein concentration (1.4CAS_high). In the emulsion with casein at pH 4.5

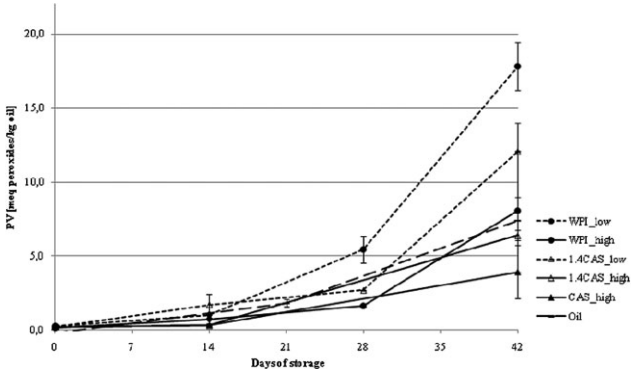

Figure 2. Peroxide values in neat oil and in emulsions prepared with protein-based emulsifiers. For interpretation of sample names, please refer to Table 1.

(1.4CAS_low) the PV increased to 12.1 meq peroxides/kg oil during storage. For the emulsions prepared with whey proteins, at pH 7.0 (WPI_high) PV increased from 0.2 to 8.1 meq peroxides/kg oil and at pH 4.5 (WPI_low) PV increased from 0.3 to 17.8 meq peroxides/kg oil during the 42 days of storage. At day 0, none of the emulsions had a PV significantly different from neat oil. At day 42 the PV of the samples were in the order CAS_high^{a} = 1.4CAS_high^{a} ≤ neat oil^{a,b} = WPI_high^{a,b} ≤ 1.4CAS_low^{b} < WPI_low^{c}. Thus, both protein emulsions at pH 4.5 (1.4CAS_low and WPI_low) had significantly higher PVs at day 42 than their corresponding emulsions at the high pH.

3.3.2 Secondary volatile oxidation products

The amount of pentanal increased significantly during storage for all emulsions prepared with proteins (Fig. 3A), being three- to fourfold higher at day 42 than at day 0. Heptanal increased only significantly for the emulsion with a low concentration of casein at pH 7.0 (1.4CAS_high; Fig. 3B), whereas, concentrations of both 2,4-heptadienals increased significantly for both emulsions with whey proteins (WPI_low and WPI_high) and the emulsion with casein at pH 4.5 (1.4CAS_low; Fig. 3C and D). *t,c*-2,4-Heptadienal increased threefold from day 0 to 42 in the three emulsions, whereas, *t,t*-2,4-heptadienal increased fourfold in WPI_low and doubled in WPI_high and 1.4CAS_low. When comparing the individual emulsions at day 0 no significant differences were observed for pentanal or *t,t*-2,4-heptadienal. However, WPI_low had a significantly higher amount of heptanal than all other emulsions, and both WPI_low and 1.4CAS_low had a significantly higher amount of *t,c*-2,4-heptadienal than the three other emulsions. Similar to the findings for PV, at day 42 WPI_low had a significantly higher amount of the two 2,4-heptadienals than all other emulsions with proteins. Furthermore, 1.4CAS_low had a higher amount of *t,c*-2,4-heptadienal than the other two CAS emulsions and WPI_high (Fig. 3C). Generally, the emulsions prepared at high pH (CAS_high, 1.4CAS_high, and WPI_high) had lower concentrations of the volatiles than the emulsions prepared at low pH (1.4CAS_low and WPI_low) at day 42.

3.4 Lipid oxidation in emulsions prepared with phospholipids

3.4.1 Peroxide values

PV in all emulsions prepared with PL increased significantly during storage, with the main increase between day 28 and 42. Furthermore, PL emulsions had higher initial PVs than

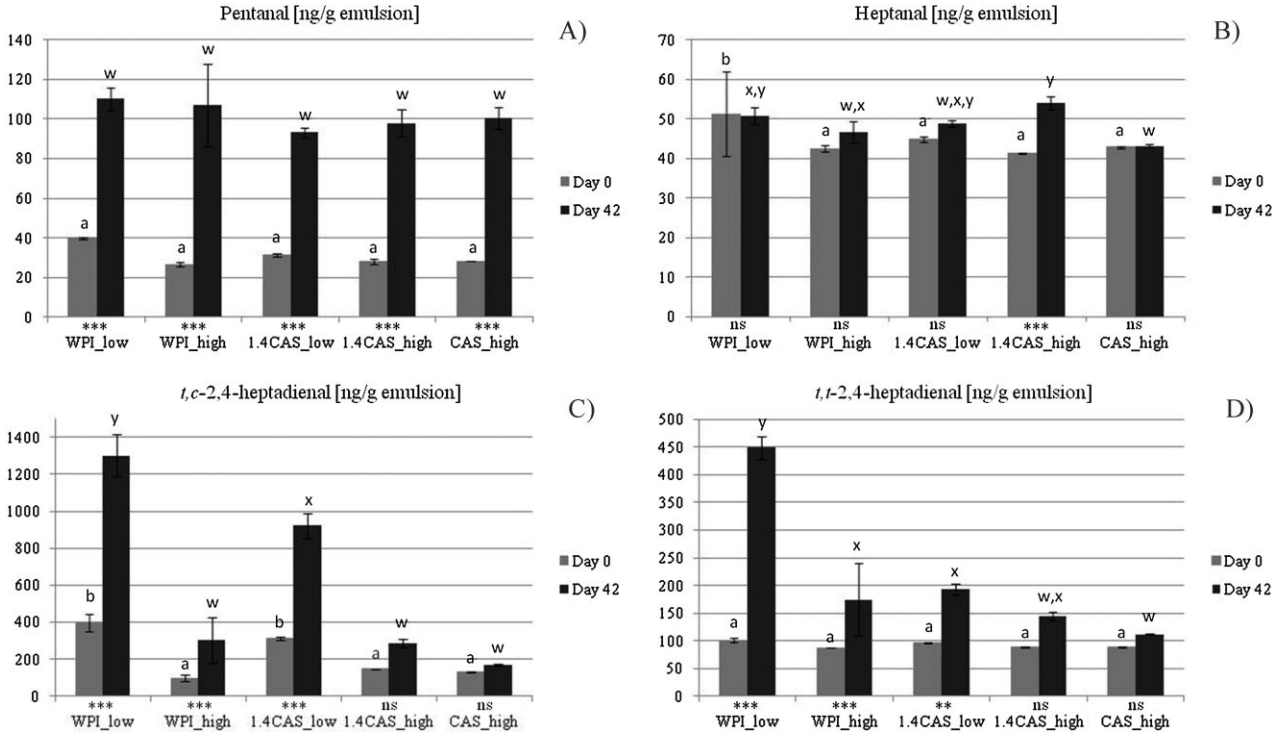

Figure 3. Volatiles in emulsions prepared with protein-based emulsifiers. For interpretation of sample names, please refer to Table 1. Stars indicate significant differences between day 0 and 42 for the individual samples (ns: not significantly different). Letters a and b indicate significant differences between samples at day 0 and letter w, x and y between samples at day 42 ($p < 0.05$). The values are not exact, due to a parallel shift of the calibration curves, making the axis arbitrary.

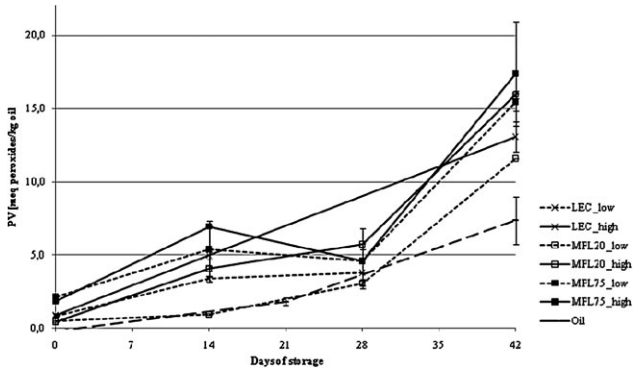

Figure 4. Peroxide values in neat oil and in emulsions prepared with PL-based emulsifiers. For interpretation of sample names, please refer to Table 1.

emulsions prepared with proteins (Fig. 4). This was particularly the case for the emulsions prepared with the milk PL rich emulsifier (MPL75_low and MPL75_high) that had initial PVs of 2.2 and 1.9 meq peroxides/kg oil, respectively. MPL75_high was the PL emulsion that oxidized the most during storage, resulting in a PV of 17.4 meq peroxides/kg oil at day 42. MPL75_low had a PV of 15.5 meq peroxides/kg oil at day 42. For the emulsions prepared with the more protein rich milk PL, PV increased from 0.5 to 12.0 meq peroxides/kg oil in the emulsion prepared at pH 4.5 (MPL20_low) and from 0.4 to 16.0 meq peroxides/kg oil in the emulsion prepared at pH 7.0 (MPL20_high) during the 42 days of storage. The two emulsions prepared with lecithins from soy (LEC_low and LEC_high) both had initial PVs of 0.9 meq peroxides/kg oil. In the emulsion prepared at pH 7.0 (LEC_high), PV increased to 13.1 meq peroxides/kg during storage. Unfortunately the similar emulsion prepared at pH 4.5 (LEC_low) was not physically stable after day 28. Hence, no data are available after this time point. At day 28, PV in LEC_low was 3.8 meq peroxides/kg oil, which was equal to the PV of the other PL emulsions prepared at low pH. At day 0, none of the emulsions were significantly different from each other or from neat oil. At day 42 all emulsions had significantly higher PVs than neat oil, and the order was neat oil^{a} < MPL20_low^{b} ≤ LEC_high^{b,c} ≤ MPL75_low^{c,d} ≤ MPL20_high^{d} = MPL75 high^{d}.

3.4.2 Secondary volatile oxidation products

During storage a significant increase in all four volatiles was observed for the two emulsions prepared with the milk PL rich emulsifier, MPL75 (Fig. 5), with the amount of the two 2,4-heptadienals increasing the most. The emulsions pre-

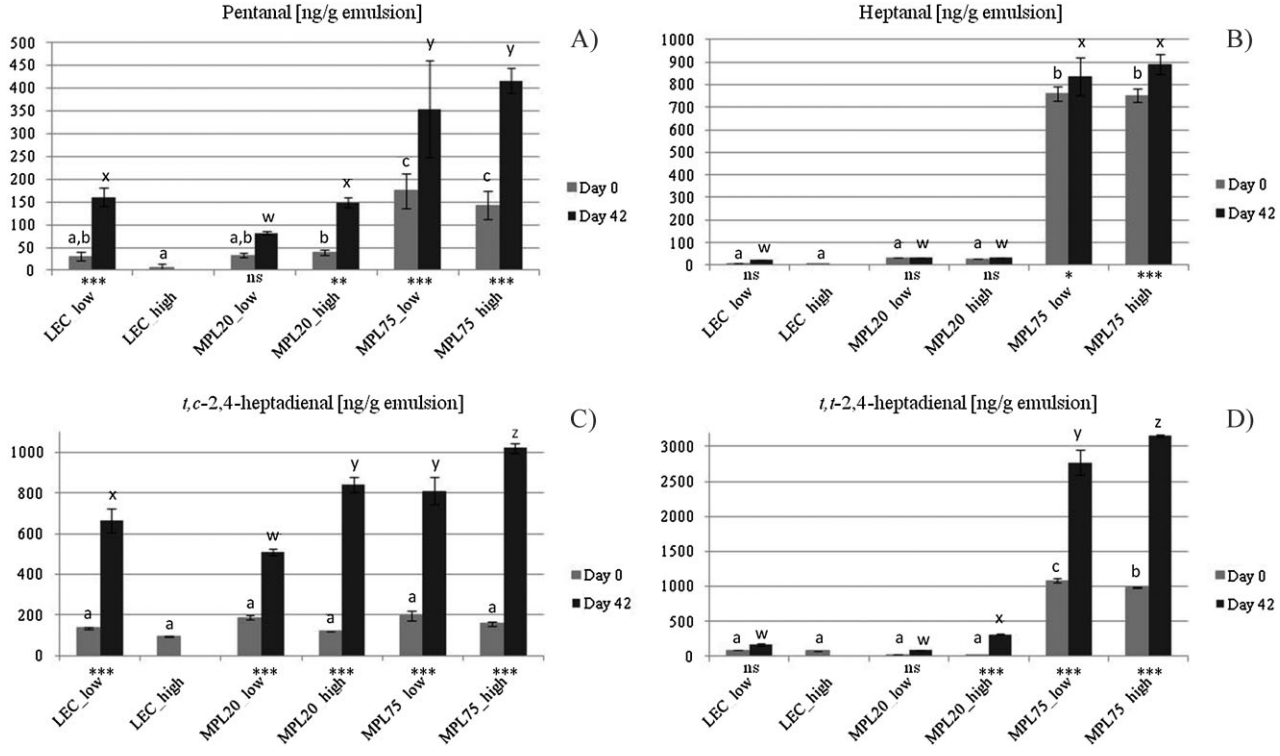

Figure 5. Volatiles in emulsions prepared with PL-based emulsifiers. For interpretation of sample names, please refer to Table 1. Stars indicate significant differences between day 0 and 42 for the individual samples (ns: not significantly different). Letters a–c indicate significant differences between samples at day 0 and letter w, x, y and z between samples at day 42 ($p < 0.05$). The values are not exact, due to a parallel shift of the calibration curves, making the axis arbitrary.

pared at pH 4.5 (MPL75_low) increased fourfold in *t*,*c*-2,4-heptadienal and the emulsion prepared at pH 7.0 (MPL75_high) increased sixfold. Both emulsions with MPL75 increased approximately threefold in *t*,*t*-2,4-heptadienal. Concentrations of *t*,*c*-2,4-heptadienal also increased significantly in all other emulsions during storage (Fig. 5C). A significant increase in pentanal was, furthermore, seen for LEC_low and MPL20_high (Fig. 5A), and in *t*,*t*-2,4-heptadienal for MPL20_high during storage (Fig. 5D). The latter increasing by 21 times the amount found at day 0. Similar to the findings for PV, MPL20_low had a significantly lower amount of pentanal and *t*,*c*-2,4-heptadienal than all other emulsions at day 42. Generally, emulsions made with lecithins from soy or the protein rich milk PL (LEC_low, MPL20_low, and MPL20_high) had lower concentrations of volatiles at day 42 than emulsions made with the milk PL rich emulsifier (MPL75_low and MPL75_high).

4 Discussion

4.1 Structure of the emulsions

4.1.1 Droplet sizes and viscosities

The droplet sizes were estimated from confocal micrographs (Fig. 1A). In general, these results confirmed the data obtained by laser diffraction (Table 3), except for droplet sizes in 1.4CAS_high, which were estimated to be slightly smaller than those observed by laser diffraction. Small emulsion droplets have previously been suggested to increase lipid oxidation [5]. However, in the present study no obvious correlation was observed. For emulsions with proteins the two emulsions with the smallest droplets were the one oxidizing the least (CAS_high) and the one oxidizing the most (WPI_low). Thus, other factors seem to influence lipid oxidation in the protein emulsions more, than the actual droplet size. PL emulsions had in general smaller mean droplet sizes at high pH than at low pH, and were also more oxidized at high pH. However, comparison of the three samples prepared at the same pH showed no correlation between droplet size and lipid oxidation. It is therefore suggested that differences in the emulsifier properties are more important for lipid oxidation than droplet sizes in these emulsions.

From cryo-SEM micrographs it seemed that some of the oil droplets were located so close that they were actually connected. However, this is most likely an artifact from the freezing process, since confocal micrographs showed that oil droplets were uniformly distributed in the emulsions. It is though noteworthy, that emulsion droplets are very closely packed, especially when droplets were small as in MPL20_high and CAS_high. This close packaging of emulsion droplets can result in particle jamming and thereby also influence what was observed as differences in the initial viscosities of the emulsions. Hence, when relating initial viscosities to droplet sizes, it was observed, that for PL emulsions an increase in droplet size led to a decrease in initial viscosity. For protein emulsions on the other hand, no such relation was observed. Hence, proteins in the water phase might have influenced the initial viscosity more than droplet size and particle jamming [11].

The staining in the confocal micrographs showed that protein was present in the water phase in all four emulsions imaged. This was even observed in the emulsion with the milk PL rich emulsifier (MPL75_high), for which the total protein content was below 0.1%. Besides the influence on initial viscosities, proteins in the water phase can also have an antioxidative effect [9], as will be further discussed later. However, the staining was not quantitative, and it is therefore not possible to determine the amount of protein in the water phase relative to the amount at the interfacial layer from the confocal imaging.

Even though no consistency relationship was found between droplet size, viscosities, and lipid oxidation, some tendencies were observed. Firstly for the protein-based emulsions, the use of CAS instead of WPI, or an increase in CAS concentration decreased droplet sizes but increased both initial viscosities and oxidative stability. However, a decrease in pH and a simultaneous increase in viscosity decreased the oxidative stability in protein-based emulsions. Secondly for the PL, the more oxidized high pH emulsions had the highest initial viscosities but the smallest mean droplet sizes. Based on this, it seems that the effect of viscosity and particle jamming on lipid oxidation depend on the type of emulsifier, and for the proteins also on pH.

4.1.2 Surface morphology

Another interesting observation from the cryo-SEM micrographs was the difference in surface morphology and fracture planes between the emulsion with the milk PL rich emulsifier (MPL75_high) and the other three emulsions (1.4CAS_high, CAS_high, and MPL20_high). It seems that the surface at MPL75_high has a more rough structure, and contains several layers (see arrows in Fig. 1B). However, it is currently not known whether these structures represent real multiple layers of emulsifier or if the observed layers are resulting from growing ice crystals during the freezing process. The difference in structure is reproducible, however. The tendency of samples to break along the interfacial layer in MPL75 emulsions is also reproducible and indicates that the planes of weakness is different in this type of emulsion compared to the others. Since both the difference in surface morphology and fracture planes are only observed for MPL75_high and not MPL20_high it could be speculated that these are features related to a high PL content in the emulsifier.

4.2 Lipid oxidation in emulsions versus in neat oil

Regarding lipid oxidation, the goal of using an emulsion as delivery system for fish oil to foods is to obtain a protection of

the oil against lipid oxidation by the emulsifier. Yet, it is also important that the delivery system is oxidatively stable before it is incorporated into the food product. However, in the present study only the emulsions with protein-based emulsifiers at pH 7.0, tended to oxidize less or equally to neat oil. All emulsions prepared with PL and the emulsions with proteins prepared at low pH oxidized more than neat oil. The better oxidative stability of neat oil than emulsions could result from absence of the physical stress during emulsion production. Even though precautions have been taken to minimize the risk of initiating lipid oxidation during the production (limited oxygen availability and cooling), the emulsions have undergone harsher production conditions compared to the neat oil that has been poured directly into the glasses for storage. The differences observed between emulsions in their oxidative stabilities are discussed throughout the next sections.

4.3 Lipid oxidation in emulsions prepared with proteins

Only a few significant differences between the emulsions prepared with proteins were observed, but there was a clear tendency of an increased oxidation when the emulsions were prepared at pH 4.5 as compared to preparation at pH 7.0. Moreover, there was a tendency toward more oxidation in WPI emulsions than in CAS emulsions, and also of a better oxidative stability the higher the concentration of CAS. The emulsion with the best oxidative stability throughout storage was prepared with 2.8% CAS at pH 7.0 (CAS_high).

4.3.1 Concentration of emulsifier (1.4CAS_high vs. CAS_high)

In the present study, the emulsion with 1.4% protein had a mean droplet size approximately twice as large (1.4CAS_high $\approx$ 21 μm) as the emulsion with 2.8% protein (CAS_high $\approx$ 9 μm). This finding is in agreement with previous findings by Fang and Dalgleish [29], where droplet size has been shown to decrease up to a concentration of 1% protein in emulsions with 20% oil. However, it is interesting, that the emulsion with the larger droplets oxidized more than the emulsion with the smaller droplets, indicating that other factors are assumed to influence lipid oxidation more, as discussed in the following.

In the above-mentioned study on 20% soya oil-in-water emulsions [29] it was shown that at a concentration of casein below 0.7% (corresponding to an emulsifier to oil ratio of 1:29), the oil droplets were not entirely covered with casein. In the present study the emulsion with 1.4% casein had an emulsifier to oil ratio of 1:50, and the oil droplets in the emulsion with 2.8% casein had an emulsifier to oil ratio of 1:25. However, since the oil droplet sizes were very different in the two studies (D3,2 $\leq$ 0.39 μm in the study by Fang and Dalgleish [29] compared to D3,2 $\geq$ 8.99 μm in the present study), it can only be speculated whether the droplets in the present study were fully covered. The possible lack of full coverage could explain why the emulsion with 1.4% of casein (1.4CAS_high) seemed less oxidatively stable than the emulsion with 2.8% of casein (CAS_high). In the same study by Fang and Dalgleish [29] it was suggested that addition of more protein would lead to a protrusion of protein into the water phase, which would change the thickness of the interfacial layer from 5 to 10 nm. A thicker interfacial barrier could therefore also help to explain the better oxidative stability of CAS_high.

Another explanation could be a metal chelating effect of the proteins shown in the confocal micrographs to be present in the water phase of the emulsions. This antioxidative effect of proteins has previously been shown in a study comparing washed and unwashed 10% fish oil-in-water emulsions with WPI, soy protein isolate, and CAS [9]. However, a quantification of proteins would be needed to argue whether more protein is present in the water phase in CAS_high than in 1.4CAS_high.

4.3.2 The difference between types of proteins (WPI_high vs. CAS_high)

In the present study a significant difference between the two emulsions prepared with whey proteins (WPI_high) and caseins (CAS_high) was only observed for *t*,*t*-2,4-heptadienal at day 42, where WPI_high had a higher amount than CAS_high. However, during storage WPI_high increased significantly in both PV and three of the four volatiles from day 0 to 42 as compared to CAS_high that only increased significantly in pentanal. This indicated that WPI_high was less stable toward oxidation than CAS_high.

These findings are in agreement with several studies on the oxidative stability of less concentrated emulsions prepared with CAS and WPI [30–32]. In these studies, CAS seemed in general to protect the emulsions better than WPI against lipid oxidation, irrespective of pH. The better oxidative stability of casein over whey protein emulsions were in these studies suggested to be mainly related to their differences in amino acid residues. Whereas, CAS contains many phosphorylated serine residues with a metal ion chelating capacity [9], WPI contains more sulfhydryl groups that can have a free radical scavenging effect [10]. It could therefore be hypothesized that the presence of metal ions is more important for oxidation than free radicals in this emulsion system. Yet, only endogenous metal ions were present, and this suggests that several other factors influence lipid oxidation as well.

Another difference between the two proteins is their structures. Thus, the protein components in WPI are globular and the protein components in CAS are unstructured, and thereby more flexible. In a previous study on WPI and CAS emulsions, it was shown that less CAS than WPI is

needed for full coverage of the droplets due to the flexible nature of CAS [6]. This could explain why smaller droplets are produced in CAS_high (D3,2 = 8.69 μm), than in WPI_high (20.86 μm). Unfortunately, possible structure differences at the interface between emulsions with WPI and CAS were not investigated in the present study. However, other types of advanced microscopy will be tried in the future, to possibly obtain even more detailed micrographs of the structure at the interfacial layer.

4.3.3 Changing pH

At the low pH in this study, the proteins were below their isoelectric points and the surface charge was therefore positive. Nevertheless, these emulsions oxidized more than the emulsions at high pH with negatively charged droplets. Similar results were obtained in a study on 10% oil emulsions with 1% casein [28]. These results could be related to the better solubility of iron at low pH. However, only endogenous iron was available in the present study. In addition, both results from the last-mentioned study and the present one are in contrast to other studies on the effect of pH on lipid oxidation in emulsions, e.g., prepared with WPI [8, 33]. In these studies, lipid oxidation was more pronounced at pH values above the isoelectric point of the emulsifiers than below.

The reasons for these contradictory results might be preferential adsorption at the droplet surface of different proteins at different pH values and at different homogenization pressures. Thus, for WPI it has been shown that there is no preferential adsorption of either α-lactalbumin or β-lactoglobulin at pH 7, whereas, more α-lactalbumin than β-lactoglobulin is adsorbed at pH 3 [34], possibly due to a lower flexibility of the β-lactoglobulin at low pH [35]. Since β-lactoglobulin has also been shown to give more oxidative stability to an emulsion than α-lactalbumin [8], the preferential adsorption of α-lactalbumin over β-lactoglobulin at pH 3, could explain the lower oxidative stability of these low pH emulsions both in the present study, and the study be Haahr and Jacobsen [28]. However, in the two studies by Donnelly et al. [33] and Hu et al. [8] the preferential adsorption of α-lactalbumin over β-lactoglobulin at the low pH might be overruled by a possible preferential adsorption of β-lactoglobulin due to the use of a high homogenization pressure. The preferential adsorption of β-lactoglobulin over casein at high homogenization pressure has previously been shown in milk [23], and something similar could be hypothesized to have taken place in the emulsions in the two above-mentioned studies.

CAS, which is the other protein used in the present study, consists of a mix of proteins mainly α_{S1}- and β-casein. Similar to the proteins in WPI, caseins could be speculated to adsorb differently according to, e.g., pH and homogenization pressure. Such different adsorption behavior could explain the lower oxidative stability at the low pH for the CAS emulsion compared to the high pH CAS emulsion. However, this needs further investigation.

4.4 Lipid oxidation in emulsions with phospholipids

None of the emulsions prepared with PL oxidized less than neat oil, but at pH 4.5 emulsions prepared with the protein rich milk PL (MPL20) or LEC generally oxidized less or equally to the emulsions prepared with proteins (compare Fig. 2 vs. Fig. 4 and Fig. 3 vs. Fig. 5). At pH 7.0 protein-based emulsions oxidized less than all PL-based emulsions. At both pH values, emulsions with MPL75 oxidized the most. Especially the contents of secondary oxidation products were remarkably higher in MPL75 emulsions than in the other emulsions. In contrast, MPL20 emulsions seemed to oxidize the least at both pH values. At high pH, the emulsion with LEC was not physically stable throughout storage.

4.4.1 Comparison of the emulsions prepared with phospholipids

In both MPL20 and MPL75 SM, PC, and PE were the main constituents. In MPL20, PC and PE were present in more or less equal amounts, whereas, the amount of SM was a little lower (for details on PL class composition refer to Table 2). In MPL75 the amount of PE was almost half that of PC, which was then again half that of SM. LEC was differing from the two milk PL by having no SM but had PI and PC as its main constituents instead. Previous studies have shown an effect of individual PL classes on lipid oxidation. In a study on refined sardine oil, PC was shown to be the most effective individual antioxidant [14], and PE to work most effectively in synergy with α-tocopherol. Thus, in a study on the active sites of PL, it was concluded that the antioxidant activity of PC and PE should be attributed the side chain amino groups enhanced by intramolecular hydroxyl groups [36]. In another study on the antioxidant properties of PL in a refined salmon oil model system during heating, no difference in the antioxidant properties was observed between SM, PC, and PE [37], even though all of them were more active than phosphatidylserine (PS), phosphatidylglycerol (PG), and PI. On this background, it cannot be ruled out that the PL composition decreased the antioxidative properties of LEC compared to the milk PL in the present study. However, it is difficult to argue how much PL classes influenced the results in the present study, since other components in the emulsifiers such as proteins and polysaccharides might also have had an oxidatively stabilizing effect.

In addition, fatty acid composition and the quality of the emulsifier may have influenced the oxidative stability. Considering the fatty acid compositions, MPL75 contained ~61% SFA, ~27% MUFA, and ~4% PUFA as compared to MPL20, which contained ~45% SFA, ~36% MUFA, and ~12% PUFA and LEC ~19% SFA, ~24% MUFA, and

~56% PUFA. An interfacial layer with a high amount of unsaturated lipids, such as LEC, is expected to be more flexible than an interfacial layer that is highly saturated, such as the one created by MPL75. Hence, the less flexible interfacial layer in MPL75 emulsions could possibly increase the distance between individual PL molecules at the interface, and thereby decrease the oxidative stability through a lower surface coverage. The better oxidative stability of MPL20 emulsions compared to MPL75 emulsions is expected to be attributed the proteins present in MPL20, that might fill in between the PL at the interfacial layer and compensate for the otherwise low coverage. Nevertheless, the more unsaturated the lipids are, the more prone they are to lipid oxidation. Hence, it would be expected that the lipids in MPL20 and especially in LEC would oxidize faster than the ones in MPL75, in contrast to what was observed. However, the data sheet of MPL75 states that it should contain about 12% PUFA, and not 4% as was the amount determined through analysis. This could indicate that part of the PUFA have already oxidized, which is confirmed by the very high PV of this emulsifier (21.9 meq peroxides/kg oil). The poor quality of MPL75 might therefore be crucial for the obtained results, as discussed in Section 4.4.3.

4.4.2 Changing pH

Due to missing data, the pH change will not be discussed for emulsions prepared with LEC. However, both emulsions made with MPLs oxidized more at high pH than at low, with the most pronounced differences between MPL20_low and MPL20_high. The emulsion with MPL20 also changed from a negative to a positive surface charge when decreasing pH, possibly due to the proteins present in MPL20 that have a higher isoelectric point than pH 4.5. The positive surface charge at the low pH could have a positive influence on the oxidative stability by repulsion of prooxidative cationic metal ions, as has previously been shown for whey proteins [8]. In comparison, the zeta potential of MPL75_high was only slightly more negative than MPL75_low. It is not known whether this difference can be attributed to the small amount of protein in MPL75, the PL, or both. However, in the literature it is described that negatively charged PL can interact with water, which will change their packing and molecular conformation at the interface [38]. Thus, conformational changes due to more negatively charged PL at high pH could also influence lipid oxidation, in this case more than the higher viscosity observed at high pH. The possible changes in packing due to surface charge could furthermore be partly responsible for the difference in droplet size between emulsions at the two pH values. At high pH smaller oil droplets were observed for both emulsions with MPL20 and MPL75, which could also contribute to more lipid oxidation, as previously discussed.

4.4.3 The quality of the ingredients

On data sheets for the emulsifiers, LEC and MPL75, PV were reported to be below 10 and 1.5 meq/kg oil, respectively. For the other emulsifiers max PV was not reported. The PV of LEC was 1.3 meq peroxides/kg oil, and thereby found to be in accordance with that reported on the data sheet. However, with a PV measured to be 21.9 meq peroxides/kg oil in MPL75, the PV was much higher than expected from the data sheet, resulting in an emulsifier of a very poor quality. The emulsions prepared with MPL75 also had a higher PV initially than the other emulsions (though not significantly) and a significantly higher content of three of the measured volatiles (pentanal, heptanal, and *t*,*t*-2,4-heptadienal) compared to all the other samples. This supports that the quality of the emulsifier might have had an influence itself. Since lipid oxidation is a chain reaction process that propagates even faster if already formed lipid hydroperoxides are present, the high PV of the emulsifier MPL75 could partly explain the very low oxidative stability of emulsions produced with this emulsifier. The effect of ingredients quality has also previously been shown to significantly influence lipid oxidation in real food emulsions, both in the case of the fish oil [39] and the emulsifier used [40].

5 Conclusions

In the present study, it was shown, that it is possible to prepare emulsions with 70% fish oil that are physically stable for at least 42 days. Emulsions prepared with proteins at pH 7.0 oxidized less or equally to neat oil, whereas, emulsions prepared at pH 4.5 oxidized more than neat oil. All emulsions prepared with PL oxidized more than neat oil, with emulsions prepared at high pH oxidizing the most. Thus, for emulsions prepared with PL-based emulsifiers or with proteins as emulsifiers at low pH, further studies have to be performed to optimize their oxidative stability and applicability as delivery systems. Interestingly, the results showed that at low pH some PL-based emulsions (LEC and MLP 20) had better oxidative stability than protein-based emulsions. Therefore, PL deserve more attention as emulsifiers for delivery systems with high oil contents at low pH. Results also demonstrated that even in simple emulsions several factors influence lipid oxidation. Cryo-SEM microscopy of selected emulsions at high pH suggested that the surface morphology of emulsions with high content of milk PL (MPL75) was different from that of emulsions with high protein content (CAS or MPL20). However, further investigations by various microscopy techniques are necessary to confirm this finding and to provide more information about the interfacial structure to improve our understanding about oxidation events taking place at the interface.

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PAPER II

Iron-mediated lipid oxidation in 70% fish oil-in-water emulsions: effect of emulsifier type and pH

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Original article

Iron-mediated lipid oxidation in 70% fish oil-in-water emulsions: effect of emulsifier type and pH

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Summary The objective of this study was to investigate the protective effect of five different emulsifiers on iron-mediated lipid oxidation in 70% fish oil-in-water emulsions. The emulsifiers were either based on protein (whey protein isolate and sodium caseinate) or based on phospholipid (soy lecithin and two milk phospholipids with different phospholipid contents, MPL20 and MPL75). Lipid oxidation was studied at pH 4.5 and 7.0, and results were compared to lipid oxidation in neat fish oil. Results showed that all emulsions oxidised more than neat oil. Furthermore, emulsions prepared with proteins oxidised more at low pH than at high pH, and casein emulsions oxidised the least (Peroxide value (PV) at day 7 was 0.5–0.7 meq kg^{-1}). Among emulsions prepared with phospholipids, emulsions with MPL75 were the most oxidised followed by emulsions prepared with lecithin and MPL20. Thus, PV in MPL75 emulsions was 5.0–5.5 meq kg^{-1} at day 7 compared with 0.9–1.9 meq kg^{-1} in MPL20 emulsions.

Keywords Emulsifiers, lipid oxidation, milk proteins, omega 3, phospholipids, solid-phase microextraction.

Introduction

In an attempt to increase the human intake of the healthy long-chain polyunsaturated omega-3 fatty acids, incorporation of marine oils into foods has gained an increased interest during the last decade. However, these highly unsaturated lipids are prone to lipid oxidation, and their incorporation into foods is therefore limited by the development of unpleasant off-flavours. When incorporating marine oils into foods, the quality of the oil (Let *et al*., 2005), pro- and antioxidative components in the ingredients present (Jacobsen *et al*., 2001; Sørensen *et al*., 2010a), the way the oil is delivered into the food product (Let *et al*., 2007) and the processing and storage conditions of the food (Sørensen *et al*., 2010b) have been shown to influence the lipid oxidation in the final fish oil–enriched food product.

In the case of lipid autoxidation, one major issue to address is the presence of transition metal ions, because metal ions are capable of promoting autoxidation either by direct initiation and formation of lipid radicals or through Fenton-like reactions. Because transition metal ions are present in most food products, inactivation of them is important in order to avoid the development of rancidity in fish oil–enriched food products. In emulsion systems, certain emulsifiers such as sodium caseinate have demonstrated an ability to chelate metal ions (Faraji *et al*., 2004). Thus, one possible way of protecting the fish oil against oxidation, when it is added to a food, could be to prepare an emulsion with an emulsifier that has chelating properties and use it as a delivery system. However, the successful use of delivering emulsions in foods requires that emulsions can be produced with an extremely high oil content that are oxidatively stable in the presence of iron. Nevertheless, studies on highly concentrated oil-in-water emulsions are very scarce, and only one study is for the moment available on lipid oxidation in 70% oil-in-water emulsions (Horn *et al*., 2011).

An obvious choice of emulsifier for these highly concentrated emulsions would be milk proteins (casein or whey protein), as they have previously shown good protective effects against lipid oxidation (Ries *et al*., 2010; Horn *et al*., 2011; Kargar *et al*., 2011), especially when present in excess in the aqueous phase (Faraji *et al*., 2004; Kargar *et al*., 2011). Their protective effects are mainly based on a number of different mechanisms related to their ability to shield the oil from the pro-oxidative transition metal ions. Thus, milk proteins have been shown to be capable of creating a physical barrier around the oil droplets that could protect the lipids from

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metal ions in the water phase (Hunt & Dalgleish, 1994). In addition, particularly casein has been shown to possess metal-chelating properties, most likely due to its content of phosphorylated serine residues (Faraji *et al.*, 2004). Furthermore, protein-stabilised emulsion droplets are also hypothesised to repel cationic metal ions owing to a positive surface charge when pH is below the isoelectric point of the protein (Hu *et al.*, 2003a). However, a potential drawback of using milk proteins is that the proteins (especially casein) tend to precipitate if pH is around its isoelectric point. Furthermore, the combination of a very high oil content and milk proteins as emulsifier results in very viscous emulsions that cannot be produced by the use of high-pressure homogenisers, and other types of emulsification equipments create very broad droplet size distributions.

Hence, an evaluation of alternative food-grade emulsifiers, such as phospholipids (PL), would be valuable, especially at low pH. The antioxidant mechanisms of PL are less well described and are still under debate, possibly because different PL classes exert different antioxidative effects. For example, phosphatidylinositol and other acidic PLs form inactive complexes with metal ions and can thereby prevent metal-catalysed oxidation. In contrast, phosphatidylcholine and phosphatidylethanolamine do not possess such metal-chelating properties (Pokorný, 1987). However, certain PLs, such as phosphatidylethanolamine, have also been shown to work synergistically with tocopherols (Ohshima *et al.*, 1993; Bandarra *et al.*, 1999; Judde *et al.*, 2003). In addition, the successful use of PLs as emulsifiers in food systems has been shown to depend both on the fatty acid composition and on their PL class composition (Wang & Wang, 2008).

On the basis of these differences in emulsifier properties, we hypothesise that protein- and PL-based emulsifiers will act differently at the interfacial layer and thereby affect lipid oxidation in emulsions differently, especially when lipid oxidation is mediated by iron addition. Furthermore, we hypothesise that pH, protein concentration and the composition of different PLs will have an influence on lipid oxidation. The aims of this study were therefore to evaluate the protective effect of five different food-grade emulsifiers on iron-mediated lipid oxidation in 70% fish oil-in-water emulsions at two pH values and to compare lipid oxidation in the emulsions with that in neat oil.

The emulsifiers investigated were whey protein isolate (WPI), sodium caseinate (CAS), soy lecithin (LEC) and two milk phospholipids with approximately 20% and 75% phospholipids (MPL20 and MPL75, respectively). Emulsions were prepared at two pH values, pH 4.5 and pH 7.0, corresponding to the pH of yoghurt and milk, respectively. For sodium caseinate, the effect of different emulsifier concentrations was also studied at the high pH.

Materials and methods

Materials

Commercial cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway) and stored at −40 °C until use. The fatty acid content of the major fatty acids was as follows (in area %): 16:0 9.2, 16:1(n-7) 8.3, 18:1(n-9) 16.7, 18:2(n-6) 1.9, 21:1(n-9) 10.6, 20:5(n-3) 9.2, 22:1(n-11) 5.8 and 22:6(n-3) 12.4. The PV and tocopherol contents were < 0.05 meq peroxides per kg oil and approximately 200 mg α-tocopherol per kg, respectively. Sodium caseinate (Miprodan® 30), WPI (Lacprodan® DI-9224) and milk phospholipids (Lacprodan® PL-20 and Lacprodan® PL-75 milk phospholipid concentrate) were kindly donated by Arla Foods Ingredients amba (Viby J, Denmark). Protein contents as reported in the data sheets are given in Table 1. Soy lecithin (SolecTM E-40-B) was donated by The Solae Company (Århus, Denmark). On the data sheet from the Solae Company, a PL content of min. 56% (as acetone insolubles) in the soy lecithin was reported (Refer to Table 1 for details on PL compositions). All other chemicals and solvents used were of analytical grade.

Preparation of emulsions and sampling

In general, the emulsions consisted of 70.0% (w/w) fish oil, 2.8% (w/w) emulsifier and 27.2% (w/w) 10 mM sodium acetate–imidazole buffer, corresponding to an emulsifier-to-oil ratio of 1:25. The only exceptions were two of the emulsions prepared with sodium caseinate, where only 1.4% (w/w) emulsifier was used and consequently they also contained more buffer (28.6%) (refer to Table 2 for study design and sample codes for the emulsions). Prior to emulsification, the emulsifiers were dispersed in either the oil or the water phase depending on their solubility. WPI, CAS and MPL20 were dispersed in the buffer, whereas MPL75 and LEC were dispersed in the fish oil. The pH of the buffer was adjusted with HCl or NaOH to reach pH values of 4.5 and 7.0 in the final emulsions. The emulsions were produced in 500 g batches in a Stephan Universal mixer (Stephan UMC5, Hameln, Germany) equipped with an emulsification blade and cooled during processing with circulating water at 0 °C. Firstly, the buffer (with or without the emulsifier) was mixed for 30 s under reduced pressure (approximately 40 kPa), and then the oil (with or without the emulsifier) was slowly added during mixing (3 min, 1200 rpm). The emulsions were mixed for additional 2 × 2 min under reduced pressure with a scrabe-down of splashes from the walls of the mixing bowl in between. Emulsions (65 g) were then added to Fe^{2+} (0.03‰ $FeSO_4 \cdot 7\ H_2O$ solubilised in water, corresponding to approximately 90 μM in the final

Table 1 Characterisation of the emulsifiers

	WPI	CAS	MPL20	MPL75	LEC
PV (meq peroxides per kg oil)	4.3 ± 0.4	2.3 ± 1.5	1.5 ± 0.3	21.9 ± 1.0	1.3 ± 0.0
Oil content (%)	0.3 ± 0.1	0.8 ± 0.1	24.1 ± 0.7	82.6 ± 0.9	85.8 ± 0.6
Fatty acid composition (%)					
Total SFA	–	–	45.2 ± 0.5	60.6 ± 0.4	19.4 ± 0.1
14:0			6.1 ± 0.1	5.6 ± 0.0	0.1 ± 0.0
16:0			24.4 ± 0.1	29.3 ± 0.2	15.7 ± 0.1
18:0			13.1 ± 0.2	19.9 ± 0.0	3.2 ± 0.0
20:0			0.4 ± 0.0	3.3 ± 0.0	0.3 ± 0.0
Total MUFA			35.9 ± 0.1	26.7 ± 0.1	23.6 ± 0.0
18:1n-9			32.5 ± 0.2	23.2 ± 0.1	21.8 ± 0.0
Total PUFA			12.9 ± 0.4	4.0 ± 0.0	56.0 ± 0.0
18:2n-6			8.0 ± 0.5	1.7 ± 0.0	50.4 ± 0.1
18:3n-3			1.1 ± 0.0	0.1 ± 0.0	5.3 ± 0.0
Protein content (%)*	92.0	93.5	53.8	3.1	–
PL content (%)*	–	–	22.6	76.0	>56
PL class composition (%)†					
GluCer	–	–	2.00 ± 0.01	3.57 ± 0.01	–
LacCer			6.76 ± 0.02	10.47 ± 0.02	–
PC			27.70 ± 0.05	21.03 ± 0.16	29.70 ± 0.40
PE			25.55 ± 0.14	13.08 ± 0.02	15.31 ± 0.06
PI			8.80 ± 0.06	6.62 ± 0.04	28.50 ± 0.10
PS			8.60 ± 0.05	6.59 ± 0.10	3.48 ± 0.03
SM			20.59 ± 0.13	38.64 ± 0.11	–
PG					1.08 ± 0.02
PA					9.71 ± 0.09
LysoPC					12.23 ± 0.28

WPI, whey protein isolate; CAS, sodium caseinate; MPL20, milk phospholipid 20%; MPL75, milk phospholipid 75%; LEC, soy lecithin; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PL, phospholipids; GluCer, glucosylceramide; LacCer, lactosylceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PG, phosphatidylglycerol; PA, phosphatidic acid; LysoPC, lysophosphatidylcholine.
Individual fatty acids are given when they constitute more than 2.0%.
*As reported on the data sheets provided by the manufacturers.
†As determined by the Laboratory of Food Technology and Engineering, Department of Food Safety and Quality, Ghent University, Belgium.

Table 2 Experimental design of the emulsions samples

	Protein-based emulsifiers			Phospholipid-based emulsifiers		
Emulsifier	**Sodium caseinate**		**Whey protein isolate (WPI)**	**Soy lecithin**	**Milk phospholipid (20%)**	**Milk phospholipid (75%)**
Emulsifier concentration	1.4%	2.8%	2.8%	2.8%	2.8%	2.8%
pH						
4.5	1.4CAS_low		WPI_low	LEC_low	MPL20_low	MPL75_low
7.0	1.4CAS_high	CAS_high	WPI_high	LEC_high	MPL20_high	MPL75_high

All emulsions were added 0.03 ‰ $FeSO_4$.

emulsion) to accelerate oxidation and 0.05% sodium azide to prevent microbial growth. Emulsions were stored in 100-mL Bluecap bottles at 19–20 °C in the dark for 1 week, and samples were taken at days 0, 2, 5 and 7 for lipid oxidation measurements. A sample of neat oil (65 g) was used for comparison; thus, the same amount of Fe^{2+} was dispersed in the oil as that in the emulsion samples, and it was stored under the same conditions. The viscosity, droplet sizes and pH were measured on the emulsions at day 1, and zeta potential was measured during storage. Where nothing else is mentioned, results are given as averages of double

determinations on the same sample. However, for the emulsion with whey protein at pH 7.0 (WPI_high), two emulsions were made, and therefore results are given as quadruple determinations (a double determination on each of the two emulsions).

Characterisation of emulsions

Droplet size

Droplet sizes were measured by laser diffraction on a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). The emulsions were pretreated according to the method described by Let *et al.* (2007). Emulsion (1 g) was dissolved in 5 g SDS buffer (10 mM NaH_2PO_4, 5 mM SDS), mixed for 30 s and then sonicated for 15 min in a waterbath at 0 °C. Droplets of the pretreated emulsions were diluted in recirculating water (3000 rpm), reaching an obscuration of 12–15%. The refractive indices of sunflower (1.469) and water (1.330) were used as particle and dispersant, respectively.

Zeta potential and pH

The surface charge of the emulsion droplets was determined by the zeta potential using a Zetasizer Nano 2S (Malvern Instruments, Ltd.) at 25 °C. Each sample was diluted in 10 mM sodium acetate–imidazole buffer (approximately 0.08 g sample in 10 g buffer) before measuring, and the zeta potential range was set to −100 to +50 mV. Results are given as averages of four or more consecutive measurements on the same sample.

For the determination of pH, emulsion and distilled water were mixed in a 1:1 ratio and pH was measured during stirring. No repetitions were made.

Viscosity measurements

The initial viscosities of the emulsions were measured using a Brookfield viscometer Model RV DV II (Brookfield Engineering Labs. Inc., Stoughton, MA, USA). The emulsions were measured using different RV spindles (no 2 for LEC_low, no 4 for milk phospholipid samples at pH 4.5 and no 5 for all protein emulsions and milk phospholipid emulsions at pH 7.0). Measurements were taken on 400 mL sample in a 600-mL beaker, at 100 rpm. During measurements, the emulsion temperature was 20.4 ± 0.3 °C.

Characterisation of the ingredients

Fatty acid composition of the fish oil and the three PL-based emulsifiers was determined on the lipid extract (prepared as described below) or directly on the oil by fatty acid methylation (AOCS Official method Ce 2-66, 1998), followed by separation through gas chromatography (HP5890A; Hewlett Packard, Palo Alto, CA, USA; Column: DBWAX, 10 m × 0.10 mm × 0.1 μm; J&W Scientific, Folsom, CA, USA) (AOCS Official method Ce 1b-89, 1998). The qualities of all five emulsifiers and the fish oil were determined by the peroxide value, using the same procedure as described for the emulsions below. Furthermore, the PL classes of the three PL-based emulsifiers, MPL20, MPL75 and LEC, were determined by the Laboratory of Food Technology and Engineering, Department of Food Safety and Quality, Ghent University, Belgium. This was done by using a HPLC method in combination with an evaporative light-scattering detector. Prevail Silica 3u was used and the PL species were eluted with a gradient mobile phase of dichloromethane, methanol and acetic acid/triethylamine buffer. Polar lipid standard solutions of milk and soy origins (Spectral Service GmbH, Köln, Germany) were also injected for quantitative determination.

Measurements of lipid oxidation

Primary oxidation products – peroxide values

For the determination of primary oxidation products in the emulsions and emulsifiers, a lipid extract was prepared according to the method described by Bligh & Dyer (1959) using 5–10 g of sample for each extraction and a reduced amount of solvent (30.0 mL of methanol and chloroform, 1:1). Peroxide values were subsequently measured on the lipid extracts or directly on the oil in the oil samples, by colorimetric determination of iron thiocyanate at 500 nm, as described by Shantha & Decker (1994).

Secondary oxidation products – solid-phase microextraction GC-MS

Approximately 1 g of emulsion, 1 mL 100 mM Tris buffer, pH 8.5, and 0.5 g NaCl were mixed on a whirly mixer for 30 s in a 10-mL vial. NaCl was added to increase the release of volatiles from the emulsion. The sample was heated for 3 min to a temperature of 60 °C, followed by extraction for 45 min while agitating the sample at 500 rpm. Headspace volatiles were extracted using a 50/30 μm DVB/CAR/PDMS SPME fiber (Supelco, Bellafonte, PA, USA) installed on a CTC Combi Pal (CTC Analytics AG, Zwingen, Switzerland). Volatiles were desorbed in the injection port of a gas chromatograph (HP 6890 Series; Hewlett Packard; Column: DB-1701, 30 m × 0.25 mm × 1.0 μm; J&W Scientific) for 60 s at 220 °C. The oven programme had an initial temperature of 35 °C for 3 min, increased with 3.0 °C min^{-1} until 140 °C, with 5.0 °C min^{-1} until 170 °C and with 10.0 °C min^{-1} until 240 °C, where the temperature was kept steady for 8 min.

The individual compounds were analysed by mass spectrometry (HP 5973 inert mass-selective detector; Agilent Technologies, Palo Alto, CA, USA; Electron ionisation mode, 70 eV, mass to charge ratio scan between 29 and 200). From a comparison of chromatograms from nonoxidised and oxidised samples, the

following volatiles were selected for quantification: pentanal (derived from the oxidation of n-6 PUFA), heptanal (derived from the oxidation of n-9 PUFA) and 2,4-heptadienal (derived from the oxidation of n-3 PUFA). In the chromatogram, two peaks were identified as 2,4-heptadienal. On the basis of results from previous studies of these two peaks (not published), the two isomers were tentatively identified as *t,c*-2,4-heptadienal and *t,t*-2,4-heptadienal. Calibration curves were made by dissolving the compounds in rapeseed oil followed by the addition of an amount corresponding to 0–1000 ng of each of the compounds to 1 g emulsion or oil. As emulsions made with different emulsifiers retained the compounds differently, calibration curves were made for the oil and for emulsions with MPL75, MPL20, LEC and WPI, individually. The latter was used for quantifying both protein-based emulsions. Calibration curves for pentanal and heptanal were parallel-shifted in order to obtain positive values; thus, amounts of volatiles are not given as exact values. Measurements were taken in triplicate on each sample. A fibre breakage necessitated the use of a second fibre to measure volatiles after day 0, and that fibre had another response than the first fibre. Therefore, data from day 0 are only used to compare individual emulsions and not changes over time. Because of the aforementioned facts, volatiles data are only shown for day 2–7.

Statistical analyses

Data were analysed by one- or two-way analysis of variance, with Bonferroni multiple comparison test as post-test (GraphPad Prism, version 4.03; GraphPad Software, Inc, La Jolla, CA, USA). All references to significant differences ($P < 0.05$) between samples or between sampling times are based on this statistical analysis of data.

Results

Characterisation of emulsions and emulsifiers

The quality and composition of the emulsifiers

The quality of the individual emulsifiers was assessed by PV. Results are listed in Table 1. PVs ranged from 1.3 meq peroxides per kg oil in LEC to 21.9 meq peroxides per kg oil in MPL75. The oil contents for LEC and MPL75 were both above 80%, whereas it was approximately 24% for MPL20 and below 1% for each of the two protein-based emulsifiers (Table 1). Fatty acid compositions for the PL-based emulsifiers showed that MPL75 contained about 60% saturated fatty acids (SFA), whereas MPL20 contained approximately the same amount of saturated and unsaturated fatty acids (SFA ~45%; MUFA/PUFA ~49%). In contrast, LEC contained about 80% unsaturated fatty acids (Table 1). Analysis of PL classes showed that MPL75 contained mainly sphingomyelin (38.64%) and phosphatidylcholine (21.03%). However, the measured amount of sphingomyelin was approximately 25% higher than that reported in the data sheet, and consequently, the amount of mainly phosphatidylethanolamine was measured to be lower (13.08%). The main constituents of MPL20 were phosphatidylcholine (27.70%), phosphatidylethanolamine (25.55%) and sphingomyelin (20.59%). In this case, the measured amounts of the different PL classes corresponded well with that reported in the data sheet. LEC contained mainly phosphatidylcholine (29.70%) and phosphatidylinositol (28.50%) (Table 1).

pH, viscosity, zeta potential and mean droplet sizes

For emulsions aimed at a pH of 4.5, the actual pH was measured to range from 3.8 to 4.7, whereas for the emulsions aimed at a pH of 7.0, the actual pH was measured to be between 6.0 and 7.1 (Table 3). The difference between the actual pH and the intended pH was found to be because of the different influence of the addition of sodium azide on pH, depending on the type of emulsifier used.

Initial viscosities were measured to compare differences between emulsions. Viscosities ranged from 146 cp for LEC_low to 3650 cp for 1.4CAS_low (data for LEC_high are missing) (Table 3). LEC_low had a consistency like milk, and 1.4CAS_low had a consistency like a mayonnaise-based dressing. The viscosities of the emulsions increased in the order: LEC_low^{a} ≤ MPL20_low^{a,b} ≤ MPL75_low^{b} < MPL75_high^{c} = 1.4CAS_high^{c} < MPL20_high^{d} = WPI_high^{d} < WPI_low^{e} < CAS_high^{f} < 1.4CAS_low^{g} (different letters indicating significant differences on a 95% level). Hence, viscosities increased with increasing pH for PL-based emulsions, whereas the opposite was the case for protein-based emulsions.

The zeta potential was negative for all emulsions except WPI_low and MPL20_low that had zeta poten-

Table 3 Physico-chemical data for the emulsions

Emulsion	pH	Viscosity (cp)	Zeta potential (mV)	D[3,2], day 1 (μm)
WPI_low	3.8	2566 ± 8	45.6 ± 2.5	12.09 ± 0.07
WPI_high	7.1	1955 ± 121	−31.4 ± 0.6	20.86 ± 0.38
1.4CAS_low	4.2	3650 ± 139	–	19.23 ± 3.41
1.4CAS_high	7.0	1230 ± 3	−40.3 ± 0.9	21.40 ± 0.05
CAS_high	7.1	3075 ± 16	−40.0 ± 0.6	8.69 ± 0.10
MPL20_low	4.1	459 ± 7	27.9 ± 2.8	20.39 ± 6.82
MPL20_high	7.0	1834 ± 37	−31.3 ± 0.6	8.19 ± 0.05
MPL75_low	4.5	479 ± 10	−38.9 ± 2.4	43.76 ± 3.28
MPL75_high	6.0	1008 ± 17	−58.1 ± 1.4	16.25 ± 0.42
LEC_low	4.7	146 ± 4	−58.7 ± 2.7	50.81 ± 0.60
LEC_high	6.5	–	−60.2 ± 1.1	13.91 ± 0.87

Please refer to Table 2 for sample codes.

tials of 45.6 and 27.9 mV, respectively. No data are available for 1.4CAS_low, but because it has been shown in previous studies that emulsions with sodium caseinate at a low pH have a positive surface charge (Haahr & Jacobsen, 2008), it is assumed that this is also the case in the present study. For WPI_high and MPL20_high, the zeta potential was approximately −30 mV; for 1.4CAS_high, CAS_high and MPL75_low, the zeta potential was approximately −40 mV; and for the two emulsions with LEC and MPL75_high, the zeta potential was approximately −60 mV (Table 3).

The mean droplet sizes were in the order MPL20_high^{a} = CAS_high^{a} ≤ WPI_low^{a,b} = LEC_high^{a,b} = MPL75_high^{a,b} = 1.4CAS_low^{a,b} ≤ MPL20_low^{b} = WPI_high^{b} = 1.4CAS_high^{b} < MPL75_low^{c} = LEC_low^{c}. The droplet size thus seemed to increase with decreasing pH for PL-based emulsions, whereas the opposite was the case for protein-based emulsions.

Lipid oxidation in neat oil

At day 0, PV in the neat oil sample was below the detection limit of 0.1 meq peroxides per kg oil. During storage, PV increased significantly to 0.6 meq peroxides per kg oil at day 7, with the main increase between day 5 and 7 (Fig. 1). No increase in the concentration of any of the volatiles was observed during storage.

Lipid oxidation in emulsions prepared with proteins

Peroxide values

PVs were in general low in all emulsions despite the addition of iron. An unexplainable, but significant decrease in PV for the emulsion with whey protein at pH 4.5 (WPI_low) and the emulsion with the low concentration of sodium caseinate at pH 7.0 (1.4CAS_high) between day 0 and 2 gave an overall

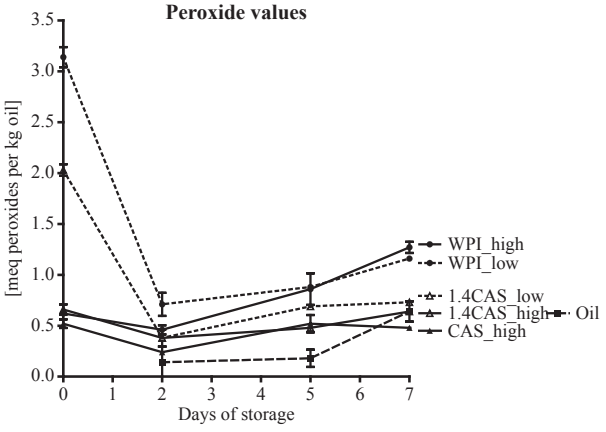

Figure 1 Peroxide values in neat oil and in emulsions prepared with protein-based emulsifiers.

significant decrease in PV from day 0 to 7 for the two emulsions as well. However, from day 2 to 7, WPI_low significantly increased from 0.7 to 1.2 meq peroxides per kg oil. PV did not change significantly in any of the emulsions at pH 7.0 with sodium caseinate (1.4CAS_high and CAS_high) throughout storage nor in the emulsion at pH 4.5 (1.4CAS_low) between day 2 and 7 (Fig. 1). The only emulsion made with protein that had a significant increase in PV throughout storage was the emulsion with whey protein at pH 7 (WPI_high). WPI_high increased from 0.6 to 1.3 meq peroxides per kg oil. At day 7, the PVs increased in the order CAS_high^{a} = neat oil^{a} = 1.4CAS_high^{a} = 1.4CAS_low^{a} < WPI_low^{b} = WPI_high^{b}.

Secondary volatile oxidation products

For emulsions prepared with whey proteins, WPI_low significantly increased in heptanal and *t,t*-2,4-heptadienal from day 2 to 7 and WPI_high significantly increased in the two 2,4-heptadienals (Table 4). The emulsion with a low concentration of sodium caseinate at pH 7 (1.4CAS_high) significantly increased in *t,t*-2,4-heptadienal during storage. Surprisingly, a significant decrease in heptanal and *t,c*-2,4-heptadienal was observed for the similar emulsion at pH 4.5 (1.4CAS_low). No significant increase in volatiles was observed for the emulsion with the high concentration of sodium caseinate (CAS_high) during storage.

When comparing individual emulsions prepared with proteins, emulsions at pH 4.5 (1.4CAS_low and WPI_low) generally had a significantly higher amount of the four measured volatiles than emulsions at pH 7.0 (1.4CAS_high, CAS_high and WPI_high) (Table 4). At pH 4.5, the emulsion with WPI had significantly more heptanal and *t,t-2*,4-heptadienal at day 7 than 1.4CAS_low, whereas 1.4CAS_low had significantly more *t,c*-2,4-heptadienal at days 2 and 5 than WPI_low. At no other sampling time point, significant differences were observed between WPI_low and 1.4CAS_low for any of the four volatiles. At pH 7.0, no significant differences were observed between the two emulsions with CAS for any of the volatiles. However, WPI_high had a higher content of pentanal at days 2, 5 and 7 and furthermore a higher content of *t,t*-2,4-heptadienal at day 7 than the two CAS emulsions as well as a higher content of heptanal than 1.4CAS_high at day 7. Taken together, WPI emulsions seemed less oxidatively stable than CAS emulsions with the most pronounced differences at high pH.

Lipid oxidation in emulsions prepared with phospholipids

Peroxide values

For emulsions made with PLs, the initial PVs were 0.8–1.0 meq peroxides per kg oil for emulsions made with LEC or MPL20, but 2.7–2.8 meq peroxides per kg oil

Table 4 Secondary volatile oxidation products in emulsion prepared with proteins (ng g^{-1})

	Pentanal			Heptanal		
	Day 2	**Day 5**	**Day 7**	**Day 2**	**Day 5**	**Day 7**
WPI_low	$66 \pm 4^{b,c}$	$62 \pm 7^{l,m}$	59 ± 2^{x}	46 ± 1^{c}	$45 \pm 0^{k,l}$	$49 \pm 1^{y,**}$
WPI_high	51 ± 15^{b}	53 ± 11^{l}	58 ± 15^{x}	43 ± 1^{b}	43 ± 2^{k}	44 ± 1^{x}
1.4CAS_low	74 ± 11^{c}	72 ± 13^{m}	66 ± 2^{x}	46 ± 2^{c}	46 ± 1^{l}	$44 \pm 0^{x,*}$
1.4CAS_high	21 ± 1^{a}	33 ± 1^{k}	33 ± 2^{w}	$41 \pm 0^{a,b}$	43 ± 0^{k}	42 ± 1^{w}
CAS_high	19 ± 1^{a}	30 ± 2^{k}	28 ± 2^{w}	41 ± 0^{a}	43 ± 1^{k}	$42 \pm 1^{w,x}$
	***t,c*-2,4-Heptadienal**			***t,t*-2,4-Heptadienal**		
WPI_low	245 ± 24^{b}	210 ± 18^{l}	218 ± 4^{x}	102 ± 2^{b}	102 ± 1^{m}	$106 \pm 0^{y,***}$
WPI_high	114 ± 5^{a}	124 ± 11^{k}	$142 \pm 7^{w,*}$	91 ± 1^{a}	94 ± 3^{l}	$97 \pm 2^{x,***}$
1.4CAS_low	309 ± 44^{c}	252 ± 6^{m}	$233 \pm 7^{x,***}$	101 ± 3^{b}	101 ± 1^{m}	99 ± 1^{x}
1.4CAS_high	136 ± 0^{a}	138 ± 10^{k}	142 ± 2^{w}	90 ± 0^{a}	$93 \pm 0^{k,l}$	$94 \pm 0^{w,**}$
CAS_high	123 ± 8^{a}	127 ± 3^{k}	142 ± 5^{w}	90 ± 1^{a}	92 ± 0^{k}	92 ± 0^{w}

Please refer to Table 2 for sample codes. For each volatile, letters indicate significant differences between samples at individual sampling days. *Significant changes from day 2 to day 7. The values for pentanal and heptanal are not exact because of a parallel shift of the calibration curves making the axis arbitrary.

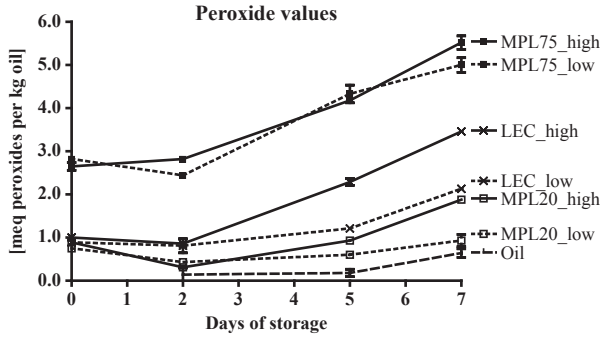

Figure 2 Peroxide values in neat oil and in emulsions prepared with phospholipid-based emulsifiers.

for emulsions made with MPL75 (Fig. 2). Throughout storage, neat oil had the significantly lowest PV, followed by the group of emulsions made with LEC and MPL20, and the highest PV was observed in the emulsions made with MPL75. During storage, only MPL20_low did not show a significant increase in PV. The other emulsions had PVs between 1.9 and 5.5 meq peroxides per kg oil at day 7. The ranking order at day 7 was Neat oil^{a} = MPL20_low^{a} < MPL20_high^{b} = LEC_low^{b} < LEC_high^{c} < MPL75_low^{d} < MPL75_high^{e} (with letters indicating significant differences).

Secondary volatile oxidation products

The two emulsions prepared with the milk PL-rich emulsifier (MPL75) were the only emulsions with PL that had significant changes in volatiles during storage. They both increased in the two 2,4-heptadienals from day 2 to 7. Furthermore, for MPL75_high, the concentration of pentanal decreased during storage (Table 5).

At low pH, MPL75_low had a significantly higher amount of all volatiles at all sampling time points, except for *t,c*-2,4-heptadienal at day 0, compared with the other emulsions (Table 5). Comparison of LEC_low and MPL20_low showed that LEC_low had a higher content of pentanal at days 2, 5 and 7, but otherwise they did not differ significantly from each other in the amounts of any of the volatiles measured. Generally, the same observations were done at high pH. For none of the volatiles, MPL20_high and LEC_high were significantly different, but MPL75_high generally had a higher amount of all volatiles than the other two emulsions.

When comparing emulsions made with the same emulsifier at different pH values, MPL20_low and MPL20_high did not differ from each other for any of the four volatiles measured. LEC_low had a significantly higher content of pentanal at days 2, 5 and 7 than LEC_high, but otherwise no differences were observed. For the two emulsions with MPL75, there was less consistency, but in the later part of the storage period MPL75_low was the most oxidised.

It should, though, be mentioned that the lack of significant differences between LEC and MPL20 samples stems from the influence of the MPL75 samples in the statistical model. MPL75 samples had much higher values and higher standard deviations. Hence, when leaving MPL75 samples out of the statistic calculations, the following results were obtained. Comparison of the two emulsions prepared with LEC, showed that LEC_low had a significantly higher content of all volatiles after day 0. Almost the same was observed for MPL20 emulsions, where MPL20_low had a signif-

Table 5 Secondary volatile oxidation products in emulsion prepared with phospholipids (ng g^{-1})

	Pentanal			Heptanal		
	Day 2	Day 5	Day 7	Day 2	Day 5	Day 7
LEC_low	463 ± 10^{b}	350 ± 23^{l}	391 ± 24^{x}	20 ± 1^{a}	26 ± 1^{k}	32 ± 1^{w}
LEC_high	145 ± 17^{a}	169 ± 10^{k}	199 ± 12^{w}	11 ± 1^{a}	15 ± 1^{k}	22 ± 2^{w}
MPL20_low	76 ± 5^{a}	99 ± 10^{k}	112 ± 6^{w}	55 ± 0^{a}	69 ± 2^{k}	84 ± 1^{w}
MPL20_high	76 ± 4^{a}	104 ± 9^{k}	105 ± 5^{w}	30 ± 0^{a}	29 ± 1^{k}	31 ± 1^{w}
MPL75_low	849 ± 223^{c}	702 ± 242^{m}	718 ± 166^{y}	2033 ± 303^{c}	1978 ± 337^{m}	1869 ± 235^{y}
MPL75_high	1033 ± 81^{d}	676 ± 18^{m}	$684 \pm 40^{y,***}$	1585 ± 49^{b}	1543 ± 58^{l}	1400 ± 42^{x}
	t,c-2,4-Heptadienal			*t,t*-2,4-Heptadienal		
LEC_low	137 ± 5^{a}	152 ± 18^{k}	207 ± 9^{w}	88 ± 1^{a}	92 ± 1^{k}	98 ± 1^{w}
LEC_high	97 ± 5^{a}	126 ± 6^{k}	178 ± 1^{w}	78 ± 0^{a}	83 ± 0^{k}	88 ± 1^{w}
MPL20_low	220 ± 4^{a}	193 ± 5^{k}	249 ± 11^{w}	24 ± 0^{a}	25 ± 0^{k}	33 ± 1^{w}
MPL20_high	132 ± 5^{a}	136 ± 5^{k}	189 ± 10^{w}	16 ± 0^{a}	18 ± 0^{k}	28 ± 1^{w}
MPL75_low	497 ± 87^{b}	1063 ± 197^{m}	$2164 \pm 296^{y,***}$	2101 ± 259^{c}	5307 ± 931^{m}	$8931 \pm 969^{y,***}$
MPL75_high	454 ± 18^{b}	748 ± 73^{l}	$1371 \pm 76^{y,***}$	1419 ± 23^{b}	2255 ± 165^{l}	$3606 \pm 127^{x,***}$

Please refer to Table 2 for sample codes. For each volatile, letters indicate significant differences between samples at individual sampling days. *Significant changes from day 2 to day 7. The values for pentanal and heptanal are not exact because of a parallel shift of the calibration curves making the axis arbitrary.

icantly higher content of three of the four volatiles measured after day 0, namely heptanal and the two 2,4-heptadienals. These findings suggest that a low pH also promoted volatiles' formation in emulsions with LEC and MPL20, similarly to what was observed for MPL75. Furthermore, a comparison of LEC samples vs. MPL20 samples showed that LEC samples in general had a significantly higher content of pentanal and *t,t*-2,4-heptadienal, whereas MPL20 samples in general had a significantly higher content of heptanal and *t,c*-2,4-heptadienal. This was observed at almost all sampling time points.

Discussion

Lipid oxidation in emulsions vs. in neat oil

Generally, conclusions made on volatiles data supported conclusions from PV data, showing that all emulsions oxidised more than neat oil. Similar results were obtained by Frankel *et al*. (2002), who compared 5% fish oil or algae oil-in-water emulsions to neat oil. However, in 70% emulsions prepared without iron addition, emulsions oxidised less or equally to neat oil, when prepared with milk proteins at pH 7 (Horn *et al*., 2011). In neat oil, transition metal ions are expected to localise at the interface between oil and air (either at the oil surface or on small air bubbles in the oil) (Frankel, 2005), or they may localise in reverse micelles present because of trace levels of, for example, free fatty acids or mono- and diacylglycerols (Chaiyasit *et al*., 2007). Hence, the transition metal ions are expected to be in close contact to the lipids in neat oil. On the contrary, in emulsions, transition metal ions are expected to localise at the interface between oil and water or in the water phase owing to their hydrophilic nature (Frankel, 2005). Furthermore, in emulsions, some emulsifiers, such as sodium caseinate, are expected to create a thick interfacial barrier (Hunt & Dalgleish, 1994) and thereby reduce direct contact between the pro-oxidative transition metal ions and lipids. However, when comparing neat oil and emulsions in the present study, results indicate that other factors than the above-mentioned may affect lipid oxidation more, for example the physical stress the lipids have been put through during emulsion production. Even though precautions have been taken to minimise the risk of initiating lipid oxidation during emulsion production (limited oxygen availability and cooling), the emulsions have undergone harsher production conditions as compared to the neat oil that has been poured directly into glasses for storage. This is confirmed by results for the initial PV, which was much lower in the neat oil (< 0.1 meq peroxides per kg oil) than in the emulsions (e.g. CAS_high: 0.5 meq peroxides per kg oil; 1.4CAS_high: 0.7 meq peroxides per kg oil). Hence, the presence of significant levels of lipid hydroperoxides in emulsions, which in the present study reacted with iron added after emulsification, seemed to explain the different order of oxidative stability for emulsions vs. neat oils compared with the order for emulsions with no iron added (Horn *et al*., 2011).

Lipid oxidation in emulsions prepared with proteins

Emulsions prepared with proteins at low pH oxidised more than emulsions prepared at high pH, especially

when considering volatiles data. At high pH, emulsions with WPI oxidised more than emulsions with CAS. At low pH, data were less consistent. No difference was observed between concentrations of CAS.

Concentration of emulsifier (1.4CAS_high vs. CAS_high)
When comparing the two emulsions with different concentrations of casein prepared at pH 7.0, no significant differences between them were observed. 1.4CAS_high had a significant increase in *t,c*-2,4-heptadienal between day 2 and 7, but otherwise no increases in neither volatiles nor PV were observed for the two emulsions. As mentioned earlier, CAS is expected to protect the emulsion against iron-mediated oxidation by its metal-chelating ability (Hu *et al*., 2003a) and/or by creating a thick physical barrier around the droplets (Hunt & Dalgleish, 1994). Because emulsions with different emulsifier contents oxidised to a similar extent, it is suggested that all metal ions were chelated even at a casein concentration as low as 1.4%. Therefore, no further protection was gained when increasing the amount of casein to 2.8%. This is supported by a study by Villiere *et al*. (2005) on the oxidative stability of sodium caseinate–stabilised emulsions where the iron-binding capacity of casein was studied within a concentration range covering the range used in the present study. Villiere *et al*. (2005) showed that when 0.7–5.2 mg L^{-1} iron was added to a solution of 1 g L^{-1} sodium caseinate, nearly all the metal was bound by the proteins. Interestingly, droplet sizes and viscosities varied significantly between the two emulsions, but apparently these differences did not result in different oxidative stabilities.

The difference between two types of protein (WPI vs. CAS)
When comparing the two emulsions with WPI and CAS prepared at pH 7, results indicated that the WPI emulsion was more oxidised than the CAS emulsion. At pH 4.5, results were less consistent, but there was a tendency towards a better oxidative stability of 1.4CAS_low than that of WPI_low.

The better protection provided by CAS is expected to be caused by its content of amino acid residues with antioxidative properties. In a previous study, Faraji *et al*. (2004) compared the metal-chelating capacity of CAS and WPI and showed that CAS binds 5.3-fold more iron than WPI. The greater metal-chelating ability of CAS was attributed to its higher content of phosphorylated serine residues. Thus, CAS has better prerequisites for protecting the oil droplets against iron-mediated lipid oxidation than WPI. In similar emulsions prepared without iron addition, the difference in lipid oxidation between WPI and CAS emulsions was less pronounced (Horn *et al*., 2011), confirming the importance of metal chelation by CAS in the present study. At day 1, droplet sizes were smaller in the WPI-stabilised emulsion than in the CAS emulsion at low pH, but were similar in WPI and 1.4CAS emulsions at high pH. Moreover, the rank order for viscosities between protein-stabilised emulsions reversed when pH was increased from 4.5 to 7. None of these pH-induced changes in physical structure and viscosity affected the rank order for lipid oxidation to any significant extent. This finding is in agreement with the conclusion made from the comparison of 1.4CAS_high and CAS_high. Taken together, this may suggest that droplet size and viscosity are less important for oxidation in protein-stabilised emulsions.

Changing pH
When comparing 1.4CAS_low with 1.4CAS_high and WPI_low with WPI_high, emulsions prepared at low pH were generally more oxidised than emulsions prepared at high pH, and an increased oxidation could be caused by the higher solubility of iron at low pH. However, other studies on both 10% and 70% oil-in-water emulsions with casein (Haahr & Jacobsen, 2008; Horn *et al*., 2011) showed similar results irrespective of iron addition. In addition, contradictory results to the above-mentioned have been obtained in studies on the influence of surface charge on lipid oxidation (Donnelly *et al*., 1998; Hu *et al*., 2003a). In the latter mentioned studies, emulsions were more oxidatively stable at low pH, and this was related to the positive surface charge of the oil droplets caused by the proteins that were below their isoelectric point. Thus, a repulsion of cationic transition metal ions was expected to cause a decrease in lipid oxidation at low pH. Horn *et al*. (2011) explained these opposing results with a possible preferential adsorption of some protein components over others related to the emulsification equipment used. Hence, the relationship between pH, surface charge and lipid oxidation is not straightforward and other factors should be considered as well.

Lipid oxidation in emulsions prepared with phospholipids

When comparing emulsions at the same pH, protein-based emulsions generally tended to oxidise less than emulsions prepared with PLs, but more than neat oil. For emulsions prepared with PLs, a similar ranking of the emulsions was observed at both low and high pH. Hence, emulsions made with MPL20 were the least oxidised, and emulsions with MPL75 were the most oxidised, with emulsions made with LEC in between. The conclusions made on the ranking of MPL20 and LEC are mainly based on PV because the pattern was less clear for the volatiles.

Emulsions prepared with MPL20
Among the emulsions prepared with PLs, MPL20 emulsions were the most oxidatively stable, as also

observed in 70% emulsions prepared without iron addition (Horn *et al*., 2011). This was somewhat surprising because LEC contained much more phosphatidylinositol than MPL20, and phosphatidylinositol has the ability to inactivate metal ions (Pokorný, 1987). However, LEC also had a much higher content of the oxidatively unstable PUFAs (approximately 56%) than MPL20 (approximately 13%). This could therefore partly explain the increased oxidation in the samples with LEC. In addition, the better oxidative stability of MPL20 emulsions could also be attributed to the proteins present in this emulsifier. In the data sheet for MPL20, it is reported that it contains 53.8% milk proteins, which may have antioxidative properties as previously suggested (Hu *et al*., 2003b; Faraji *et al*., 2004; Ries *et al*., 2010). However, the antioxidative properties of proteins might depend on the partitioning of the proteins between the interface and water phase. Thus, a prerequisite for the formation of a thick interfacial barrier (Hunt & Dalgleish, 1994) and repulsion of metal ions (Hu *et al*., 2003a) from the interface would be that proteins are present at the interface, whereas the metal-chelating properties of proteins have been mainly attributed to proteins present in the water phase (Faraji *et al*., 2004).

It is assumed that the positive zeta potential in MPL20_low was attributable to proteins at the interface, because a surface with mainly PLs as the one in MPL75_low had a negative zeta potential. Moreover, confocal microscopy of a similar emulsion with MPL20 (prepared without iron addition at pH 7.0) showed that proteins were present in the water phase (Horn *et al*., 2011). Thus, it is expected that emulsions with MPL20 have proteins both at the interface and in the water phase. For the partitioning of PLs, a study on the addition of egg-yolk lecithin to casein-stabilised oil-in-water emulsions showed that lecithin changed the thickness of the absorbed layer of casein. Therefore, it was suggested that PLs were adsorbed at the interface taking up space into which the caseins would normally have partitioned, and that this in turn would lead to the protrusion of the caseins into the water phase instead (Fang & Dalgleish, 1993; Dalgleish, 1996). The combination of proteins and PLs as in MPL20 might therefore be important for the oxidative stability of emulsions made with this emulsifier. Further investigations must, though, be done to determine the partitioning of both PLs and proteins between the water phase and the interfacial layer and the structure of the latter, when a combination of proteins and PLs is used as emulsifier.

The 53.8% proteins in MPL20 are expected to be milk proteins (casein and whey protein), and initial viscosities in the emulsions made with either WPI or CAS were shown to increase when pH was decreased, possibly due to pH-dependent structural changes in the proteins. Despite this, the viscosity of MPL20_high was almost fourfold that of MPL20_low. It could be speculated that during the production of MPL20, the milk proteins have been modified in a way that makes them react differently to a decrease in pH than WPI and CAS do on an individual basis. The lower viscosity at low pH possibly made the emulsion with MPL20 less oxidatively stable than the similar emulsion at high pH with a higher viscosity. On the other hand, neither the surface charge nor the droplet size seemed to influence the oxidative stability of emulsions with MPL20. Thus, the slightly less oxidatively stable MPL20_low had a positive zeta potential and much larger droplet sizes than MPL20_high that also had a negative zeta potential. Thus, a possible repulsion of cationic metal ions owing to a positive surface charge as previously shown (Hu *et al*., 2003a) did not contribute to a better oxidative stability of MPL20_low, neither did larger droplet sizes.

Emulsions prepared with LEC or MPL75

A comparison of emulsions prepared with LEC and MPL75, which are both emulsifiers with a lipid content of more than 80%, showed that emulsions prepared with MPL75 oxidised faster than emulsions prepared with LEC, irrespective of pH. This was despite a much higher content of the less oxidatively stable PUFA in LEC (56.0% PUFA) than in MPL75 (4.0% PUFA). However, the emulsifier MPL75 itself was more oxidised (PV 21.9 meq peroxides per kg oil) than LEC (PV 1.3 meq peroxides per kg oil), and consequently, emulsions with MPL75 also had a higher initial PV than the other emulsions. Hence, the combination of already existing lipid peroxides and the addition of transition metal ions led to an extensively accelerated lipid oxidation in emulsions prepared with MPL75, as compared to similar emulsions prepared without iron addition in the study by Horn *et al*. (2011). Already at day 0, the concentrations of the volatiles measured were higher in the present study as compared to the study on emulsions prepared without iron addition. Furthermore, despite the much longer storage period in the latter, much higher concentrations of all the measured volatiles were observed at day 7 when iron was added than at day 42 when iron was not added. In addition, the differences in the saturation of MPL75 and LEC could influence the structure of the PL at the interfacial layer and thereby affect the contact between transition metal ions and lipids/lipid hydroperoxides in the oil droplets/at the interface differently.

For both MPL75 and LEC, droplet sizes were largest and viscosities lowest at low pH (although no viscosity data were available for LEC_high). Moreover, these emulsions had lower PVs but higher contents of volatiles than the corresponding emulsions at high pH. In general, no clear relationship between droplet size, viscosities and oxidation was thus observed for these emulsions. However, it cannot be ruled out that the

large droplet size in low pH emulsions was responsible for the low PVs in these emulsions. The possible role of droplet sizes for PL emulsions was not seen in protein-based emulsions. In the latter emulsions, the structure of proteins at the interface and/or the presence of metal-chelating proteins in the aqueous phase seem to be more important.

Conclusions

In the present study, it was shown that the type of emulsifier used for preparing 70% fish oil-in-water emulsions greatly influenced iron-mediated lipid oxidation. Emulsions prepared with proteins tended to oxidise less than emulsions prepared with PLs, and all emulsions oxidised more than neat oil. For emulsions prepared with proteins, lipid oxidation was faster at low pH than at high pH. Furthermore, emulsions prepared with WPI oxidised more than emulsions prepared with CAS, and the differences were more pronounced at high pH. The better oxidative stability of CAS emulsions was suggested to be because of its content of metal-chelating proteins. Among emulsions prepared with PLs, emulsions with MPL20 oxidised the least, LEC slightly more, and emulsions with MPL75 the most. The better stability of MPL20 emulsions was suggested to be because of a favourable combination of proteins and PL.

Results demonstrate that many factors are to be taken into consideration when working with high-fat fish oil-in-water emulsions, especially when the emulsions are going to be used as delivery systems in foods with a high metal ion content. Furthermore, it is important to beware that emulsions prepared with a high metal ion content oxidise more than neat oil, despite the use of, for example, a metal-chelating emulsifier such as casein.

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PAPER III

The choice of homogenisation equipment affects lipid oxidation in emulsions

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The choice of homogenisation equipment affects lipid oxidation in emulsions

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ABSTRACT

Milk proteins are often used by the food industry because of their good emulsifying properties. In addition, they can also provide oxidative stability to foods. However, different milk proteins or protein components have been shown to differ in their antioxidative properties, and their localisation in emulsions has been shown to be affected by the emulsification conditions. The objective of this study was to investigate the influence of homogenisation equipment (microfluidizer vs. two-stage valve homogeniser) on lipid oxidation in 10% fish oil-in-water emulsions prepared with two different milk proteins. Emulsions were prepared at pH 7 with similar droplet sizes. Results showed that the oxidative stability of emulsions prepared with sodium caseinate was not influenced by the type of homogeniser used. In contrast, the type of homogenisation equipment significantly influenced lipid oxidation when whey protein was used as emulsifier, with the microfluidizer resulting in lower levels of oxidation.

1. Introduction

Lipid oxidation in emulsions is expected to be initiated at the interface between oil and water. The emulsifier used to build the interfacial layer might therefore be crucial for the resulting oxidative stability. The type of emulsifier determines the structure and thickness of the interfacial layer (Hunt & Dalgleish, 1994a) and emulsifiers can furthermore have different antioxidative properties (Faraji, McClements, & Decker, 2004; Haahr & Jacobsen, 2008). Moreover, the oil droplet size has in some cases been shown to influence lipid oxidation (Jacobsen et al., 2000; Kargar, Spyropoulos, & Norton, 2011; Sun & Gunasekaran, 2009). This was explained by the fact that smaller droplets have a larger contact area between prooxidative transition metal ions in the water phase and lipid hydroperoxides present at the oil–water interface. Oil droplet size is known to be influenced by different factors such as the type and concentration of the emulsifier used (Horn et al., 2011; Qian & McClements, 2011), the emulsification equipment and the homogenisation conditions (Dalgleish, Tosh, & West, 1996; Let, Jacobsen, Sorensen, & Meyer, 2007; Robin, Remillard, & Paquin, 1993).

The bovine milk proteins, caseins and whey proteins, are commonly used as emulsifiers by the food industry because of their good emulsifying and physically stabilising properties. Whereas casein consists of mainly four different components (α_{s1}-, α_{s2}-, β- and κ-casein), whey protein has two major protein components (α-lactalbumin and β-lactoglobulin). During emulsification they easily adsorb at the surface of the oil droplets with the more hydrophobic amino acid regions projecting into the water phase, and the less hydrophobic regions facing the oil phase (Krog, 2004). However, the surface hydrophobicities of individual protein components have been suggested to be of less importance for the adsorption rate and emulsifying abilities than the flexibility of the conformational structure (Shimizu, Kamiya, & Yamauchi, 1981). The caseins are very flexible in nature, due to the lack of higher structures (Creamer, 2003). Hence, in low concentration casein is expected to stretch over the oil droplet surface, whereas in high concentration the casein molecules are expected to form more compact structures at the interfacial layer and they therefore protrude longer into the water phase. This will result in the formation of interfacial layers ranging from 5 to 10 nm (Fang & Dalgleish, 1993). In contrast, the flexibility of whey proteins depend on a reduction of their tertiary and quartenary structures, as influenced by e.g. emulsion formation, protein concentration, pH or heating (Fang & Dalgleish, 1997, 1998; Lee, Lefèvre, Subirade, & Paquin, 2007).

In addition to the structure of the proteins, also the amino acid compositions of the two types of proteins give them different antioxidative properties. Caseins, but not whey proteins, contain several phosphorylated serine residues that have been suggested

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to possess metal chelating effects (Diaz, Dunn, McClements, & Decker, 2003; Hekmat & Mcmahon, 1998). In contrast whey proteins have more sulfhydryl groups that are suggested to scavenge free radicals (Elias, McClements, & Decker, 2005; Tong, Sasaki, McClements, & Decker, 2000).

If the pH of the emulsion is above or below the isoelectric point of the milk proteins, the interfacial layer will be electrically charged, which can prevent droplet flocculation by electrostatic repulsion. Moreover, the surface charge can influence lipid oxidation, with positive charge giving rise to reduced oxidation due to repulsion of the cationic transition metal ions (Hu, McClements, & Decker, 2003a).

In the industry two kinds of high pressure homogenisation systems are widely used: The valve homogeniser and the microfluidizer (Schultz, Wagner, Urban, & Ulrich, 2004). Both devices are mainly used for secondary homogenisation, meaning the disruption of larger droplets into smaller ones in an already prepared premix. The valve homogeniser has a pump that pulls the emulsion into a chamber on its backstroke, and then forces it through a narrow valve at the end of the chamber on its forward stroke. In the valve, intense disruptive forces will cause the larger droplets to break down into smaller ones. The microfluidizer also has a pump that drives the emulsion through to a chamber with a very small passage, in which the droplets are forced to collide, and thereby reduce in size (Schultz et al., 2004). A previous study on milk has shown that homogenisation in a microfluidizer resulted in a different location of the individual protein components at the interfacial layer or in the aqueous phase, when compared to homogenisation in a valve homogeniser (Dalgleish et al., 1996). Furthermore, structural differences in the milk proteins present at the interface were observed between milk homogenised in the two equipments.

Based on these previous studies, it was hypothesised that emulsification by different homogenisation equipments would influence the structure of the proteins at the interfacial layer and their partitioning into the aqueous phase, and that these differences would affect lipid oxidation. Furthermore, it was hypothesised that whey protein emulsions would oxidise more than casein emulsions when iron was added, due to the metal chelating effect of phosphorylated serine residues in casein. Thus, the aim of this study was to compare lipid oxidation in 10% fish oil-in-water emulsions prepared on a microfluidizer or a two stage high pressure valve homogeniser. Emulsions were made with either 1% sodium caseinate or whey protein isolate as emulsifier, and lipid oxidation was catalysed by iron addition. Emulsions were characterised, visually inspected using cryogenic transmission electron microscopy (cryo-TEM) and followed by oxidation stability studies.

2. Materials and methods

2.1. Materials

Commercial cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway) and stored at −40 °C until use. The fish oil was described by its fatty acid composition, content of tocopherols and peroxide value (PV). The fatty acid composition was determined by preparation of methyl esters (AOCS, 1998b) that was in turn analysed by gas chromatography (AOCS, 1998a). The content of the major fatty acids was (in area% of total fatty acids) as follows: 14:0 3.0, 16:0 8.9, 16:1(n−7) 8.2, 18:1(n−9) 16.0, 18:1(n−7) 5.2, 18:4(n−3) 2.5, 20:1(n−9) 11.6, 20:5(n−3) 9.3, 22:1(n−11) 6.1 and 22:6(n−3) 11.6. The levels of tocopherols were determined by HPLC (AOCS, 1998c). Tocopherol contents were 207 ± 16 μg α-tocopherol/g oil and 100 ± 1 μg γ-tocopherol/g oil. The initial PV of the fish oil was <0.1 meq peroxides/kg oil, as determined by the method described in Section 2.4.1. Sodium caseinate (Miprodan® 30) and whey protein isolate (Lacprodan® DI-9224) were kindly donated by Arla Foods Ingredients amba (Viby J, Denmark). Data sheets from Arla reported a protein content of 93.5% in sodium caseinate and 92% in whey protein isolate. All other chemicals and solvents used were of analytical grade.

2.2. Preparation of emulsions and sampling

Emulsions were prepared with 10.0% (w/w) fish oil, 89.0% (w/w) 10 mM sodium acetate imidazole buffer (pH 7.0) and either 1.0% (w/w) sodium caseinate or 1.0% (w/w) whey protein isolate. Emulsions were produced in batches of 600 g. Prior to emulsification the protein was dissolved in the buffer overnight, and fish oil was then added slowly during mixing at 16,000 rpm (Ystral mixer, Ballrechten-Dottingen, Germany). The fish oil was added during the first minute of mixing, and the total mixing time was 3 min. Secondary homogenisation was done either on a microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75 μm), or a two-valve high-pressure homogeniser (Panda 2K, GEA, Niro Soavi, Parma, Italy). The experimental design with sample code names, applied pressures and number of passes in the homogeniser is listed in Table 1. The differences in homogenisation pressures and number of passes were used to obtain equal droplet size distributions for emulsions prepared at the two different emulsification equipments by the same emulsifier. Emulsions were added 100 μM $FeSO_4$ to accelerate lipid oxidation and 0.05% (w/w) sodium azide to prevent microbial growth. Emulsions were stored in 100 ml Bluecap bottles at room temperature (19–20 °C) in the dark for 14 days. Samples were taken at day 0, 4, 7, 10 and 14. Measurements of viscosity and pH were done at day 0, droplet sizes were measured at day 1 and 14. Two emulsions of each type were prepared.

2.3. Characterisation of the emulsions

2.3.1. Droplet size, viscosity and pH

Droplets were measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). Emulsion was diluted in recirculating water (3000 rpm), until it reached an obscuration of 12–14%. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Measurements were made in duplicate. The initial emulsion viscosities were determined using a Brookfield viscometer Model RV DV II (Brookfield Engineering Labs. Inc.,

Table 1
Experimental design.

Emulsion	Emulsifier	Homogeniser	Pressure	No of passes[a]
CAS_M	Sodium caseinate	Microfluidizer	10.000 psi (69 MPa)	3
CAS_H	Sodium caseinate	Valve homogeniser	80/8 MPa	4
WPI_M	Whey protein isolate	Microfluidizer	10.000 psi (69 MPa)	3
WPI_H	Whey protein isolate	Valve homogeniser	50/5 MPa	3

[a] Number of passes run on the homogeniser.

Stoughton, MA, USA). The emulsions were measured using RV spindle no 1 rotating with 100 rpm, and measurements were done on 400 ml sample in a 600 ml beaker at room temperature. Determination of pH was performed during stirring of the emulsion. Viscosity and pH was determined once on each emulsion replicate.

2.3.2. Microscopy

The samples were prepared according to the procedure outlined by Bellare, Davis, Scriven, and Talmon (1988) by the following procedure: A small amount of sample (5 μl) was put on glow discharged lacey carbon film supported by a copper grid. The grids were blotted with filter paper in a humidity controlled chamber with relative humidity close to 100% to avoid evaporation of the sample during preparation and then plunged into liquid ethane. The vitrified samples were stored under liquid nitrogen until microscopy at −180 °C in a TEM (Philips CM120 BioTWIN Cryo) at 120 kV. Image analysis for distance between droplets was performed manually in Image J (http://rsb.info.nih.gov/ij/).

2.4. Measurements of lipid oxidation

2.4.1. Primary oxidation products – peroxide values

A lipid extract (a duplicate on each emulsion replicate) was prepared from each emulsion according to a modified form of the method described by Bligh and Dyer (1959) using 10 g emulsion and a reduced amount of solvent (30.0 ml methanol and chloroform, 1:1). Peroxide values were subsequently determined on this lipid extract, or directly on the oil used for preparation of the emulsions, by colorimetric determination of iron thiocyante at 500 nm as described by Shantha and Decker (1994). Measurements were made in duplicate.

2.4.2. Secondary oxidation products – SPME GC–MS

Approximately 1 g of emulsion was weighted out in a 10 ml vial (in triplicate on each emulsion replicate). The sample was heated for 5 min to a temperature of 60 °C. Extraction of headspace volatiles was done by the use of an 85 μm Carboxen/PDMS SPME fiber (Supelco, Bellafonte, PA, USA) installed on a CTC Combi Pal (CTC Analytics AG, Zwingen, Switzerland). Extraction was done at 60 °C for 45 min while agitating the sample at 500 rpm. Volatiles were desorbed in the injection port of a gas chromatograph (HP 6890 Series, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m × 0.25 mm × 1.0 μm; J&W Scientific, CA, USA) for 60 s at 230 °C. The oven programme had an initial temperature of 35 °C for 3 min, increasing with 3.0 °C/min until 140 °C, with 5.0 °C/min until 170 °C and with 10.0 °C/min until 240 °C, where the temperature was kept for 8 min. The individual compounds were analysed by mass-spectrometry (HP 5973 inert mass-selective detector, Agilent Technologies, Palo Alto, CA, USA). The MS was operating in the electron ionisation mode at 70 eV and mass to charge ratios between 29 and 200 were scanned. From a comparison of chromatograms from non-oxidised and oxidised samples, the following volatiles were selected for quantification: propanal, 2-butenal, 1-penten-3-one, pentanal, hexanal, 2-hexenal, heptanal, 4-heptenal, *t*,*t*-2,4-hexadienal, 2,4-heptadienal and 2,6-nonadienal. In the chromatograms two peaks were identified as 2,4-heptadienal. From previous studies of these two peaks (not published) it was concluded that they represent the two isomers *t*,*c*-2,4-heptadienal and *t*,*t*-2,4-heptadienal. Calibration curves were made by dissolving the compounds in rapeseed oil followed by the addition of an amount of this oil corresponding to 0–400 ng of the volatile compounds to 1 g emulsion made with WPI as emulsifier. Measurements were made in triplicate on each sample.

2.5. Statistical analyses

All data except microscopy data were analysed by one- or two-way analysis of variance (ANOVA) with Bonferronis multiple comparison test as post test (GraphPad Prism, version 4.03, GraphPad Software Inc., La Jolla, CA, USA). Sample replicates ($n = 2$) were tested by the use of average data from measurement replicates (n as stated in the method descriptions). Results from analyses that were only performed at one sampling time point were subjected to one-way ANOVA and results from analyses that were performed several times during storage were subjected to two-way ANOVA. All references to significant differences ($p < 0.05$) between samples or between sampling times, are based on this statistical analysis of data. The microscopy data for droplet distances were centred and tested pair-wise with the non-parametric two-sample Kolmogorov–Smirnov test. Confidence level was set to 0.05. Statistical analysis was performed with R (R version 2.14.0 Copyright © 2011 The R Foundation for Statistical Computing, ISBN 3-900051-07-0). Furthermore, oxidation data were subjected to multivariate data analysis (LatentiX, version 2.00, The MathWorks Inc., Frederiksberg, Denmark). A principal component analysis (PCA) was carried out with the eight emulsions as objects and peroxide values and volatiles data as variables (65 variables in total), with one variable representing the average of a given peroxide value or volatile at a specific sampling time point. Data were autoscaled in order to make the variables contribute equally to the model, and the PCA model was validated systematically segmented, according to replicates of emulsions.

3. Results

3.1. Characterisation of the emulsions

3.1.1. Viscosity, pH and droplet size

The initial viscosities of the emulsions did not differ significantly, and ranged from 12.3 to 14.4 cP (Table 2). pH values were determined to be 7.0–7.1 (Table 2). Droplet size distributions were monomodal and similar for all emulsions (data not shown), with only small variations in the mean droplet sizes. At day 1 mean droplet sizes were 0.126 and 0.130 μm for CAS_M and CAS_H, respectively, and 0.144 and 0.147 μm for WPI_M and WPI_H, respectively (Table 2). Thus, emulsions prepared with the same emulsifier were not significantly different from each other at day 1. A similar observation was done at day 14, even though the mean droplet size for WPI_M increased significantly during storage to 0.168 μm.

3.1.2. Microscopy

Cryo-TEM images of the four different emulsions are shown in Fig. 1. The oil droplets appear as well-defined round dark objects on a lighter background, which is the amorphous water phase. The thick dark bands in the micrographs are the lacey carbon film. It can be seen that the oil droplets are perfectly round shaped, and positioned in the film according to size. The distribution of the oil droplets according to size is a consequence of the constraints caused by the geometry of the vitrified liquid film which is a meniscus with the thickest part at the edge of the carbon film. The oil droplets were non-aggregated and especially for oil droplets emulsified with CAS the droplets all seemed to be evenly distributed in the film with mean distances of 28 ± 13 and 24 ± 12 nm for CAS_M and CAS_H, respectively (Fig. 1A and B). On the contrary, in the whey protein emulsions the droplets were located in a less ordered way with more varying distances between oil droplets. Mean distances were 19 ± 19 and 21 ± 17 nm for WPI_M and WPI_H, respectively (Fig. 1C and D). Differences in the distances were found to be statistically significant between emulsions with

Table 2
pH, viscosity and mean droplet sizes in the emulsions. For interpretation of sample names, please refer to Table 1.

Emulsion	pH[a]	Viscosity[a] [cp]	D[3,2], day 1[a] [μm]	D[3,2], day 14[a,b] [μm]
CAS_M	7.1 ± 0.0^{y}	14.4 ± 1.2^{xx}	0.126 ± 0.004^{x}	$0.130 \pm 0.007^{ns,x}$
CAS_H	$7.0 \pm 0.0^{x,y}$	12.3 ± 0.1^{x}	$0.130 \pm 0.001^{x,y}$	$0.128 \pm 0.001^{ns,x}$
WPI_M	$7.0 \pm 0.0^{x,y}$	13.1 ± 0.4^{x}	$0.144 \pm 0.001^{y,z}$	$0.168 \pm 0.005^{*ns,y}$
WPI_H	7.0 ± 0.0^{x}	12.9 ± 0.6^{x}	0.147 ± 0.011^{z}	$0.158 \pm 0.000^{ns,y}$

[a] For each column letters x–z indicate significant differences between samples ($p < 0.05$).
[b] Significant differences between day 1 and 14 are indicated by either ns: not significantly different or $^{*}p < 0.05$.

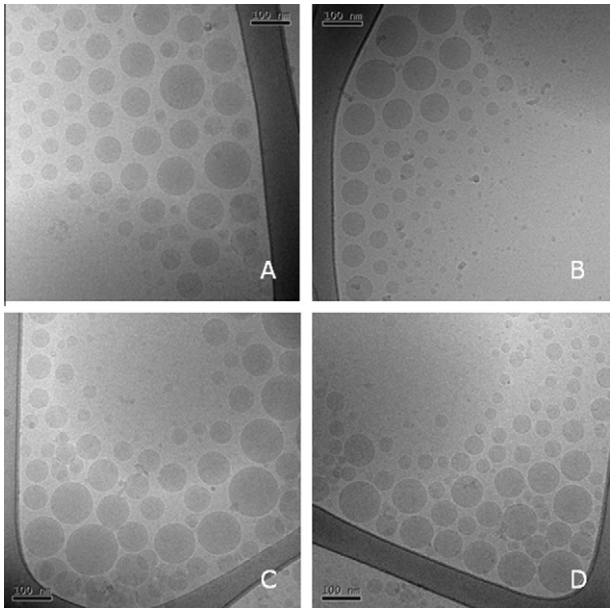

Fig. 1. Cryo-TEM images: (A) CAS_M, (B) CAS_H, (C) WPI_M and (D) WPI_H. In CAS emulsions, the oil droplets are located with a distance between them; whereas WPI emulsion droplets are located very close, almost overlapping.

different emulsifiers (p = 2E-12 between CAS_H and WPI_H; and p = 2E-15 between CAS_M and WPI_M) and the distances were thus significantly smaller for WPI emulsions. We found no statistically significant difference between CAS_H and CAS_M (p = 0.3). There was, however a significant difference between WPI_H and WPI_M (p = 9E-4). Diameters of the oil droplets in the micrographs were measured and D[3,2] was calculated to be 0.093, 0.102, 0.101 and 0.072 μm for CAS_M, CAS_H, WPI_M and WPI_H, respectively. These data were in good agreement with the data obtained from the laser diffraction measurement and in general confirmed that droplet sizes were similar in all four emulsions.

3.2. Lipid oxidation in emulsion

3.2.1. General

A PCA model was calculated to get a visual overview of all the variables at the same time, and to interpret correlations between the variables. In the PCA, the first principal component (PC1) explained 59% of the variance and the second principal component (PC2) explained 13%. The scores plot showed that all emulsions with CAS were located in the 2nd and 3rd quadrant and WPI emulsions in the 1st and 4th quadrant (Fig. 2A). CAS emulsions had more or less similar values for PC1 whereas the emulsions with WPI prepared on the valve homogeniser were located further to the right in the plot than the WPI emulsions prepared on the microfluidizer. Since PC1 explained most of the variation in the data, these findings suggest that homogenisation conditions affected whey protein emulsions but not casein emulsions.

3.2.2. Peroxide values

The PCA loadings plot showed a cluster of peroxide values in mainly the 2nd quadrant, indicating higher PVs in the emulsions with CAS than in the emulsions with WPI (Fig. 2B). The raw data confirmed this interpretation of the model (Fig. 3) for all other sampling time points than day 0. At day 0, the initial PVs were 2.5 and 3.7 meq peroxides/kg oil for WPI_H and WPI_M, respectively and 4.5 and 4.8 meq peroxides/kg oil for CAS_M and CAS_H, respectively. Later in the storage period, the differences between samples increased. CAS_M had significantly higher PV from day 4 to 14 than WPI_M, and CAS_H had a significantly higher PV than WPI_H at day 10. At day 14, the ranking order of the emulsions were $WPI\_M^{a} \leq WPI\_H^{a,b} \leq CAS\_H^{b,c} \leq CAS\_M^{c}$ (with letters indicating significant differences at a 95% level) (Fig. 3).

As indicated from the similar location at PC1 in the PCA plot, PVs in the two emulsions prepared with CAS did not differ significantly from each other throughout storage, however, they had higher PVs than WPI_M and WPI_H. In accordance with these observations from the PCA plot, a trend was observed from PV data, towards a higher PV in WPI_H than in WPI_M after day 4. However, from univariate statistics PV was only found to be significantly higher in WPI_H at day 7.

3.2.3. Secondary volatile oxidation products

The PCA loadings plot also showed a cluster of volatiles in mainly the 1st and 4th quadrant (Fig. 2B), indicating a higher concentration of secondary volatile oxidation products in the emulsions prepared with WPI than in the emulsions prepared with CAS. This was supported by raw data, showing a significant increase during storage in more of the quantified volatiles for WPI emulsions than for CAS emulsions, as illustrated in Fig. 4, which shows two of the volatiles quantified, 2-hexenal and *t,t*-2,4-heptadienal, representing oxidation products of omega-3 fatty acids. Furthermore, the increase in the concentrations of volatiles during storage was generally faster in WPI emulsions than in CAS emulsions as observed in Fig. 4b. All emulsions increased significantly in their contents of 2-butenal, 1-penten-3-one, 2-hexenal, 4-heptenal and *t,t*-2,4-heptadienal from day 0 to day 14. Both WPI emulsions furthermore increased significantly in pentanal and *t,c*-2,4-heptadienal, and WPI_H also in 2,4-hexadienal and 2,6-nonadienal. CAS_M was the only emulsion that had an increase in the content of propanal from day 0 to 14.

When comparing the two emulsions produced with the microfluidizer, WPI_M had a significantly higher content of 6 volatiles (2-butenal, pentanal, hexanal, 2-hexenal, heptanal and *t,t*-2,4-heptadienal) on day 10 than CAS_M. Furthermore, WPI_M also had a significantly higher content of 2-hexenal and *t,t*-2,4-heptadienal at day 4, 7 and 14. Otherwise no significant differences were observed between the two emulsions prepared on the microfluidizer. For the two emulsions prepared using the two-valve high pressure homogeniser, WPI_H generally had a significantly higher content than CAS_H of all the volatiles except propanal, hexanal and heptanal at most sampling time points after day 4. Thus, emulsions prepared using different emulsifiers were

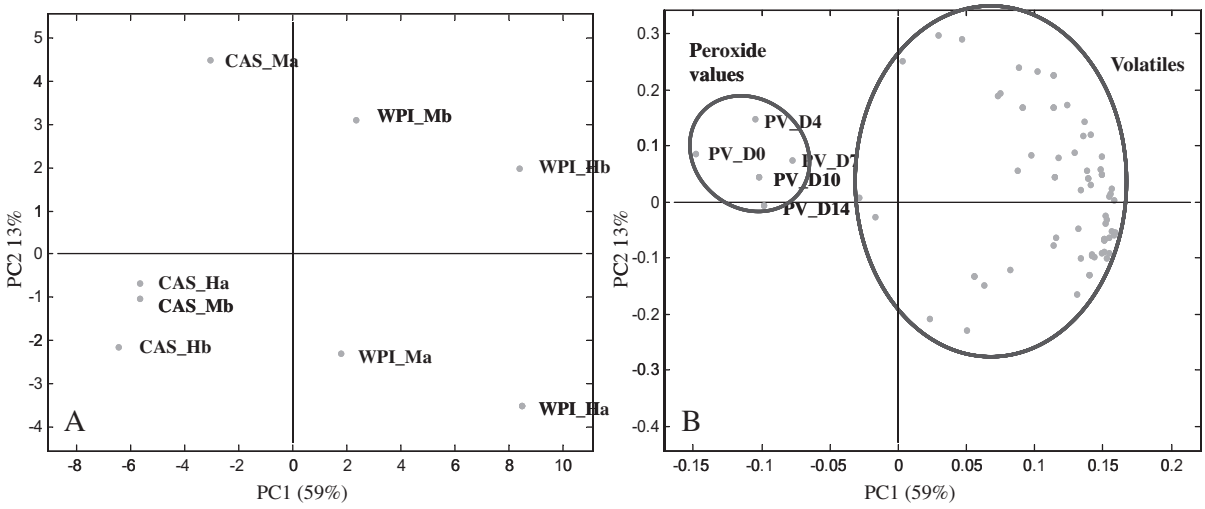

Fig. 2. PCA plot: scores plot to the left (A) with all sample replicates (a and b depict replicates of each treatment). Loadings plot to the right (B) with peroxide values mainly in the 2nd quadrant (D0–14 indicate sampling days 0–14), and volatiles mainly in the 1st and 4th quadrant.

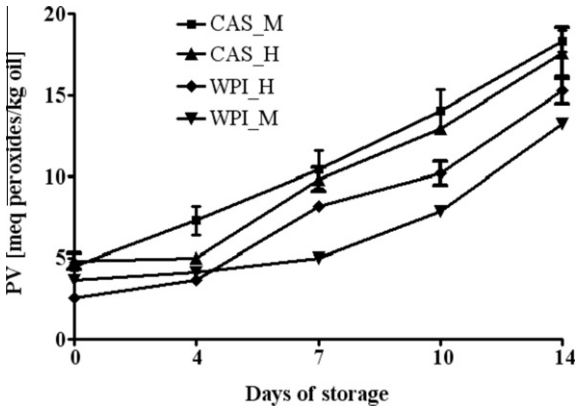

Fig. 3. Primary oxidation products: peroxide values in 10% (w/w) fish oil-in-water emulsions during storage at ~20 °C, prepared with 1% (w/w) caseinate (CAS) or whey protein isolate (WPI) as emulsifier on a microfluidizer (M) or a two-stage valve homogeniser (H). Data points represent means (n = 2) ± standard deviations.

more different when prepared on the valve homogeniser compared to the microfluidizer.

Comparison of the two emulsions prepared with CAS showed no significant differences at any sampling time point for any of the volatiles measured, which was in accordance with the observations from the PCA scores plot. On the other hand, amongst the two emulsions prepared with WPI, WPI_H had a significantly higher content of 8 (2-butenal, 1-penten-3-one, 2-hexenal, 4-heptenal, 2,4-hexadienal, *t,c*-2,4-heptadienal, *t,t*-2,4-heptadienal and 2,6-nonadienal) of the 12 measured volatiles, compared to WPI_M, in the last part of the storage period.

4. Discussion

4.1. Comparison of the two types of milk proteins: caseins and whey proteins

When comparing the emulsions prepared on the same homogenisation equipment with different milk proteins, WPI emulsions had lower PVs than CAS emulsions. However, WPI emulsions had a significantly higher content of most secondary volatile oxidation products. This was most pronounced when emulsions were prepared on the valve homogeniser. Thus, the lower PV in WPI emulsions appeared to be related to the decomposition of lipid hydroperoxides. Overall, WPI emulsions thus oxidised more than CAS emulsions.

The better oxidative stability of CAS stabilised emulsions compared to WPI stabilised emulsions is in accordance with other studies comparing lipid oxidation in emulsions prepared with whey proteins or caseins (Allen & Wrieden, 1982; Faraji et al., 2004; Horn et al., 2011; Hu, McClements, & Decker, 2003b). In these studies, the differences observed between the two types of proteins are mainly suggested to be related to the structure and thickness of the interfacial layer, or to the metal chelating and free radical scavenging properties of the different proteins.

Regarding the interfacial layer, it has been shown that the interfacial layer provided by CAS is thicker than that provided by WPI when milk proteins are present in excess (Fang & Dalgleish,

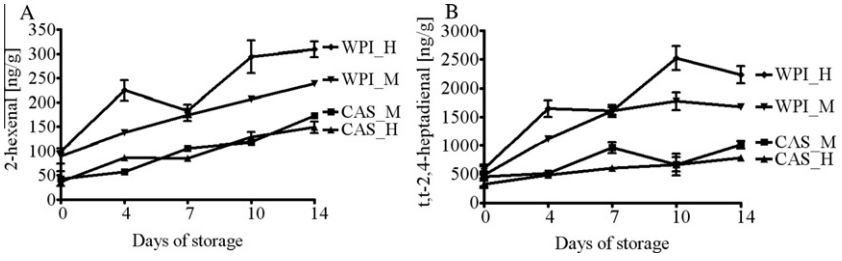

Fig. 4. Secondary volatile oxidation products: 2-hexenal and *t,t*-2,4-heptadienal in ng/g emulsion during storage at ~20 °C. Emulsions were prepared with 10% (w/w) fish oil and 1% (w/w) sodium caseinate (CAS) or whey protein isolate (WPI) as emulsifier on a microfluidizer (M) or a two-stage valve homogeniser (H). Data points represent means (n = 2) ± standard deviations.

1993; Hunt & Dalgleish, 1994a). Hence, the thicker interfacial layer provided by CAS could protect the oil/lipid hydroperoxides better from coming in close proximity to the transition metal ions in the water phase, and thereby reduce lipid oxidation.

Some interesting observations were made for the emulsions prepared with different types of milk proteins. In the images of CAS emulsions, the droplets were positioned with the largest possible distance that was allowed by the volume of the meniscus. This could indicate that the droplets are affecting each other by repulsive forces. This type of interphase behaviour has also been observed by Waninge, Kalda, Paulsson, Nylander, and Bergenståhl (2004) for membranes in multilamellar vesicles. On the other hand, WPI emulsions showed droplets lying close, almost overlapping. Yet, these differences in behaviour of the emulsions cannot be explained further by the present results, and more studies must be carried out to elucidate these observations.

In addition to a difference in thickness of the interfacial layer, the CAS and WPI are also known to differ in their amino acid compositions. Hence, WPI has been shown to possess better free radical scavenging properties than CAS, whereas CAS has been shown to have better metal chelating effects (Elias et al., 2005; Hekmat & Mcmahon, 1998). Since lipid oxidation is accelerated by iron in the present study the metal chelating effect provided by CAS present in the water phase might play a major role for the good antioxidative properties observed by this protein. Ries, Ye, Haisman, and Singh (2010) did a displacement study where the continuous phase of CAS emulsions was replaced with either reverse osmosis water or a solution of 4% protein. It was observed that replacing the water phase with reverse osmosis water increased lipid oxidation, whereas replacing with the protein solution decreased lipid oxidation. The same observations were done on WPI emulsions, however, all emulsions with WPI oxidised more than CAS emulsions independent of the water phase. In accordance with this, Faraji et al. (2004) observed that CAS can bind 5.3-fold more iron than WPI.

Emulsions with CAS and WPI prepared on the valve homogeniser were prepared using different pressures (Table 1). Thus, the differences in lipid oxidation could also be expected to be related to the homogenisation pressure applied. However, since the same differences in oxidative stability were observed when emulsions were prepared on the microfluidizer, using similar pressures, the homogenisation pressure is not suggested to play a major role for the present findings.

4.2. Comparison of the different homogenisation equipments

To obtain similar droplet sizes on the two homogenisation equipments, a higher pressure was needed to emulsify the oil droplets with CAS in the valve homogeniser (80 MPa) than in the microfluidizer (69 MPa). For emulsions with WPI the opposite was observed since a higher pressure was needed on the microfluidizer (69 MPa) than on the valve homogeniser (50 MPa).

These findings cannot readily be explained, however, in a previous study on the physicochemical properties of β-carotene emulsions, it was concluded that homogenisation on a microfluidizer was more efficient in producing emulsions with small droplet sizes than a two stage valve homogeniser (Mao, Yang, Xu, Yuan, & Gao, 2010). Thus, a higher pressure and more passes were needed on the valve homogeniser to obtain similar droplet sizes compared to production on the microfluidizer. These results are in accordance with the results obtained in the production of CAS emulsions in the present study, but not with the results for WPI emulsions. An effective emulsification is therefore suggested to be related to both how willingly the proteins adsorb to the interface, to the geometries of the interaction chambers and to the way the droplet disruption occurs in the interactions chambers of the homogenisers.

Neither PV nor secondary volatile oxidation products differed between the two emulsions prepared with CAS when homogenised on the two different equipments. In contrast, the emulsion with WPI prepared on the valve homogeniser oxidised faster during storage than the similar emulsion prepared on the microfluidizer. Especially the contents of secondary volatile oxidation products were significantly higher in the emulsion prepared on the valve homogeniser.

In milk, where both casein and whey proteins are present, a competition occurs between the two types of milk proteins to reach the surface of the oil droplets during homogenisation. Thus, it has previously been reported that when milk was homogenised on a conventional homogeniser both casein and whey proteins were present at the oil droplet interface, whereas when milk was homogenised on a microfluidizer only casein was present at the interface (Dalgleish et al., 1996). Thus, a preferential adsorption of one type of milk protein (whey proteins and caseins) over the other was found, depending on the homogeniser used. Likewise, a preferential adsorption of the different whey protein components (α-lactalbumin and β-lactoglobulin), has in previous studies been shown to exist and depend upon the total protein concentration (Fang & Dalgleish, 1997; Fang & Dalgleish, 1998; Ye, 2008) and of pH (Fang & Dalgleish, 1997; Hunt & Dalgleish, 1994b; Yamauchi, Shimizu, & Kamiya, 1980), and in some of these studies furthermore been related to structural changes at the interface. Lee et al., 2007 compared their own results to results obtained in the above-mentioned studies, and suggested that the choice of homogenisation equipment could have had an influence on the structural differences of the interfacial proteins observed in the studies. Combined with the differences in the antioxidative properties of β-lactoglobulin and α-lactalbumin previously observed (Allen & Wrieden, 1982; Hu et al., 2003a), the preferential adsorption of one whey protein component over the other, could explain the results in the present study. Unfortunately, it was not possible by the cryo-TEM imaging to investigate visually any differences in the structure of the emulsions when prepared on the different homogenisers. However, emulsions similar to WPI_M and WPI_H have subsequently been prepared in our laboratory, where the protein concentration in the water phase was determined spectrophotometrically and the protein composition was evaluated through SDS–page. Results from these studies confirmed the above hypotheses, since both the protein concentration and composition of the water phase differed between emulsions prepared on different homogenisers (Unpublished). The total protein content in the water phase was higher in the emulsion produced on the valve homogeniser than the emulsion produced on the microfluidizer. Further studies are on-going on the composition of the proteins in the water phase versus the interface as influenced by homogenisation conditions.

Studies on the adsorption of different casein components (α_{s1}-, α_{s2}-, β- and κ-casein) at the interface in emulsions have also shown that a competition can exist (Dickinson, Rolfe, & Dalgleish, 1988; Srinivasan, Singh, & Munro, 1999; Sun & Gunasekaran, 2009; Ye, 2008), and furthermore that the different casein components varies in their antioxidative properties (Cervato, Cazzola, & Cestaro, 1999). Nevertheless, the possible difference in the adsorption of casein components to the interface in the present study is suggested to be less important than the previously mentioned metal chelating effect that CAS posses.

Another aspect to consider, regarding the differences in lipid oxidation, is the temperature elevation in the two equipments. Mao et al. (2010) observed that the temperature elevation in the microfluidizer was less than that in the valve homogeniser. A possible heating of the fish oil could influence lipid oxidation in the emulsions. However, the temperature difference in WPI emulsions prepared on the two homogenisers were determined in the

present study to be only approximately 4 °C. Furthermore, since no difference was observed for CAS stabilised emulsions when prepared on the two homogenisers, heating is not expected to play a major role for the differences observed on lipid oxidation in the present study.

To sum up, the effects of milk proteins on lipid oxidation in emulsions have in some studies mainly been related to their effects at the interface, whereas in other studies it has mainly been related to their effects in the aqueous phase (Berton, Ropers, Viau, & Genot, 2011; Faraji et al., 2004; Kargar et al., 2011; Let et al., 2007). In the present study, milk proteins were present in excess, and it must be assumed that protein was present both at the interface and in the aqueous phase. Nevertheless, for CAS emulsions the content in the water phase seemed to be the most important, whereas for WPI emulsions the content and composition of individual whey protein components at the interface seemed to be responsible for the resulting lipid oxidation.

5. Conclusions

The use of different homogenisation equipments altered lipid oxidation in emulsions stabilised by WPI but not CAS. Emulsions with WPI oxidised more when prepared on a two-stage valve homogeniser than on a microfluidizer. The metal chelating effect of phosphorylated serine groups in CAS was hypothesised to be responsible for the lower content of volatile secondary oxidation products in CAS stabilised emulsions compared to WPI stabilised emulsions, despite the higher PV in the first-mentioned emulsion. Thus, it is suggested, that for CAS stabilised emulsions the casein proteins in the aqueous phase, and its metal chelating effect, rather than the actual composition of protein components at the interface may be the most important factor determining its oxidative stability. On the contrary, for WPI stabilised emulsions the composition at the interface seem to influence lipid oxidation the most, since it is hypothesised that the different geometries in the interaction chambers and the different droplet disruption patterns in the two equipments influences the distribution of protein components between the interface and the aqueous phase.

Some interesting features were observed regarding the localisation of oil droplets dependent on the protein used for emulsification. Thus, to elucidate the findings from the present study even further, more studies on the partitioning of different milk protein components between the interface and the water phase has to be conducted. In addition other types of electron microscopy for imaging the interfacial layer have to be evaluated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.02.184.

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PAPER IV

Effect of α-lactalbumin and β-lactoglobulin on the oxidative stability of 10% fish oil-in-water emulsions depends on pH

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Effect of α-lactalbumin and β-lactoglobulin on the oxidative stability of 10% fish oil-in-water emulsions depends on pH

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Abstract: The objective of this study was to investigate the influence of pH on lipid oxidation and protein partitioning in 10% fish oil-in-water emulsions prepared with different whey protein isolates with varying ratios of α-lactalbumin and β-lactoglobulin. Results showed that an increase in pH increased lipid oxidation irrespective of the emulsifier used. At low pH, lipid oxidation was not affected by the type of whey protein emulsifier used or the partitioning of proteins between the interface and the water phase. However, at neutral pH the emulsifier with the highest concentration of β-lactoglobulin protected better against oxidation during emulsion production, whereas the emulsions with the highest concentration of α-lactalbumin were most oxidatively stable during storage. The differences were explained by differences in the pressure and adsorption induced unfolding of the individual protein components.

Keywords: Fish oil-in-water emulsion, microfluidizer, whey protein isolate, α-lactalbumin, β-lactoglobulin

1. Introduction

An oil-in-water emulsion consists of oil droplets dispersed in an aqueous phase. The oil droplets are surrounded by an interfacial layer, which is considered crucial for the oxidative stability of the emulsion. The interfacial layer is built by the emulsifier and can vary in composition, structure and thickness dependent on both the type of emulsifier used (Hunt & Dalgleish, 1994a) and the conditions under which the emulsion is produced, e.g. the homogenization pressure (Sørensen, Baron, Let, Brüggemann, Pedersen, & Jacobsen, 2007) or pH (Hunt & Dalgleish, 1994b).

Whey protein products such as whey protein isolate (WPI) and whey protein concentrate (WPC) are commonly used emulsifiers since they have good emulsifying and stabilizing properties. Moreover, they have been shown to possess antioxidative properties (Elias, McClements, & Decker, 2005; Sun & Gunasekaran, 2009). The two major components in these whey protein products are α-lactalbumin (α-lac) and β-lactoglobulin (β-lg). These two proteins differ in their amino acid structures and thereby in their emulsifying and antioxidative properties. Hence, a higher number of proline residues in β-lg than in α-lac (8 and 2 respectively) leads to a higher hydrophobicity of β-lg, and more cysteine residues in α-lac than β-lg (8 versus 5) leads to a increased number of possible internal disulfide bridges in α-lac (Ng-Kwai-Hang, 2003).

Both whey proteins are highly structured globular proteins that undergo conformational changes upon adsorption at an interface. Fang and Dalgleish (1997, 1998) investigated these changes by Fourier transform infrared spectroscopy in soy bean oil emulsions with either β-lg or α-lac (prepared from WPI products) and suggested that the structural changes following adsorption were concentration dependent. In the study on β-lg the authors suggested that at low concentration (1% β-lg to 20% oil) the proteins had to stretch over the interface whereby they changed conformation. In contrast, when the proteins were present in excess (2% β-lg to 20% oil) they did not have to stretch over the interface and therefore they did not differ in conformation from the native protein in solution (Fang et al., 1997). Two other studies on emulsions prepared with similar protein to oil ratios as the first mentioned (1:20) also observed that the structure of the whey proteins upon adsorption greatly differed from that of the native protein in solution (Corredig & Dalgleish, 1995; Lee, Lefèvre, Subirade, & Paquin, 2007). It was furthermore suggested that the adsorption related conformational changes corresponded to the initial conformational changes taking place as a result of heat denaturation (Lee et al., 2007). In a slightly different matrix (50% miglyol emulsion with β-lg purified from milk) the conformational changes upon adsorption were related to changes in the α-helix structure of the protein (Dufour, Dalgalarrondo, & Adam, 1998).

The conformational changes and preferential adsorption of individual whey protein components have furthermore been observed to depend on pH. In emulsions prepared with whey proteins, the total protein adsorption was shown to be highest at pH 5 (Shimizu, Kamiya, & Yamauchi, 1981). In addition, these authors observed a pH dependent preferential adsorption of the individual protein components to the interface. The adsorption of β-lg decreased when pH decreased from 9 to 3, whereas adsorption of α-lac increased in the same pH range. The decrease in the adsorption of β-lg was later ascribed to pH dependent structural changes in the β-lg molecule (Shimizu, Saito, & Yamauchi, 1985). In accordance with these findings on the preferential adsorption of individual protein components Hunt and Dalgleish (1994b) reported a preferential adsorption of α-lac over β-lg at pH 3 in emulsions prepared from soy bean oil and WPI.

As previously mentioned, lipid oxidation in emulsions is affected by the structure and composition of the interface. Hence, the pH dependent adsorption of α-lac and β-lg at the interface can potentially affect lipid oxidation. However, the effect of pH on lipid oxidation has only been investigated in emulsions with WPI and pure β-lg, but not in emulsions with a high concentration of α-lac. From the available studies, it is clear that results on simple 5-30% o/w emulsions stabilised by whey proteins, are consistent and show an increase in oxidative stability with decreasing pH, independent of the oil type used or the method for determining lipid oxidation (Donnelly, Decker, & McClements, 1998; Hu, McClements, & Decker, 2003; Kellerby, McClements, & Decker, 2006). The better oxidative stability obtained when pH was below the pI of the protein was suggested to be related to the positive surface charge of the oil droplets and thereby a possible repulsion of transition metal ions. In addition, it has been suggested that at neutral pH the iron ions are likely to precipitate at the oil droplet surface and thereby promote oxidation by their close proximity to the lipids (Mancuso, McClements, & Decker, 1999).

Studies that have compared the antioxidative effects of the individual protein components, α-lac and β-lg are scarce. However, one study compared 5% salmon oil-in-water emulsions prepared with 0.2% α-lac, β-lg or WPI at pH 3 and observed that α-lac provided less oxidative stability to the emulsion than β-lg (Hu et al., 2003). Both individual protein components were though better than WPI. The factors suggested to be responsible for these differences were the amino acid compositions and the thickness and packaging of proteins on the emulsion droplet surface. In another study, where Cu^{2+}-mediated oxidation was investigated in a model system for milk (pH 6.8), α-lac was shown to possess a slightly better antioxidative effect than whole whey (Allen & Wrieden, 1982). Hence, further studies are needed to elucidate the exact antioxidative mechanisms of individual protein components, and their influence on the oxidative stability of emulsions at different pH values.

On this background, it was hypothesized that pH will influence the adsorption of α-lac and β-lg at the interface in oil-in-water emulsions and that the different adsorption patterns will affect lipid oxidation. The aim of this study was therefore to compare lipid oxidation in 10% fish oil-in-water emulsions prepared with either one of two commercially available whey protein isolates or a combination of them as influenced by pH. The WPI products had different ratios of α-lac to β-lg. The concentrations of α-lac and β-lg in the two emulsifiers were 22-24% and 48-25%, respectively, in WPIβ, and ~60% and 20-25% respectively, in WPIα. Emulsions were made at pH 4.0 and 7.0 with 1% protein as emulsifier. Lipid oxidation was catalyzed by iron addition and followed for 14 days.

2. Materials and methods

2.1 Materials

The fish oil used was commercial cod liver oil provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway). The oil was stored at -40°C until use. The fatty acid composition was determined by preparation of methyl esters (AOCS, 1998a) that was in turn analysed by gas chromatography (AOCS, 1998b). The content of the major fatty acids was (in %) as follows: 14:0 3.0, 16:0 8.9, 16:1(n-7) 8.2, 18:1(n-9) 16.0, 18:1(n-7) 5.2, 18:4(n-3) 2.5, 20:1(n-9)11.6, 20:5(n-3) 9.3, 22:1(n-11) 6.1 and 22:6(n-3) 11.6. Tocopherol content was determined by HPLC (AOCS, 1998c), and levels were 207 ± 16 μg α-tocopherol/g oil and 100 ± 1 μg γ-tocopherol/g oil. The initial PV of the fish oil was 0.1 ± 0.0 meq peroxides/kg oil, as determined by the method described in section 2.4.1. Whey protein isolate (Lacprodan® DI-9224) and α-lac enhanced whey protein isolate (Lacprodan® ALPHA-20) were kindly donated by Arla Foods Ingredients amba (Viby J, Denmark). Data sheets reported a protein content of 88-94% in both whey protein isolates, and the manufacturer specified that Lacprodan® DI-9224 contained 22-24% α-lac and 48-52% β-lg, whereas Lacprodan® ALPHA-20 contained 60% α-lac and 20-25% β-lg. All other chemicals and solvents used were of analytical grade.

2.2 Preparation of emulsions and sampling

Emulsions were prepared at pH 4.0 and 7.0 with 10.0% (w/w) fish oil, 89.0% (w/w) 10 mM sodium acetate imidazole buffer and either 1.0% (w/w) Lacprodan® DI-9224 (emulsions named WPIβ), 1.0% Lacprodan® ALPHA-20 (emulsions named WPIα) or 0.5% of each (emulsions named WP). The water phase was prepared by dispersing the protein in the buffer and adjusting the pH. A premix (600 g) was made by adding the fish oil slowly to the water phase during the first minute of mixing at 16,000 rpm (Ystral mixer, Ballrechten-Dottingen, Germany). The total mixing time was 3 minutes.

The premix was afterwards homogenized on a microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75 μm). Homogenization was carried out at a pressure of 10,000 psi, running 3 passes. Emulsions were added 100 μM $FeSO_4$ to accelerate lipid oxidation and 0.05% (w/w) sodium azide to prevent microbial growth. Emulsions (65 g) were stored in closed 100 mL bottles at room temperature (19-20°C) in the dark for 14 days, with one bottle (in replicate) of each emulsion for each sampling time point. Samples were taken at day 0, 5, 9 and 14 for measurements of lipid oxidation. Viscosities were determined at day 2 and 14, pH was measured at day 1, and droplet sizes were measured at day 1 and 14. A similar set of emulsions were prepared for protein analysis. Those emulsions were stored for 5 days before centrifugation as described in section 2.3.2.

2.3 Characterization of the emulsions

2.3.1 Droplet size, viscosity and pH

Droplet size distributions were determined in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) by laser diffraction. Emulsion was suspended directly in recirculating buffer (10 mM sodium acetate imidazole, pH 4.0 or 7.0 respectively; 3000 rpm, 12-14% obscuration). The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Emulsion viscosities were determined on 16 mL emulsion by the use of a Stresstech rheometer (Reologica Instruments AB, Sweden) mounted with a CC25 bob cup system in a temperature controlled vessel. The temperature was equilibrated to 20°C over 180 sec before measurement. Measurements were carried out in the shear stress range 0.0245-0.9903Pa. Determination of pH was done during stirring of the emulsion.

2.3.2 Protein composition of emulsifiers and the water phase of the emulsions

Emulsions (~20g) were centrifuged for 50 min at 45,000 g and 10°C (Sorvall RC-6 PLUS, Thermo Fisher Scientific, Osterode, Germany; rotor SS-34) and the water phase was extracted by the use of a syringe. The obtained water phase was then subjected to ultracentrifugation (Beckman Ultracentrifuge L8-60M, Fullerton, CA; rotor 21102) for 60 min at 70,000 g and 15°C, and once again the water phase was extracted by the use of a syringe. The water phase was diluted 1:9 in 10 mM sodium acetate imidazole buffer (pH 4.0 or 7.0 dependent on the pH of the emulsion). The total protein concentration was determined by the use of a BCA protein assay reagent kit (Pierce, ThermoScientific, Rockford, IL, USA) and measured on a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 562 nm.

To separate the individual protein components in the extracted water phases SDS-page was conducted. The water phases were diluted in 10 mM sodium acetate imidazole buffer (pH 4.0 or 7.0 dependent on the pH of the emulsion) to a concentration of 1 mg protein/mL, and then diluted 1:1 with 10% DTT/Laemlli buffer (63 mM Tris-Cl pH 6.8, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue), and boiled for 3 min. Samples were centrifuged for 3 min at 12,000 rpm (13684g) (Biofuge pico, Heraeus, Osterode, Germany). Samples (10 µL) were loaded on NuPage 10% Bis-Tris gel (Novex, Invitrogen, Life Technologies Ltd, Paisley , UK), and run in MES running buffer for 35 min at 200V. The gels were stained with Coomassie Brilliant Blue R-250 (CBB) and the individual protein spots were qualitatively assessed. Selected protein spots were furthermore identified as described in section 2.2.1.

2.2.1 Protein identification

Protein bands of interest were excised (OneTouch Plus spotpicker, 1.5 mm, The gel Company, San Francisco, CA, USA) and the bands were washed first in water and then acetonitrile. Bands were then dried and in-gel digested overnight at 37 °C with trypsin (porcine graded modified trypsin, Promega, Southampton, UK) and finally redisolved in 0.1% trifluoroacetic acid (TFA). The supernatants were collected and loaded on an Anchorchip^{TM} target (Bruker-Daltonics, Bremen, Germany) as previously described (Zhang et al., 2007). Mass to charge ratios were acquired using an Ultraflex II mass spectrometer (Bruker-Daltonics, Bremen, Germany) in positive reflector mode. Searches were performed using MASCOT as search engine against the NCBInr database (NCBI Mammalia 20120623) and the significance threshold were set at 0.05 resulting in scores greater than 43 being significant. Prior to any search the software PeakErazor (http://gpmaw) (Hjerno & Højrup, 2004) were used to remove peaks originating from trypsin or contaminants.

2.4 Measurements of lipid oxidation

2.4.1 Primary oxidation products – peroxide values

A lipid extract was prepared from 10 g emulsion according to a modified form of the method described by Bligh and Dyer (1959) and by a reduced amount of solvent (30.0 ml methanol and chloroform, 1:1). Subsequently, peroxide values were determined on this lipid extract, or directly on the oil used for emulsion production, by colorimetric determination of iron thiocyanate at 500 nm as described by Shantha and Decker (1994).

2.4.2 Secondary oxidation products – SPME GC-MS

Emulsion (1 mL) was poured into a 10 mL vial, and preheated for 5 min to a temperature of 60°C. Extraction of headspace volatiles was done by the use of an 85µm Carboxen/PDMS SPME fiber (Supelco, Bellafonte, PA, USA) installed on a CTC Combi Pal (CTC Analytics AG, Zwingen, Switzerland). Extraction was done for 55 min while agitating the sample at 500 rpm. The desorption of volatiles from the fiber to the injection port of a gas chromatograph (HP 6890 Series, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m x 0.25 mm x 1.0 µm; J&W Scientific, Folsom, CA, USA) was done for 60 sec at 230°C. The oven program had an initial temperature of 35°C for 3 min, increasing with 3.0°C/min until 140°C, with 5.0°C/min until 170°C and with 10.0°C/min until 240°C, where the temperature was kept steady for 8 min. The individual compounds were analyzed by mass-spectrometry (HP 5973 inert mass-selective detector, Agilent Technologies, Palo Alto, CA, USA). From a comparison of chromatograms from non-oxidised and oxidised samples, the following volatiles were selected for quantification: 2-butenal, 1-penten-3-one, hexanal, 2-hexenal, 4-heptenal, 2,4-hexadienal, 2,4-heptadienal and 2,6-nonadienal. In the chromatograms two peaks were identified as 2,4-heptadienal. From previous studies of these two peaks (not published) it is anticipated that they represent the two isomers *t,c*-2,4-heptadienal and *t,t*-2,4-heptadienal. Calibration curves were made by dissolving the compounds in rapeseed oil followed by the addition of an amount of this oil corresponding to 2-800 ng of the compounds to 1 g emulsion (WP_7 frozen right after production and thawed prior to use). Measurements were made in triplicate on each sample.

2.5 Statistical analyses

All data were analysed by one- or two-way analysis of variance with Bonferronis multiple comparison test as post test (GraphPad Prism, version 4.03, GraphPad Software Inc, La Jolla, CA USA). Averages of the experimental double/triple determinations were used for each replicate. All references to significant differences ($P < 0.05$) between samples or between sampling times, are based on this statistical analysis of data. Oxidation data were furthermore subjected to multivariate data analysis (LatentiX, version 2.00, The MathWorks Inc, Frederiksberg, Denmark). A principal component analysis (PCA) was carried out with all replicates (12 emulsions) as objects and peroxide values and volatiles data as variables, with one variable representing the average of a given peroxide value or volatile at a specific sampling time point. The data was autoscaled in order to make the variables contribute equally to the model, and the PCA model was validated systematically segmented, according to the replicates of the emulsions. A second model was also calculated with the six different emulsions as objects (averages of the replicates) and peroxide values and volatiles data as variables. Data were autoscaled, and the PCA model was validated by full cross validation.

Overall, samples were similarly distributed in both models, and therefore the model with averages is shown for simplicity.

3 Results

3.1 Characterization of the emulsions

3.1.1 Droplet size, viscosity and pH

The pH values were measured to be 3.9 or 7.0-7.2 (Table 1), thus, within the two pH values samples did not differ significantly from each other. The viscosities were measured to be in the range of 3.24-4.19 mPa·s, and did not differ between samples (Table 1). All emulsions demonstrated Newtonian behaviour in the measured shear stress range. Mean oil droplet sizes ranged from 0.122 μm to 0.156 μm at day 1 and from 0.123 μm to 0.203 μm at day 14 (Table 1). The rank order was WPIα_7 < WP_7 < WPIβ_7 < WPIα_4 < WP_4 < WPIβ_4 at both measurement days. As shown in Table 1, significant differences existed between emulsions but in general differences were small. All emulsions prepared at pH 4 and WPIβ_7 significantly increased in mean droplet size during the 14 days of storage, but no visible phase separation occurred.

3.1.2 Proteins in the water phase

The SDS-page gels are shown in Fig. 1. The band appearing at around 17-18 kDa was identified as β-lg and the band appearing around 13-1 4 kDa as α-lac (A table containing accession number, Mascot score and sequence coverage for the identified proteins is included as a supplementary). In all emulsions prepared at pH 4, the majority of the proteins in the water phase were β-lg. In comparison, emulsions prepared at pH 7 had less β-lg and more α-lac in the water phase. At both pH values the concentration of α-lac in the water phase increased with the increase in the total concentration of α-lac in the emulsifier used. At pH 7 some indistinct bands for higher molecular weight compounds also appeared on the gels. The protein contents in these bands were too low for identification, but they most likely represented other proteins such as BSA that have a molecular weight of 66.3 kDa.

3.2 Lipid oxidation in emulsions

To get a visual overview of all the samples and to interpret correlations between variables, a PCA model was calculated (Fig. 2). The first principal component (PC1) explained 76.6% of the variance, and the second principal component (PC2) explained 13.4%. The scores plot (Fig. 2A) showed that emulsions prepared at pH 4 were located to the left in the 2nd quadrant and very close together. Thus, emulsions at pH 4 did not differ in the oxidation products measured. Emulsions prepared at neutral

pH were located to the right, in the 1st (WPIβ_7) and the 4th quadrant (WPIα_7 and WP_7), thus larger variations were observed between these samples than between samples prepared at pH 4. Hence, PC1 explains the variation in the samples related to pH, whereas PC2 explains the variation in the samples at pH 7 related to emulsifier.

3.2.1 Peroxide values

The PCA loadings plot (Fig. 2B) showed that all PV results were located in the 4th quadrant with PV, day 14 above a group of PV measurements from the other sampling days (day 0, 5 and 9). This indicated that all emulsions at pH 7 had higher PV than emulsions prepared at pH 4. Among emulsions prepared at pH 7, the loadings plot pointed towards a higher PV in WPIα_7 and WP_7 in the first part of the storage period, which was confirmed by raw data (Table 2). Moreover, in accordance with the location of PV_D14 near WPIα_7, PV was highest in this sample at day 14 followed by WPIβ_7 and WP_7. Interestingly, WPIα_7 had a significantly higher PV than the rest of the samples already at day 0, and at day 14 the rank order of the emulsions were $WP\_4^{a} = WPI\alpha\_4^{a} = WPI\beta\_4^{a} \leq WP\_7^{a,b} \leq WPI\beta\_7^{b} \leq WPI\alpha\_7^{c}$ (with letters indicating significant differences at a 95% level). PV in all samples significantly increased between day 0 and 14, with a relative increase in the order: WPIα_7 < WPIα _4 < WP_4 < WPIβ_4 < WP_7 < WPIβ_7. Hence, despite the higher initial PV in WPIα_7, this sample had the lowest relative increase during storage.

3.2.2 Volatiles secondary oxidation products

Nine different volatiles were quantified and included in the PCA model. The volatiles selected were all expected to derive from oxidation of mainly n-3 or n-6 fatty acids. In Fig. 3a and 3b raw data for hexanal (derived from oxidation of n-6) and *t,c*-2,4-heptadienal (derived from oxidation of n-3) are shown, respectively.

The PCA loadings plot (Fig. 2B) showed that volatiles measured at day 0 were spread out mainly in the 1st and 2nd quadrant. All other volatiles were located in the right side of the plot, indicating a higher concentration of secondary volatile oxidation products in the emulsions prepared at pH 7 than in the ones prepared at pH 4 at day 5, 9 and 14. These observations were in accordance with raw data (represented by hexanal and t,c-2,4-heptadienal in Fig. 3a and 3b, respectively). None of the quantified volatiles differed in concentration between any of the samples at day 0. Neither did the concentrations of any of the volatiles among samples prepared at pH 4 at any other sampling time point (Data not shown). In addition, the general picture from raw data was that emulsions prepared at pH 7 had higher concentrations of volatiles in the later part of the storage period than emulsions prepared at pH 4.

As mentioned, no differences were observed between samples prepared at pH 4. However, emulsions prepared at pH 7 were located differently in the PC2 direction in the scores plot. The loadings plot did not show any overall systematic behaviour according to sampling time point or type of volatile in the direction for PC2, but as hexanal and 2,4-hexadienal at day 5, 9 and 14 were located in the positive direction for this PC, the concentrations of these volatiles were expected to be higher in WPIβ_7 than in WP_7 and WPIα. This was confirmed from raw data that showed that for both these two volatiles WPIβ_7 had significantly higher concentrations than the other two samples prepared at pH 7 both at day 9 and day 14 (as also observed in Fig. 3a). From the raw data it could furthermore be observed that WPIβ_7 had a significantly higher content of 2-butenal, 2-hexenal, *t,c*-2,4-heptadienal and 2,6-nonadienal than all other samples at day 14. In addition WPIβ_7 had a significantly higher concentration of 4-heptenal and *t,t*-2,4-heptadienal than WP_7 at day 14, whereas the concentrations of these volatiles did not differ significantly between WPIα_7 and WPIβ_7 at this sampling time point. Overall, it was observed concentrations of hexanal, 2-hexenal and 4-heptenal increased significantly during storage in all emulsions and concentrations of 2-butenal, 1-penten-3-one, 2,4-hexadienal, *t,c*-2,4-heptadienal, *t,t*-2,4-heptadienal and 2,6-nonadienal increased significantly in all emulsions prepared at pH 7.

The overall conclusions from volatiles data were that samples prepared at pH 4 had much lower concentrations of all volatiles than emulsions prepared at pH 7 during storage. Among samples prepared at pH 4 no differences were observed in oxidation, whereas WPIβ_7 was the most oxidized sample among samples prepared at pH 7. PV in this sample also increased most during storage.

4 Discussion

4.1 The effect of pH

In previous studies, the total adsorption of whey proteins to the interface, and in particular the preferential adsorption of individual whey protein components has been observed to be pH dependent (Hunt et al., 1994b; Lee, Subirade, & Paquin, 2008; Shimizu et al., 1981). Shimizu et al. (1981) reported that the highest total protein adsorption of whey proteins occurred at pH 5 (7.65 mg/m^2), and that the concentration of adsorbed β-lg decreased from 61.6% at pH 9 to 12.9% at pH 3. In contrast, the concentration of adsorbed α-lac increased from 9.9% at pH 9 to 48.3% at pH 3. The results obtained in the present study confirmed this finding, as more β-lg was present in the water phase at pH 4 than at pH 7 (Fig. 1). Shimizu et al. (1985) later did a follow-up study where they suggested that the decrease in the adsorption of β-lg at low pH was caused by structural changes in the β-lg molecule that would lower its flexibility and thereby its emulsifying properties. This was, however, not clear from the results of oil droplet sizes in the present study. Even though WPIβ_4 had

larger droplets than WPIα_4 (Table 1), which could indicate a better emulsifying ability of WPIα at low pH, a similar observation could also be done at neutral pH. Hence, the possible better emulsifying capacity of WPIα was not pH dependent, but was observed at both pH values. It should though be mentioned, that the emulsifying capacity as reflected in smaller oil droplet sizes was in general slightly better for all emulsifiers at neutral pH than at low pH.

With regard to the oxidative stability of the emulsions, lipid oxidation was much lower at pH 4 than at neutral pH in agreement with several other studies (Berton, Ropers, Viau, & Genot, 2011; Donnelly et al., 1998; Hu et al., 2003; Kellerby et al., 2006). At low pH emulsion droplets were expected to carry a positive surface charge and thereby repel the transition metal ions present in the aqueous phase and this is most likely a major factor contributing to the increased oxidative stability at low pH. In addition, it is suggested that a higher concentration of β-lg in the water phase of emulsions prepared at pH 4 may have contributed to the better oxidative stability at this pH, as previously shown by Elias et al. (2005).

4.2 Emulsions at pH 4

Interestingly, regardless of the type of emulsifier used, and the ratios of α-lac and β-lg, the oxidative stability was observed to be similar in all emulsions prepared at low pH. This observation was in contrast to the results obtained by Hu et al. (2003) on the comparison of 5% emulsions stabilized by α-lac, β-lg or WPI. The differing results could be caused by the different protein to oil ratios used in the two studies or the different homogenization conditions. Hu et al. (2003) prepared emulsions with a much lower protein to oil ratio (1:25) than the ratio used in the present study (1:10). Furthermore, Hu et al. (2003) prepared emulsions by the use of a 2-stage valve homogenizer as compared to the use of a microfluidizer in the present study. The use of a 2-stage valve homogenizer for emulsion preparation has in a study by our research group been shown to increase lipid oxidation due to a lower total adsorption of protein (Horn, Nielsen, Jensen, Horsewell, & Jacobsen, 2012). Hence, the lower protein to oil ratio and the use of a 2-stage valve homogenizer could have led to a general lower coverage at the interface by protein, and thus increased the differences between individual whey protein components in the study by Hu et al. (2003). It is also noteworthy that Hu et al. (2003) stored their emulsions at 37˚C, whereas emulsions were stored at 19-20˚C in the present study.

4.3 The difference between emulsions at pH 7

In contrast to the results obtained at pH 4, emulsifier dependent differences were observed in the oxidative stability of emulsions prepared at pH 7. The results are, however, not straightforward. Already at day 0 WPIα_7 had a significantly higher PV than the other two samples (WP_7 and

WPIβ_7), and this ranking remained throughout storage. However, from the relative increase in PV and volatiles data WPIβ_7 was shown to oxidize by far the most during storage. Hence, these results indicated that a high concentration of β-lg improved the protection during production of the emulsion, and a high concentration of α-lac improved the protection against lipid oxidation during storage. This difference is difficult to explain. However, the several different factors that can influence the unfolding and exposure of individual antioxidative amino acid residues have most likely influenced the obtained results.

Previous studies on pasteurized milk (Needs, Capellas, Bland, Manoj, Macdougal, & Paul, 2000) and raw skim milk (Huppertz, Fox, & Kelly, 2004) have reported β-lg to be more sensitive towards pressure treatments than α-lac. In pasteurized milk, a pressure treatment of 600 MPa for 15 min led to a denaturation of 92.3% of the β-lg but only 15.4% of the α-lac. Similarly, the pressure treatments of raw skim milk from 0 to 800 MPa showed that denaturation of β-lg occurred at pressures > 100 MPa, whereas denaturation of α-lac occurred at pressures≥ 400 MPa. Thus, obviously, the pressure treatment induced in the present study at 69 MPa has most likely not affected the unfolding of α-lac during emulsion production. The effect on β-lg unfolding might be more speculative. Scollard. Beresford, Needs, Murphy, and Kelly (2000) reported no denaturation of β-lg in raw milk upon pressure treatment at 50 MPa, but extensively denaturation at 300 MPa. However, pressures in between these values were not investigated. Huppertz et al. (2004) reported an increased denaturation of β-lg when raw milk (pH 6.7) was pressure treated at > 100 MPa at 20°C. At pH 7, the denaturation was shown to increase compared to pH 6.7 at pressure treatments above 250 MPa, however, pressures in between 0 and 250 MPa were not investigated at this pH (Huppertz et al., 2004). Stapelfeldt and Skibsted (1999) suggested that the pressure induced denaturation of β-lg occurred in three stages. These authors observed an increased thiol activity and a pressure induced partial collapse of the inner calyx in β-lg already at the lowest stage (< 50MPa). Pressure induced structural changes were also suggested by Lee et al. (2007) when studying adsorbed and non-adsorbed proteins in 10% soy oil-in-water emulsions prepared with whey protein isolate at 50 MPa. Hence, it could be speculated whether the better protective effect of β-lg during emulsion production in the present study could be related to its higher pressure sensitivity and the possibility of a pressure induced change in its structure. The increased thiol activity suggested by Stapelfeldt et al. (1999) could possibly increase the ability of β-lg to donate a hydrogen atom to radicals produced during the homogenization process in the present study. On the contrary, the less pressure sensitive α-lac might not have unfolded to a similar degree under the high-pressure treatment and thereby its radical scavenging effects might not have been increased similarly as that of β-lg and this could explain the higher PV observed in WPIα_7 at day 0. Studies on the pressure induced structural changes of β-lg

are, however, not consistent, and results are mainly obtained in milk or on proteins solubilised in water. Hence, further studies are needed for the complete understanding of the influence of β-lg under pressure induced emulsion production.

Turning to the better oxidative stability of WPIα_7 during storage than WPIβ_7, this could either be attributed the protein composition in the water phase or the proteins adsorbed at the interface. Considering the water phase, results from SDS-page showed that both α-lac and β-lg were present in the water phase in all samples. In addition, more α-lac and slightly more β-lg was observed in the water phase for WPIα_7 than for WP_7 and WPIβ_7 (Fig. 1). Even though the differences for concentrations of β-lg were very small, it could not be ruled out that β-lg in the water phase could have influenced the oxidative stability as suggested for emulsions at pH 4. A protective effect by α-lac in the water phase seems more unlikely due to the reduced pressure induced unfolding and exposure of antioxidative amino acid residues as discussed above for this protein component.

The differences in the oxidative stability among samples prepared at pH 7 could also exist as a result of the protein composition at the interface. Previous studies have suggested that there is no preferential adsorption of the different whey protein components at neutral pH (Dickinson, Rolfe, & Dalgleish, 1989; Hunt et al., 1994b) or a slight predominance for the adsorption of β-lg (Ye, 2008). Hence, it is hypothesized that the composition of adsorbed proteins more or less reflects the protein composition of the emulsifier used. This means that the concentration of β-lg at the interface must be higher for WPIβ_7 than for the other emulsions prepared at pH 7. As WPIβ_7 was also the most oxidized sample, this implied that β-lg at the interface did not protect emulsion against oxidation to the same degree as β-lg in the water phase. Several studies have reported that structural changes occur when whey proteins adsorb to an interface (Dufour et al., 1998; Fang et al., 1997; Lee et al., 2007). Zhai, Wooster, Hoffmann, Lee, Augustin, & Aguilar (2011) more specifically reported a loss of globular structure in β-lg upon adsorption to an interface. Hence, this might explain the above-mentioned differences in the protective effects of β-lg in the water phase and at the interface observed in this study. More studies are however needed to elucidate this and to fully understand the present results.

5 Conclusions

The overall conclusions from the present study were that the oxidative stability of the emulsions were lower at pH 7 than at pH 4. The better oxidative stability at low pH was ascribed to a positive surface charge of the proteins and a high concentration of β-lg in the water phase. However, at low pH the ratio of α-lac versus β-lg in the emulsifier did not influence lipid oxidation. At neutral pH the

protective effect of β-lg was higher during high pressure-induced homogenization, whereas α-lac improved the oxidative stability during storage. These differences were most likely attributed differences in the unfolding of the two protein components during high pressure treatment and upon adsorption, which might have exposed antioxidative amino acid residues to a different extent.

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Table 1. Physico-chemical data for emulsions.

Emulsion[a]	pH	Viscosity[b] [mPa·s]	D[3,2], day 1[c] [µm]	D[3,2], day 14[c] [µm]	Water phase protein[b] [mg/mL]
WPIβ_4	3.9 ± 0.1[a]	3.75 ± 0.12	0.156 ± 0.003 [e]	0.203 ± 0.013 [d]	5.31 ± 0.15
WP_4	3.9 ± 0.0[a]	4.19 ± 0.20	0.150 ± 0.006 [d,e]	0.185 ± 0.008 [c]	4.90 ± 0.03
WPIα_4	3.9 ± 0.0[a]	3.71 ± 0.00	0.140 ± 0.003 [c,d]	0.174 ± 0.004 [c]	4.63 ± 0.07
WPIβ_7	7.1 ± 0.0[b]	3.37 ± 0.06	0.129 ± 0.001 [b,c]	0.156 ± 0.002 [b]	4.89 ± 0.25
WP_7	7.2 ± 0.0[b]	3.26 ± 0.01	0.126 ± 0.005 [a,b]	0.137 ± 0.008 [a]	5.05 ± 0.27
WPIα_7	7.0 ± 0.0[b]	3.24 ± 0.02	0.122 ± 0.001 [a]	0.123 ± 0.001 [a]	4.88 ± 0.13

[a]Sample names are given as a combination of the type of emulsifier used (WPIβ: whey protein isolate, WPIα: whey protein isolate enhanced with α-lactalbumin or WP: a combination of whey protein isolate and whey protein isolate enhanced with α-lactalbumin in a ratio of 1:1) and the pH as postfix (4 or 7).
[b]None of the samples were significantly different from each other ($P > 0.05$).
[c]For each column letters a-e indicate significant differences between samples ($p < 0.05$).

Table 2. Peroxide values (meq lipid hydroperoxides/kg oil) in emulsions during storage for 14 days, and the relative increase between day 0 and day 14 (Δ PV).

Emulsion[a]	Day 0[b]	Day 5	Day 9	Day 14	Δ PV
WPIβ_4	3.3 ± 0.0 [a]	7.5 ± 0.2 [a]	9.7 ± 0.6 [a,b]	14.3 ± 0.2 [a]	11.0
WP_4	2.9 ± 0.1 [a]	7.1 ± 0.1 [a]	9.1 ± 2.7 [a,b]	13.8 ± 0.1 [a]	10.9
WPIα_4	3.4 ± 0.3 [a]	6.8 ± 0.1 [a]	8.3 ± 0.2 [a]	13.8 ± 0.3 [a]	10.4
WPIβ_7	3.0 ± 0.2 [a]	8.7 ± 0.3 [a,b]	9.8 ± 0.1 [a,b]	17.3 ± 0.6 [b]	14.3
WP_7	4.0 ± 0.5 [a]	10.1 ± 1.2 [b]	11.9 ± 2.4 [b,c]	15.4 ± 0.1 [a,b]	11.4
WPIα_7	10.0 ± 0.6 [b]	11.0 ± 0.0 [b]	13.7 ± 0.8 [c]	19.9 ± 0.5 [c]	9.9

[a]Sample names are given as a combination of the type of emulsifier used (WPIβ: whey protein isolate, WPIα: whey protein isolate enhanced with α-lactalbumin or WP: a combination of whey protein isolate and whey protein isolate enhanced with α-lactalbumin in a ratio of 1:1) and the pH as postfix (4 or 7).
[b]For each column letters a-c indicate significant differences between samples ($p < 0.05$).

Figure 1. Proteins in the water phase of emulsions as determined on SDS-page gels. L1 and L8: Molecular weight standards; L2 and L3: WPIβ_4: L4 and L5: WP_4; L6 and L7: WPIα_4; L9 and L10: WPIβ_7: L11 and L12: WP_7; L13 and L14: WPIα_7. Sample names are given as a combination of the type of emulsifier used (WPIβ: whey protein isolate, WPIα: whey protein isolate enhanced with α-lactalbumin or WP: a combination of whey protein isolate and whey protein isolate enhanced with α-lactalbumin in a ratio of 1:1) and the pH as postfix (4 or 7).

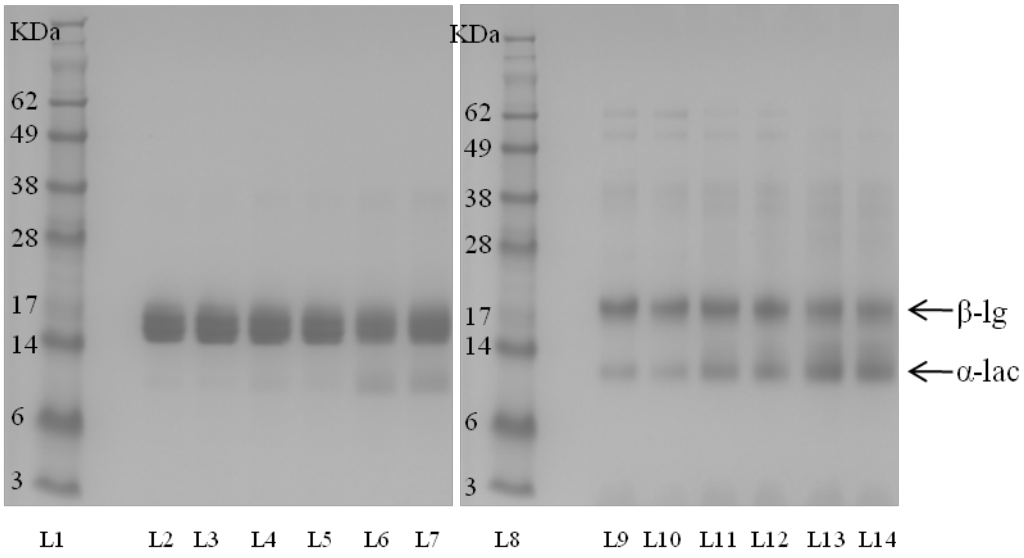


Figure 2. A PCA plot. Scores plot (A) and loading plot (B). PC1 explained 76.6% of the variance, and PC2 explained 13.4%. Sample names are given as a combination of the type of emulsifier used (WPIβ: whey protein isolate, WPIα: whey protein isolate enhanced with α-lactalbumin or WP: a combination of whey protein isolate and whey protein isolate enhanced with α-lactalbumin in a ratio of 1:1) and the pH as postfix (4 or 7). Variable names are given as a combination of the type of volatile quantified (2B: 2-butenal, 1P3O: 1-penten-3-one, HX: hexanal, 2HX: 2-hexenal, 4H: 4-heptenal, 24HX: 2,4-hexadienal, tc24H: *t,c*-2,4-heptadienal, tt24H: *t,t*-2,4-heptadienal or 26N: 2,6-nonadienal) or PV, and the sampling time point as postfix (D0, D5, D9 or D14).

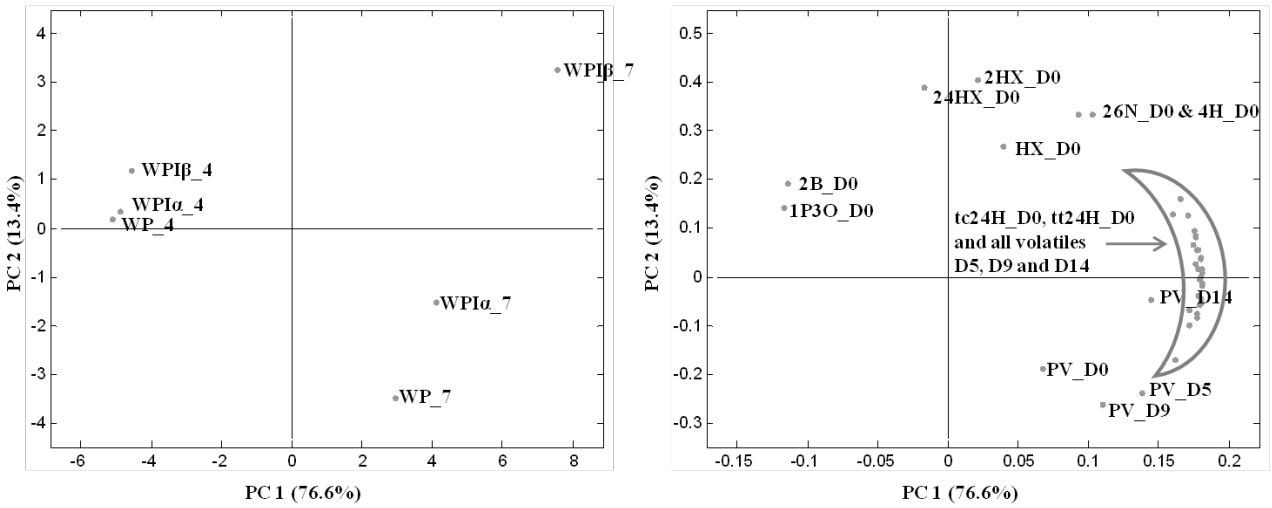
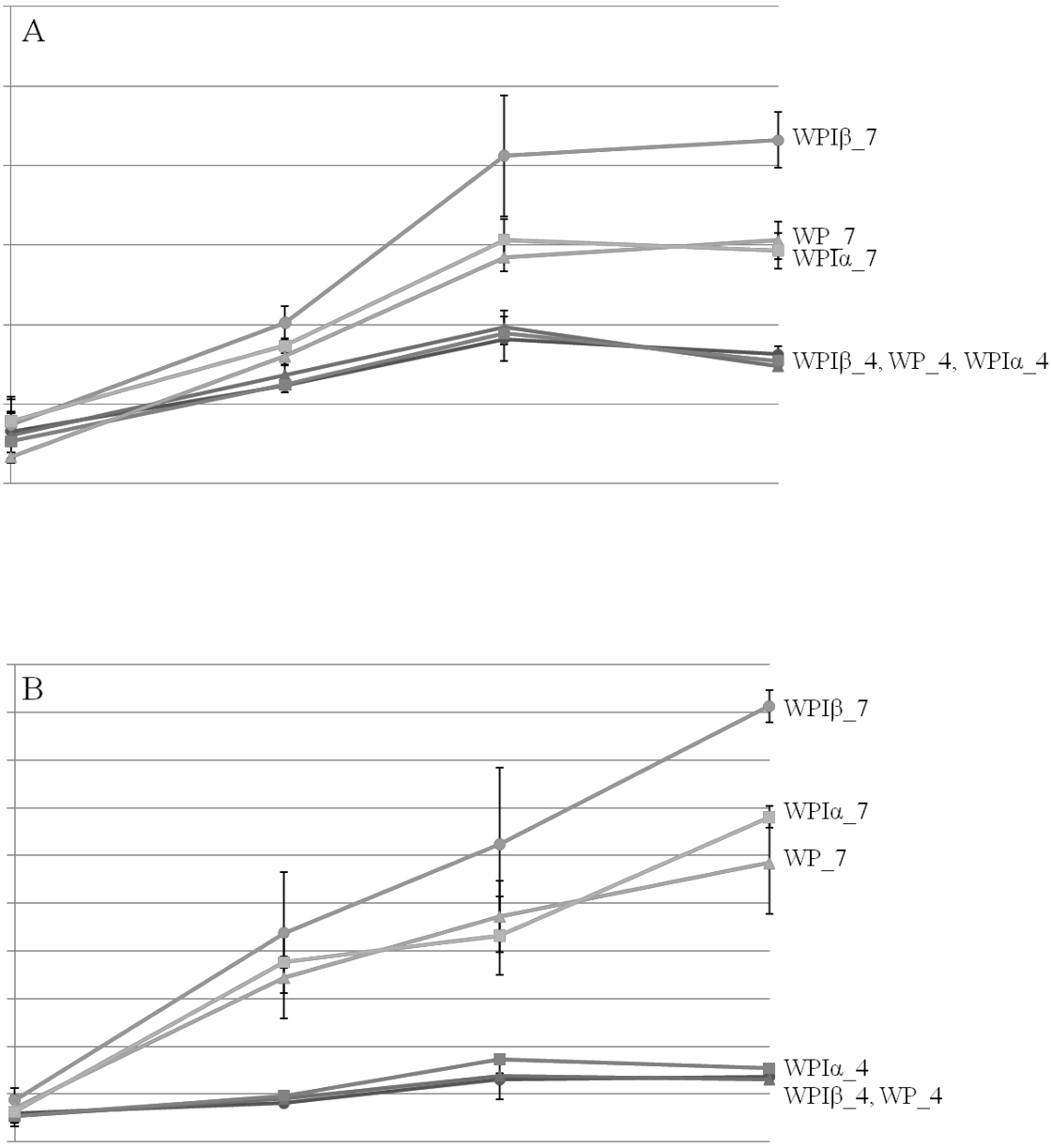


Figure 3. Concentrations of hexanal (A) and of *t,c*-2,4-heptadienal (B) in emulsions during storage for 14 days (n=3). Standard deviations are given by vertical lines.



PAPER V

Homogenization pressure and temperature affect protein partitioning and oxidative stability of emulsions

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Homogenization pressure and temperature affect protein partitioning and oxidative stability of emulsions

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Abstract: The oxidative stability of 10% fish oil-in-water emulsions was investigated for emulsions prepared under different homogenization conditions. Homogenization was conducted at two different pressures (5 MPa or 22.5 MPa), and at two different temperatures (22 °C and 72 °C). Milk proteins were used as emulsifier. Hence, emulsions were prepared with either a combination of α-lactalbumin and β-lactoglobulin or with a combination of sodium caseinate and β-lactoglobulin. Results showed that an increase in pressure increased the oxidative stability of emulsions with caseinate and β-lactoglobulin, whereas it decreased the oxidative stability of emulsions with α-lactalbumin and β-lactoglobulin. For both types of emulsions the partitioning of proteins between the interface and the aqueous phase appeared to be important. The effect of pre-heating the aqueous phase with the milk proteins prior to homogenization did not have any clear effect on lipid oxidation in any of the two types of emulsions.

Keywords: Omega-3 emulsion, sodium caseinate, α-lactalbumin, β-lactoglobulin, homogenization conditions

1. Introduction

Lipid oxidation in emulsions is generally considered an interfacial phenomenon. Hence, the properties of the emulsifier at the interfacial layer are important for the oxidative stability of an emulsion. Moreover, when emulsifier is present in excess, it can exert antioxidative effects by its presence in the aqueous phase as well [1]. The type of emulsifier and the partitioning of the emulsifier components between the interface and the aqueous phase are therefore expected to be crucial for the resulting lipid oxidation.

In food emulsions, bovine milk proteins are commonly used as emulsifiers because of their good emulsifying and physically stabilizing properties. Bovine milk proteins include a wide range of components within two main groups, i.e. caseins and whey proteins. These protein components differ in their structural and antioxidative properties. In general caseins are considered to be flexible molecules, since they lack stable secondary and tertiary structures, whereas whey proteins are globular and highly structured. The tertiary structure of whey proteins is partly due to the presence of cysteine residues that form disulfide bridges [2], and highly influenced by various conditions, such as pH, temperature and whether the protein is unadsorbed or adsorbed to a surface [3, 4]. Extraction and purification processes of the milk to obtain milk protein emulsifier products may also affect the protein´s structures resulting in different properties of the purified emulsifiers compared to the compounds that they are derived from, e.g. sodium caseinate and casein [5].

The structural differences between caseins and whey proteins can possibly affect the thickness and coverage of the interfacial layer in milk protein stabilized emulsions and can thereby influence the resulting lipid oxidation. In addition to the structure of the proteins, also the amino acid compositions of the two types of proteins will affect their antioxidative properties. Caseins, but not whey proteins, contain several phosphorylated serine residues that have been suggested to possess metal chelating properties [6, 7]. In contrast, whey proteins have sulfhydryl groups that are suggested to scavenge free radicals [8]. However, studies on blocking sulfhydryl groups in whey proteins and dephosphorylation of caseins have revealed that for both caseins and whey proteins the antioxidative mechanisms are much more complex and not solely restricted to the number of phosphorylated serine residues or sulfhydryl groups [9, 10].

Food emulsions with a low oil content are often produced by the use of high pressure homogenizers [11]. In high pressure homogenizers, the main parameter that can be varied is the pressure applied. Increasing the pressure or the number of passes through the interaction chamber reduces the size of the oil droplets during homogenization [12]. A reduction in oil droplet size increases the total surface

area of the oil droplets, and this has been hypothesized to increase lipid oxidation [13]. Nevertheless, lipid oxidation studies on emulsions prepared with caseinate, Tween20 or whey protein concentrate have not been able to confirm a relationship between oxidative stability, pressure and droplet size [14, 15]. Moreover, studies on fish oil-enriched milk have shown that an increase in pressure during homogenization decreased oil droplet sizes, but increased the oxidative stability due to an exchange of milk protein components between the aqueous phase and the interfacial layer [16, 17]. The same authors also observed that heating the milk prior to homogenization from 50 °C to 72 °C led to an increase in the adsorption of β-lactoglobulin to the interface [17]. This was explained by a temperature dependent unfolding of β-lactoglobulin. Hence, for fish oil enriched milk it was concluded that the unfavorable decrease in oil droplet size and harsh production conditions, was less important for the oxidative stability than a favorable protein composition at the interface overcame

On this background, we hypothesized that dependent on the emulsifier used the homogenization pressure would influence the partitioning of protein components between the interfacial layer and the aqueous phase in emulsions. Moreover, we hypothesized that an increase in temperature would influence the unfolding of whey proteins and thereby their antioxidative activity. The aim of this study was therefore to compare lipid oxidation in 10% fish oil-in-water emulsions prepared on a two-stage valve homogenizer at pressures of 5 or 22.5 MPa and different temperatures (room temperature ~ 22 °C, or 72 °C). Emulsions were made with either 1% whey protein isolate, or a mix of sodium caseinate and β-lactoglobulin (9:1) corresponding to the ratio in milk. The whey protein used was a mix of two commercially available whey protein isolates. The purpose of mixing two types of whey protein isolates was to have an emulsifier with almost equal amounts of α-lactalbumin and β-lactoglobulin. Emulsions were characterized by droplet size and viscosity, and lipid oxidation was followed during storage for 14 days. In addition, protein compositions in the aqueous phase were determined.

2. Materials and methods

2.1 Materials

The fish oil used was commercial cod liver oil provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway). The fish oil was stored at -40 °C until use. The content of the major fatty acids given in area % was as follows: 14:0 3.0%, 16:0 8.9%, 16:1(n-7) 8.2%, 18:1(n-9) 16.0%, 18:1(n-7) 5.2%, 18:4(n-3) 2.5%, 20:1(n-9)11.6%, 20:5(n-3) 9.3%, 22:1(n-11) 6.1% and 22:6(n-3) 11.6% (as determined by GC analysis of methyl esters of fatty acids [18, 19]). Tocopherol contents were 207 ± 16 μg α-tocopherol/g oil and 100 ± 1 μg γ-tocopherol/g oil (as determined by HPLC [20]). The initial

PV was measured to be < 0.1 meq peroxides/kg oil (as determined by the method described in section 2.4.1). Sodium caseinate, CAS (Miprodan® 30), whey protein isolate, WPI (Lacprodan® DI-9224), whey protein isolate enhanced with α-lactalbumin, WPIα (Lacprodan® ALPHA-20), and a non-commercial purified β-lactoglobulin, Lg, were kindly donated by Arla Foods Ingredients amba (Viby J, Denmark). Specifications from the manufacturer reported a protein content of 88-94% in all protein emulsifiers. Furthermore, WPI contained 22-24% α-lactalbumin and 48-52% β-lactoglobulin, WPIα contained 22-60% α-lactalbumin and 20-25% β-lactoglobulin, whereas Lg contained 7% α-lactalbumin and 76% β-lactoglobulin. CAS contained a combination of α_{s1}-, α_{s2}-, β- and κ-caseins. All other chemicals and solvents used were of analytical grade.

2.2 Preparation of emulsions, storage and sampling

Eight emulsions were prepared (Table 1) with 10% (w/w) fish oil, 1% (w/w) emulsifier and 89% (w/w) sodium acetate imidazole buffer (10 mM, pH 7.0). The above-mentioned protein emulsifiers were used in combination. Hence, either a combination of WPI and WPIα (ratio 1:1) or a combination of CAS and Lg (ratio 9:1) were used for preparing the different emulsions. Proteins were dispersed in the buffer overnight under stirring (5 °C). On the day of emulsion preparation, the protein/buffer solution was either left to heat to room temperature (~ 22 °C) for a few hours or heated on a heating plate to 72 °C (for approximately 12 min). Hereafter, a premix was prepared by adding the fish oil slowly to the protein/buffer solution during mixing at 16,000 rpm (Ystral mixer, Ballrechten-Dottingen, Germany) for a total of three minutes. The oil was added during the first minute of mixing. The addition of oil only influenced the temperature slightly. A second homogenization step was carried out in a two-stage valve Rannie homogenizer (APV, Albertslund, Denmark) at a pressure of either 5 MPa or 22.5 MPa in the first valve and 0.5MPa and 2.5MPa, respectively, in the second valve. Emulsions were homogenized with three passes (1 L/min) through the homogenizer. After homogenization emulsions were added 0.05% sodium azide to prevent microbial growth. Emulsions (65 g) were stored in closed 100 mL Bluecap bottles at room temperature (20.2 °C ± 0.2 °C) in the dark for up to 14 days, with one bottle of emulsion for each sampling time point.

2.3 Characterization of the emulsions

2.3.1 pH, viscosity, and droplet size of emulsion

The pH was measured in the emulsions at day 0 and 14, at room temperature, directly in the sample during stirring (pH meter, 827 pH Lab, Methrom Nordic ApS, Glostrup, Denmark).

Viscosities of the emulsions were measured at day 1 and 14 using a stress controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden) equipped with a CC25 standard bob cup system in a temperature vessel. Measurements (15 mL emulsion) were done at 20 °C (equilibration time 5 min) by a linear increase in shear stress from 0.01 to 1.64 Pa. Viscosities are given as the average viscosity of the linear part of the plot of shear stress versus viscosity and are expressed in mPa·s. Viscosities were measured twice on each emulsion.

Droplet sizes were measured at day 1 and 14 by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK), and distributions in volume % as well as droplet mean diameters were calculated. Emulsion droplets were suspended in recirculating water (3000 rpm), reaching an obscuration of 13-18%. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively.

2.3.2 Protein content in the aqueous phase

Emulsions (~20 g) were centrifuged for 50 min at 45,000 g and 10 °C (Sorvall RC-6 PLUS, Thermo Fisher Scientific, Osterode, Germany; rotor SS-34) and the water phase was extracted by the use of a syringe. The obtained water phase was then subjected to ultracentrifugation (Beckman Ultracentrifuge L8-60M, Fullerton, CA; rotor 21102) for 60 min at 70,000 g and 15 °C, and once again the water phase was extracted by the use of a syringe. The water phase was diluted 1:9 in 10 mM sodium acetate imidazole buffer (pH 7.0). The total protein concentration was determined by the use of a BCA protein assay reagent kit (Pierce, ThermoScientific, Rockford, IL, USA) and a spectrophotometric determination at 562 nm.

To separate the individual protein components in the extracted water phases SDS-page was conducted. The water phases were diluted in 10 mM sodium acetate imidazole buffer (pH 7.0) to a concentration of approximately 1 mg protein/mL, and then diluted 1:1 with 10% DTT/Laemlli buffer (63 mM Tris-HCl pH 6.8, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue), and boiled for 3 min. Samples were centrifuged for 3 min at 12,000 rpm (Biofuge pico, Heraeus, Osterode, Germany). Samples (10 µL) were loaded on NuPage 10% Bis-Tris gels (Novex, Invitrogen, Life Technologies Ltd, Paisley PA4RF, UK), and run in MES running buffer for 35 min at 200 V. The gels were stained with Coomassie Brilliant Blue R-250 and the individual protein spots were assessed by the use of QuantityOne 4.0 (Bio-Rad, Hercules, CA, USA).

2.4 Measurements of lipid oxidation

2.4.1 Lipid extraction and peroxide values

A lipid extract was prepared from each emulsion according to a modified version of the method described by Bligh and Dyer [21] using 10 g emulsion and a reduced amount of solvent (30.0 mL methanol and 30.0 mL chloroform). Peroxide values were subsequently determined in this lipid extract or directly in the oil samples by colorimetric determination of iron thiocyanate at 500 nm as described by Shantha and Decker [22].

2.4.2 Secondary oxidation products

Volatile secondary oxidation products were analyzed according to the method described by Let et al. [23]. Approximately 4 g of emulsion and 30 mg internal standard (4-methyl-1-pentanol, 30 μg/g water) were weighted out in a 100 mL purge bottle. The bottle was heated in a water bath at 45 °C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile secondary oxidation products were trapped on Tenax GR tubes. The volatiles were desorbed again by heat (200 °C) in an Automatic Thermal Desorber (ATD-400, Perkin Elmer, Norwalk, CN), cryofocused on a cold trap (-30 °C), released again (220 °C), and led to a gas chromatograph (HP 5890IIA, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m x 0.25 mm x 1.0 μm; J&W Scientific, Folsom, CA, USA). The oven program had an initial temperature of 45 °C for 5 min, increasing with 1.5 °C/min until 55 °C, with 2.5 °C/min until 90 °C, and with 12.0 °C/min until 220 °C, where the temperature was held for 4 min. The individual compounds were analyzed by mass-spectrometry (HP 5972 mass-selective detector, Agilent Technologies, Palo Alto, CA, USA; Electron ionisation mode, 70 eV; mass to charge ratios between 30 and 250). From a comparison of chromatograms from non-oxidised and oxidised samples, the following volatiles were selected for quantification: butanal, pentanal, 1-penten-3-ol, 1-penten-3-one, hexanal, 2-hexenal, and 2,4-heptadienal. Calibration curves were made by dissolving the selected volatile compounds in 96% ethanol, and diluting to concentrations in the range 25-500 ng/μL. These solutions were injected (1 μL) directly on the Tenax tube (in triplicate) using a small syringe (Hamilton syringe 7105N, Bonaduz, Switzerland). Ethanol was subsequently removed by nitrogen (purge flow 50 mL/min, 5 min).

2.5 Statistical analyses

Data were analysed by one or two-way analysis of variance with Bonferronis multiple comparison test as post test (GraphPad Prism, version 4.03, GraphPad Software Inc., La Jolla, CA, USA). For volatiles data, all samples and all sampling time points were included in the two-way ANOVA carried out on each individual volatile compound. However, only day 0 and day 14 are shown in Table 3. All references to significant differences ($p < 0.05$) between samples or between sampling times, are based on these statistical analyses of data.

3 Results

3.1 Characterization of the emulsions

3.1.1 pH and viscosity

The pH ranged from 6.6 to 6.9 (Table 2) in emulsions at day 0 and from 6.7 to 6.9 at day 14 (Data not shown). The viscosity of the emulsions was in the range from 2.90 to 3.18 mPa·s, with no significant increases during storage. At day 1, the viscosity in the four samples with WP did not differ significantly. However, among the samples with LgCAS some variations were observed. Emulsions prepared at high pressure were slightly more viscous than their corresponding emulsions prepared at low pressure (Table 2), with a significant difference between emulsions prepared at 72 °C. At day 14 differences were less clear and the rank order of the emulsions were WP_high^{a} ≤ WP_high72^{ab} = LgCAS_low^{ab} ≤ LgCAS_low72^{abc} = WP_low72^{abc} ≤ LgCAS_high^{bc} = WP_low^{bc} ≤ LgCAS_high72^{c}.

3.1.2 Droplet sizes

Droplet size distributions are shown in Figure 1 and mean oil droplet sizes in Table 2. At day 1 mean oil droplet sizes (expressed as D[3,2]) ranged from 548 nm to 711 nm in emulsions prepared at low pressure, and from 220 nm to 362 nm in emulsions prepared at high pressure. At the same sample time point, mean oil droplet sizes (expressed as D[4,3]) ranged from 1566 nm to 2258 nm in emulsions prepared at low pressure and from 423 nm to 782 nm in emulsions prepared at high pressure. The mean oil droplet size did only increase significantly during storage in LgCAS_low, when the size was expressed as D[4,3], otherwise no increases in mean droplet sizes were observed. Droplet size distributions were in general bimodal, especially for emulsions prepared at high pressure. At low pressure the two peaks were less clearly distinguished. An increase in temperature and especially in pressure moved the distributions toward smaller particle sizes. The effect of pressure was confirmed from mean oil droplet sizes that showed the following rank order at day 1 for D[4,3] WP_high72^{a} = LgCAS_high72^{a} = WP_high^{a} = LgCAS_high^{a} < LgCAS_low^{b} = LgCAS_low72^{b} = WP_low72^{b} = WP_low^{b}. The effect of temperature on mean oil droplet sizes was not significant despite the observed change in the shape and to some extent position of the droplet size distributions (Figure 1).

3.1.3 Protein content in the aqueous phase

The total protein content in the aqueous phase of the emulsions ranged from 3.3-4.3 mg/mL (Table 2). Thus, in general a high pressure resulted in a lower concentration of proteins in the aqueous phase

than a low pressure when emulsions with the same emulsifier and at the same temperature were compared. For emulsions prepared at low pressure an increase in temperature increased the protein content in the aqueous phase significantly. In contrast, for emulsions prepared at high pressure no difference was observed when emulsions were prepared with LgCAS, but when prepared with WP an increase in temperature decreased the concentration of protein in the aqueous phase.

SDS-page of the protein compositions in the aqueous phase showed that in both WP and LgCAS emulsions the concentration of β-lactoglobulin was slightly lower when emulsions were prepared at high pressure (Figure 2). In WP emulsions the opposite of what was observed for concentrations of β-lactoglobulin was evident for concentrations of α-lactalbumin. Similarly was the concentration of casein in emulsions prepared with LgCAS higher when emulsions were prepared at the highest pressure. As the only sample LgCAS_low72 had equal concentrations of β-lactoglobulin and casein in the aqueous phase (approximately 30% of each) and it furthermore also contained approximately 20% α-lactalbumin. A comparable observation was done in a similar emulsion prepared at a pressure of 12.5 MPa and 72 °C (Data not shown). An increase in pressure thus led to a decrease in α-lactalbumin in the aqueous phase when emulsions were prepared at 72 °C. The temperature effect on the composition of proteins in the aqueous phase was not clear as a temperature increase in some cases led to increased concentrations of e.g. β-lactoglobulin in the aqueous phase and in other cases the opposite was observed.

3.2 Lipid oxidation in emulsions

3.2.1 Peroxide values (PV)

PV increased significantly in all samples during storage. However, PV did not differ significantly between WP samples until day 14. At day 14 the rank order was WP_high72^{a} = WP_high^{a} < WP_low72^{b} = WP_low^{b} (Figure 3a). Hence, an increase in pressure reduced the development in PV, whereas an increase in temperature did not change PV significantly. In contrast to the samples prepared with WP, samples prepared with LgCAS were already significantly different at day 0 (Figure 3b). At day 0 the rank order was LgCAS_high^{a} < LgCAS_low72^{b} = LgCAS_high72^{b} < LgCAS_low^{c}. PV in LgCAS samples though developed at different rates, and at day 14 the rank order was LgCAS_high72^{c} < LgCAS_high^{d} < LgCAS_low72^{e} < LgCAS_low^{f}. Thus, increasing both temperature and pressure during emulsion production decreased PV. These observations were in accordance with results from a pre-experiment carried out prior to the current study, where three sampling timepoints were included. In addition, in the current experiment data were recorded on emulsions prepared at intermediate pressures (12.5 and 15 MPa) and the results obtained were in agreement with the abovementioned patterns (Data not shown).

Comparison between samples produced under the same conditions but with different emulsifiers (LgCAS versus WP) showed that emulsions with LgCAS had significantly higher PV than WP emulsions already at day 0, except emulsions prepared at high pressure/room temperature, which were not significantly different. At day 14 all emulsions with LgCAS had significantly higher PV than all emulsions with WP.

3.2.2 Volatile secondary oxidation products

The concentrations of pentanal, 1-penten-3-ol and 2-hexenal increased significantly in all samples during storage (Table 3). The concentrations of hexanal furthermore increased in all samples prepared with WP and in three of the four samples prepared with LgCAS (not LgCAS_low72). Butanal concentrations increased in all samples prepared with LgCAS and in three of the four samples prepared with WP (not WPlow_72). The concentrations of 1-penten-3-one increased in all samples prepared with LgCAS, but only in WP samples prepared at room temperature (WP_low and WP_high). Concentrations of 2,4-heptadienal did not increase significantly in any WP samples but in all LgCAS samples except LgCAS_high72.

Between WP samples none of the volatiles differed significantly in concentrations at day 0, but over time the concentrations of the seven volatiles quantified developed differently among the four samples (Table 3). The concentrations of butanal, pentanal, 1-penten-3-ol and 2-hexenal increased the most in the samples prepared at high pressure whereas the temperature did not have any clear effect. In contrast, 1-penten-3-one only increased in the samples prepared at room temperature, but the increase was modest. For hexanal, only WP_low had a significantly higher concentration than the other WP samples at day 14, whereas no significant differences were observed for the concentration of 2,4-heptadienal between any of the WP samples at day 14. Thus, the main differences observed from volatiles data were related to the increase in pressure, which resulted in increased concentrations of some volatiles. No clear effect of temperature was observed.

For the LgCAS emulsions at day 0, the concentration of pentanal and hexanal were significantly higher in LgCAS_low72 than in the two emulsions prepared at high pressure (LgCAS_high and LgCAS_high72). For hexanal the concentration in LgCAS_low72 was even significantly higher than the concentration in the similar emulsions prepared at room temperature (LgCAS_low). None of the other volatiles showed significant differences at day 0. Concentrations of 1-penten-3-ol, 1-penten-3-one, hexanal, 2-hexenal and 2,4-heptadienal increased more during storage in samples prepared at

low pressure than in samples prepared at high pressure (Table 3). The concentration of butanal was similar for all samples whilst only LgCAS_low72 had a significantly higher concentration of pentanal when compared to the other samples at day 14. Hence, the overall conclusion on the effect of pressure treatment on volatiles data in LgCAS emulsions was the opposite of what was concluded from WP emulsions. A slower development in the concentrations of the majority of the volatile secondary oxidation products was observed at increased pressure. The impact of temperature on the development of secondary oxidation products was, however, not clear in LgCAS emulsions either.

Confirming results from PV data, LgCAS emulsions in general had higher concentrations of the volatiles quantified than the similar emulsions prepared with WP (for five of the seven volatiles).

4 Discussion

The observed decrease in oil droplet size due to an increase in homogenization pressure independent of emulsifier was in accordance with previous studies [12, 16, 24]. Furthermore, Let et al. [16] reported a slight decrease in oil droplet size in fish oil enriched milk when temperature was increased from 50 °C to 72 °C prior to homogenization. In the present study, a slight difference between emulsions homogenized at different temperatures was observed from inspecting droplet size distributions, however, mean oil droplet sizes (D[4,3]) did not differ significantly. Despite small differences in the viscosities between samples at day 1, no correlations could be observed between viscosity and lipid oxidation in general.

In the present study, an increase in homogenization pressure decreased the concentration of proteins in the aqueous phase. This is contradictory to results obtained by Liu et al. [25] whom observed that an increase in pressure (between 0 and 160 MPa) led to a higher solubility of whey proteins in aqueous solution. These authors explained their results by an increased exposure of hydrophilic parts of amino acids towards water upon high pressure treatment. In our study however, much lower pressures were applied, and furthermore, in our emulsions a lipophilic surface of the oil droplets competes and attracts exposed hydrophobic parts of the proteins. This most likely explains the observed lower solubility of proteins in the aqueous phase in our study.

Regarding the adsorption of individual protein components to the interface in the present study, an increase in homogenization pressure decreased the concentration of β-lactoglobulin in the aqueous phase and thus increased the adsorption of β-lactoglobulin to the interface irrespective of the emulsifier used to prepare the emulsions. Similar effects were observed in a previous study on fish

oil enriched milk [17]. In contrast, the effect of temperature on the adsorption of β-lactoglobulin to the interface was emulsifier dependent in the present study. Hence, in WP emulsions an increase in temperature decreased the adsorption of β-lactoglobulin when emulsions were homogenized at low pressure, but not at high pressure. In LgCAS emulsions an increase in temperature increased adsorption of β-lactoglobulin at both pressures in accordance with the observations from the milk study [17]. However, at low pressure an increase in temperature reduced the adsorption of α-lactalbumin and thereby led to a total increase in the proportion of whey proteins relative to the proportion of caseins in the aqueous phase. These results cannot be explained from the current literature or from the present data, and further studies are needed to confirm the data and explain the mechanisms behind the observations.

The effect of homogenization conditions on lipid oxidation differed depending on the emulsifier used and will therefore be discussed separately for the two emulsifiers in the following.

4.1 Lipid oxidation in emulsions prepared with whey proteins (WP)

The effect of homogenization temperature and pressure in emulsions prepared with WP was not clear, but the tendency was towards a more pronounced effect of pressure than of temperature. Thus, a higher pressure led to lower PV, but also a higher concentration of volatile secondary oxidation products.

A low PV and a high concentration of volatiles could be the result of a fast degradation of lipid hydroperoxides in these emulsions, caused by exposure to transition metal ions. When pressure was increased, droplet sizes decreased, whereby the total droplet surface area increased. This has previously been hypothesized to increase lipid oxidation due to increased exposure of lipid hydroperoxides towards transition metal ions in the aqueous phase [26-28]. Results from studies on the influence of droplet size on lipid oxidation are, however, unclear and in general other factors are most often concluded to influence lipid oxidation more than the actual droplet size [17, 29-31]. Hence, the droplet size might not be the sole explanation for the results obtained in the present study.

Besides the differences in oil droplet size, the protein composition in the aqueous phase was also slightly different when emulsions were prepared at different pressures. At low pressure, more β-lactoglobulin was present in the aqueous phase than at high pressure. Hence, it could be speculated that the antioxidative activity of individual whey protein components differed when present at the interface or in the aqueous phase and that this could explain the increase in concentrations of volatile oxidation products at high pressure. Structural changes have been observed upon adsorption of β-lactoglobulin to an interface [3, 32]. In the study by Zhai et al. [32] a loss of globular structure in β-lactoglobulin was observed upon adsorption. This could potentially change the accessibility of amino

acid residues with antioxidative properties. To fully explain the present results, more studies are needed on the unfolding of whey proteins under different conditions.

With regards to homogenization temperature, this seemed to have little impact on the oxidative stability and this was somewhat surprising as results in milk showed that heating to 72 °C decreased lipid oxidation [16]. In addition, Kiokias et al. [15] showed a reduction in conjugated diene formation when 30% sunflower o/w emulsions were stabilized by heat-treated whey protein concentrate instead of native whey protein concentrate. In their study, the oxidative stability was increased in the temperature range from 60 °C to 80 °C. At 80°C the whey proteins were expected to have all there reduced sulfhydryls in the reactive form, and no beneficial effect of further heating was observed. However, a study on the addition of native or pre-heated β-lactoglobulin to the aqueous phase of Brij-stabilized 5% menhaden oil-in-water emulsions showed that to decrease lipid hydroperoxides and TBARS formation, β-lactoglobulin should be pre-heated to 95 °C [33]. Preheating to 70 °C did not have any effect as compared to native β-lactoglobulin, even though the exposure of cysteine and thereby sulfhydryl residues were highest at 70 °C. In addition, the same authors showed that the ability to scavenge free radicals was better for β-lactoglobulin pre-heated to 70 °C than for native β-lactoglobulin [33]. Hence, the fact that heat treatment had only a slight impact on lipid oxidation in emulsions prepared with WP in the present study is difficult to explain, and studies of heat treatment to higher temperatures would be valuable.

4.2 Lipid oxidation in emulsions prepared with casein and β-lactoglobulin (LgCAS)

The emulsions produced with a combination of CAS and Lg were prepared using a ratio of casein and β-lactoglobulin close to that found in milk. The effect of homogenization pressure on the oxidative stability was in accordance with results obtained in milk [16]. Thus, despite a decrease in oil droplet size and an increased total surface area of the oil droplets, lipid oxidation was decreased when emulsions were produced at high pressure. In milk, it was suggested that a more optimal partitioning of proteins between the interface and the aqueous phase was responsible for the higher oxidative stability when emulsions were produced at a high pressure than at a low pressure [16, 17]. Similar results were obtained in the present study, where the concentration of CAS was higher in the aqueous phase when emulsions were produced at high pressure, and similarly the concentration of β-lactoglobulin was lower. The presence of CAS in the aqueous phase has previously been shown to provide a good antioxidative effect by effectively chelating transition metal ions both in emulsions [1] and algal oil enriched milk [34].

In milk, an increased concentration of β-lactoglobulin at the interface upon increasing the homogenization temperature was mainly ascribed to the unfolding of β-lactoglobulin which increases its ability to adsorb to the interface [17]. In the present study, the effect on lipid oxidation of

increasing temperature was not clear, but an increase in pressure decreased the concentration of β-lactoglobulin in the aqueous phase. The increase in pressure might therefore in itself have led to unfolding of the protein and in turn increased adsorption of β-lactoglobulin at the oil-water interface even when the proteins were not heated prior to homogenization. Structural changes in β-lactoglobulin due to high pressure homogenization have previously been suggested by Stapelfeldt and Skibsted [35] as well as Lee et al. [36]. If increased pressure led to unfolding of β-lactoglobulin this could explain why an increase in temperature when homogenizing at high pressure did not have any additional effect on lipid oxidation, but it cannot explain why temperature did not have any effect at low pressure. Further studies are needed to elucidate this matter.

4.3 The emulsifier dependent effect of homogenization pressure and temperature

Despite more or less similar conclusions from the effect of pressure and temperature on protein compositions in the aqueous phase and droplet size distributions for WP and LgCAS emulsions, their oxidative stability differed significantly. Hence, LgCAS emulsions oxidized more than WP emulsions under the conditions applied in the present study. The better oxidative stability of WP emulsions was surprising, since emulsions prepared with casein in previous studies have been shown to increase the oxidative stability compared to emulsions prepared with whey proteins [31, 37-40]. However, these emulsions were prepared with casein only and did not contain any β-lactoglobulin and this may have influenced the results. In addition, the opposite effect on the oxidative stability of emulsions with different emulsifier combinations was observed when increasing the homogenization pressure. Hence, this indicates that when CAS is present (as in LgCAS) it is most beneficial to have this protein in the aqueous phase and β-lactoglobulin at the interface, whereas when CAS is not present (as in WP), it is more beneficial to have β-lactoglobulin in the aqueous phase and α-lactalbumin at the interface. An antioxidative effect of β-lactoglobulin in the aqueous phase of Brij-stabilized emulsions has been shown by Elias et al. [8], and was suggested to mainly depend on a radical scavenging effect of cysteine and tryptophan residues. The same authors later reported that β-lactoglobulin may possess both radical scavenging activity and have metal chelating properties when present in the aqueous phase of Brij-stabilized emulsions [33]. These observations support the observations in the present study on the importance of β-lactoglobulin in the aqueous phase.

From the present data it can be concluded that an increase in homogenization pressure increased oxidative stability of LgCAS emulsions, whereas the opposite was observed for WP emulsions. An increase in temperature had only minor effects on the oxidative stability, and no clear conclusions on its effect could be drawn from the present results. In WP emulsions the combination of β-lactoglobulin in the aqueous phase and larger oil droplet sizes seemed to decrease lipid oxidation. In

LgCAS emulsions, casein present in the aqueous phase had an antioxidative effect and the oil droplet size did not seem to influence lipid oxidation. The combination of whey protein isolates (WPI and WPIα (1:1)) used in the present study was observed to be preferential over a mix of purified Lg and CAS (1:9), with respect to obtaining oxidatively stable emulsions.

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Table 1. Experimental design.

Sample code	Emulsifier [%]	Pressure [MPa]	Temperature [°C]
WP_high72	0.5 WPI + 0.5 WPIα	22.5	72
WP_high	0.5 WPI + 0.5 WPIα	22.5	22
WP_low72	0.5 WPI + 0.5 WPIα	5	72
WP_low	0.5 WPI + 0.5 WPIα	5	22
LgCAS_high72	0.1 Lg + 0.9 CAS	22.5	72
LgCAS_high	0.1 Lg + 0.9 CAS	22.5	22
LgCAS_low72	0.1 Lg + 0.9 CAS	5	72
LgCAS_low	0.1 Lg + 0.9 CAS	5	22

Table 2. Physico-chemical data for the emulsions. Letters indicate significant differences between samples for each column ($p > 0.05$). For interpretation of sample codes refer to Table 1.

Sample code	pH	Viscosity [mPa·s]		Droplet size D[3,2] [nm]		Droplet size D[4,3] [nm]		Protein in aqueous phase [mg/mL]
	Day 0	Day 1	Day 14	Day 1	Day 14	Day 1	Day 14	Day 1
WP_high72	6.9	2.94 ± 0.00^{a}	2.94 ± 0.08^{ab}	220 ± 3^{a}	212 ± 10^{a}	423 ± 3^{a}	417 ± 13^{a}	3.3 ± 0.0^{a}
WP_high	6.8	2.96 ± 0.00^{a}	2.90 ± 0.02^{a}	258 ± 1^{ab}	255 ± 5^{a}	580 ± 2^{a}	590 ± 6^{a}	3.8 ± 0.0^{c}
WP_low72	6.9	2.98 ± 0.10^{a}	3.02 ± 0.00^{abc}	707 ± 58^{f}	681 ± 50^{e}	2019 ± 42^{b}	2033 ± 68^{b}	4.3 ± 0.0^{e}
WP_low	6.8	2.94 ± 0.02^{a}	3.10 ± 0.06^{bc}	711 ± 50^{f}	617 ± 24^{d}	2258 ± 33^{b}	2222 ± 27^{b}	3.9 ± 0.1^{cd}
LgCAS_high72	6.9	3.18 ± 0.01^{c}	3.16 ± 0.14^{c}	283 ± 3^{b}	255 ± 17^{a}	526 ± 3^{a}	492 ± 8^{a}	3.3 ± 0.1^{a}
LgCAS_high	6.8	3.16 ± 0.13^{bc}	3.08 ± 0.08^{bc}	362 ± 4^{c}	343 ± 2^{b}	782 ± 4^{a}	774 ± 2^{a}	3.3 ± 0.1^{a}
LgCAS_low72	6.6	3.01 ± 0.05^{ab}	3.02 ± 0.05^{abc}	601 ± 52^{e}	596 ± 42^{cd}	1812 ± 70^{b}	1864 ± 23^{b}	4.0 ± 0.0^{d}
LgCAS_low	6.8	3.03 ± 0.02^{abc}	2.97 ± 0.01^{ab}	548 ± 48^{d}	545 ± 38^{c}	1566 ± 37^{b}	2473 ± 1776^{b}	3.5 ± 0.0^{b}

Table 3. Concentration of volatile secondary oxidation products in ng/g emulsion. Letters indicate significant differences between samples for each column ($p > 0.05$). nd: not detected. Standard deviations < 0.5 are stated as 0.

	Butanal		Pentanal		1-penten-3-ol		1-penten-3-one		Hexanal		2-hexenal		2,4-heptadienal	
	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
WP_high72	nd	5 ± 2^{cd}	1 ± 0^{a}	11 ± 1^{d}	2 ± 0^{a}	29 ± 2^{c}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	3 ± 0^{a}	nd	8 ± 0^{b}	12 ± 0^{a}	13 ± 0^{a}
WP_high	nd	6 ± 0^{d}	1 ± 0^{a}	10 ± 1^{cd}	2 ± 0^{a}	25 ± 2^{bc}	2 ± 0^{a}	3 ± 0^{b}	2 ± 0^{a}	3 ± 0^{a}	nd	8 ± 0^{b}	12 ± 0^{a}	13 ± 0^{a}
WP_low72	nd	1 ± 1^{a}	1 ± 0^{a}	7 ± 1^{a}	2 ± 0^{a}	15 ± 1^{a}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	3 ± 0^{a}	nd	7 ± 0^{a}	12 ± 0^{a}	13 ± 1^{a}
WP_low	nd	3 ± 0^{b}	1 ± 0^{a}	9 ± 0^{bc}	2 ± 0^{a}	20 ± 1^{ab}	2 ± 0^{a}	3 ± 0^{b}	2 ± 0^{a}	4 ± 0^{b}	nd	7 ± 0^{a}	12 ± 0^{a}	13 ± 1^{a}
LgCAS_high72	nd	3 ± 0^{b}	1 ± 0^{a}	8 ± 1^{ab}	5 ± 1^{a}	38 ± 3^{d}	2 ± 0^{a}	3 ± 0^{b}	2 ± 0^{a}	4 ± 0^{b}	nd	9 ± 0^{c}	12 ± 0^{a}	14 ± 1^{ab}
LgCAS_high	nd	4 ± 0^{bc}	1 ± 0^{a}	9 ± 1^{bc}	2 ± 0^{a}	39 ± 3^{d}	2 ± 0^{a}	4 ± 0^{c}	2 ± 0^{a}	4 ± 0^{b}	nd	9 ± 0^{c}	12 ± 0^{a}	16 ± 1^{b}
LgCAS_low72	nd	4 ± 0^{bc}	3 ± 0^{b}	11 ± 2^{d}	4 ± 0^{a}	56 ± 8^{e}	2 ± 0^{a}	6 ± 1^{e}	6 ± 0^{c}	6 ± 1^{c}	nd	10 ± 1^{d}	12 ± 1^{a}	22 ± 4^{c}
LgCAS_low	nd	3 ± 0^{b}	2 ± 0^{ab}	9 ± 1^{bc}	6 ± 1^{a}	69 ± 5^{f}	3 ± 0^{b}	5 ± 0^{d}	4 ± 0^{b}	6 ± 0^{c}	nd	10 ± 0^{d}	13 ± 0^{a}	24 ± 1^{c}

Figure 1. Droplet size distributions in emulsions (n=2). For interpretation of sample codes refer to Table 1.

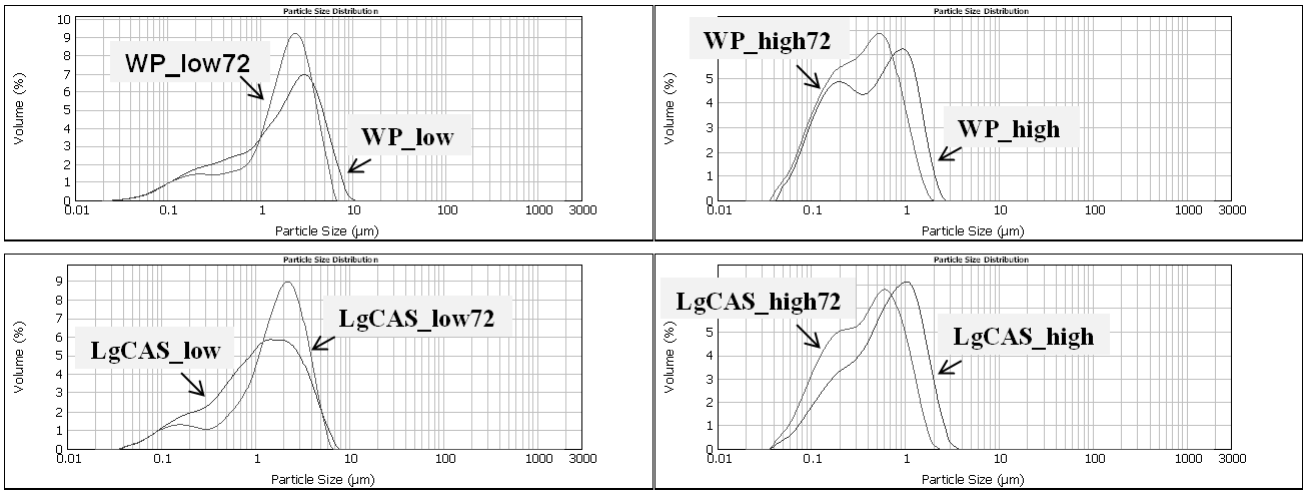


Figure 2. Protein composition in the aqueous phase as determined by SDS-page (β-lg: β-lactoglobulin; α-lac: α-lactalbumin). Lane 1: Molecular weight standard (SeeBlue ® Plus2 Prestained Standard); Lane 2: WP_low; Lane 3: WP_low72; Lane 4: WP_high; Lane 5: WP_high72; Lane 6: Molecular weight standard (SeeBlue ® Plus2 Prestained Standard); Lane 7: LgCAS_low; Lane 8: LgCAS_low72; Lane 9: LgCAS_high; Lane 10: LgCAS_high72. For interpretation of sample codes, please refer to Table 1.

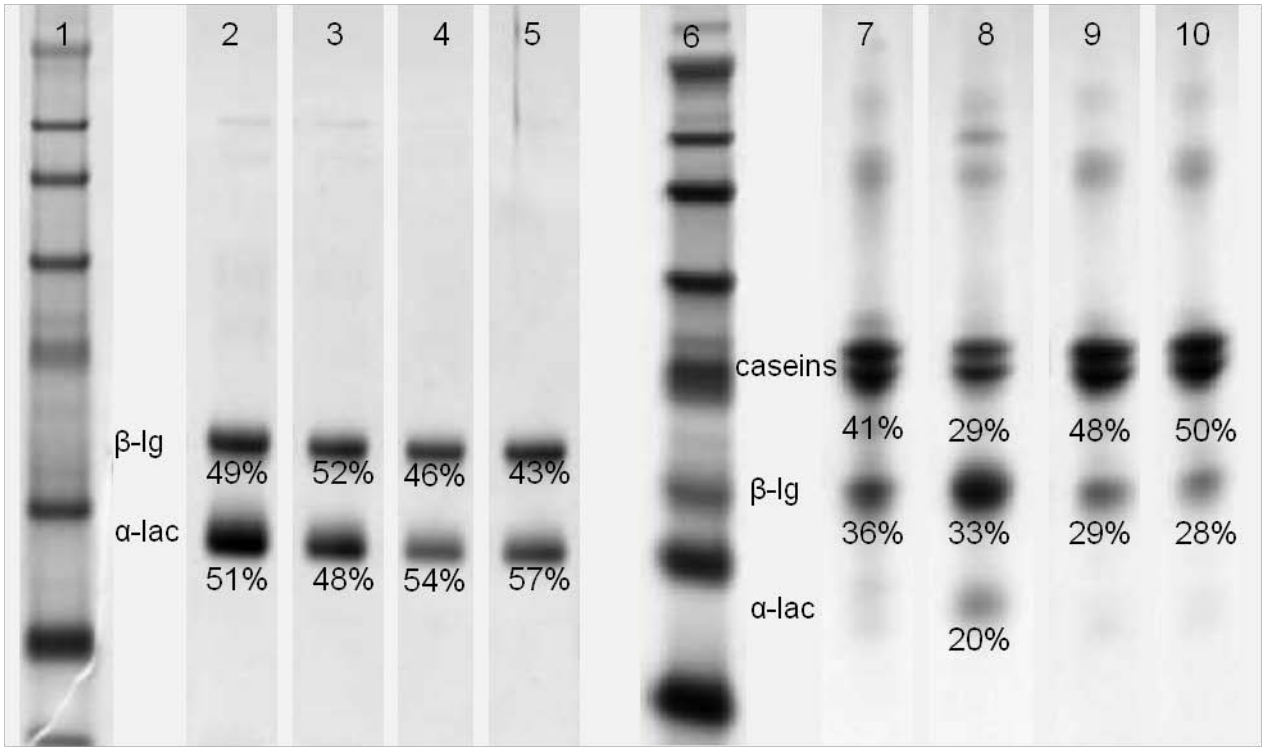
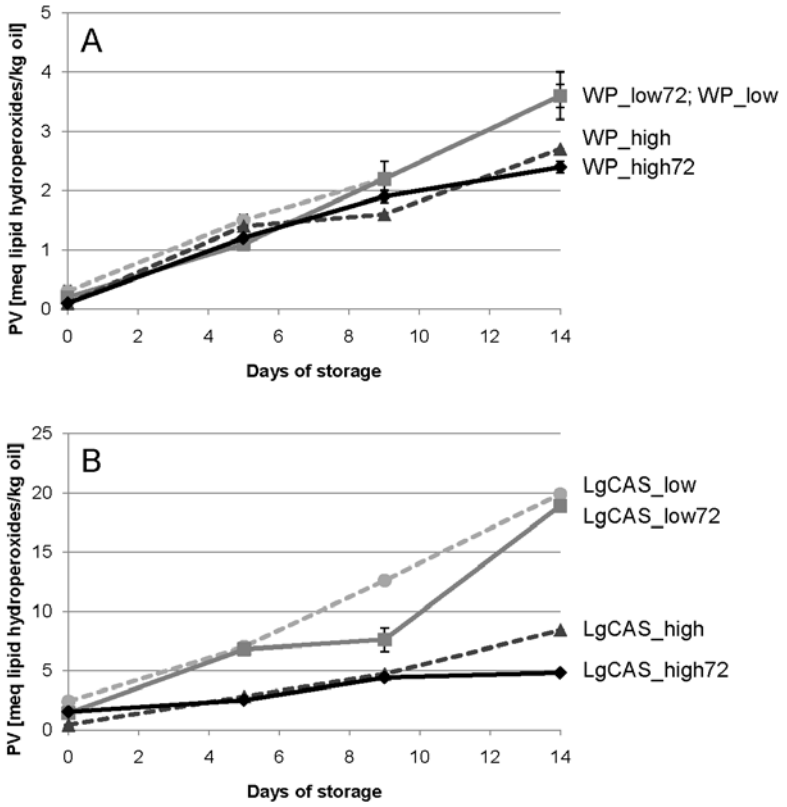


Figure 3. Peroxide values in emulsions as determined during storage (n=3). Standard deviations are given by vertical bars. For interpretation of sample codes, please refer to Table 1.



PAPER VI

Effect of emulsifier type, pH and iron on oxidative stability of 5% fish oil-in-water emulsions

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Effect of emulsifier type, pH and iron on oxidative stability of 5% fish oil-in-water emulsions

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Running title: Oxidative stability of fish oil emulsions

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Key words: lipid oxidation, zeta potential, volatile oxidation products, milk proteins, phospholipids

ABBREVIATIONS USED

LC PUFA, long-chain polyunsaturated fatty acids; $FeSO_4$, iron sulphate; PV, peroxide value; PCA, principal component analysis; PC1, principal component 1; PC2, principal component 2; pI, isoelectric point; Whey, whey protein isolate emulsifier and emulsions made with this; Cas, caseinate emulsifier and emulsions made with this; Lec, lecithin emulsifier and emulsions made with this; MPL20, milk phospholipid emulsifier with 20% phospholipid and emulsions made with this; MPL75, milk phospholipid emulsifier with 75% phospholipid and emulsions made with this

Summary. The effect on lipid oxidation of using different emulsifiers in 5% fish oil-in-water emulsions was investigated. Emulsions with protein based emulsifiers; whey protein isolate (Whey) and sodium caseinate (Cas) were the most oxidatively stable, followed by phospholipid based emulsifiers; soy lecithin (Lec) and two milk phospholipid concentrates (MPL20 and MPL75), independent of pH (3 or 7), presence of added iron or emulsifier concentration with few exceptions. Increase in emulsifier concentration generally increased the oxidative stability at pH 7 particularly in emulsions with iron, whereas at low pH, the effect depended on both iron addition and emulsifier type. Addition of iron only changed the order of stability, amongst the emulsions produced with different emulsifiers, at neutral pH. Moreover, the effect of pH depended on both emulsifier concentration and iron addition. Iron addition resulted in faster oxidation at pH 7 than at pH 3. In emulsions without iron and with low emulsifier concentration, pH 7 emulsions oxidized faster for MPL20, Whey and Cas emulsions. When high emulsifier concentration were used, oxidation was most pronounced at pH 7 in Lec, Cas, Whey and MPL20 emulsions, and at pH 3 in MPL75 emulsions.

Practical applications: The overall conclusion from this study was that the oxidative stability of 5 % o/w emulsions depended on both emulsifier type, concentration, pH and iron content. Although this finding was observed in simple o/w emulsions, the same conclusion is most likely also valid in more complex food emulsions with similar or higher lipid contents such as milk drink, dressing etc. Hence, in such foods the emulsifier and the emulsifier concentration should be carefully chosen in order to minimize lipid oxidation.

1. Introduction

Due to the health beneficial effects of omega-3 long-chain polyunsaturated fatty acids (LC PUFA) [1], a high intake of fish and fish-products is recommended by various health organisations. However, despite increased awareness by consumers, the intake of fish and fish-products in the Western population is still lower than recommended. Introduction of fish-oil-enriched foods on the market may help to increase the intake of omega-3 LC PUFA. However, addition of these oxidatively unstable fatty acids will lead to increased lipid oxidation in food products to which they are added, unless precautions are taken. Prooxidative compounds, such as transition metal ions, may be present in the matrix to which the oil is added and this is particularly a challenge in omega-3 PUFA rich foods. This is due to the fact that trace metals mainly exert their prooxidative effect by decomposing already existing lipid hydroperoxides and peroxides stemming from omega-3 PUFA are more susceptible to decomposition than peroxides from less unsaturated fatty acids [2]. Particularly, traces of iron are present in most food products. It may stem from the oil itself, as well as from the other ingredients in the product or from processing equipment.

Several of the food products to which omega-3 PUFA have been added are emulsions. Since most foods themselves are oil-in-water (o/w) emulsions, this emulsion type may be used as delivery systems when oil is added to such food products. The use of o/w emulsions as delivery systems may confer the additional benefit of protecting the oil from oxidation. This has been observed in some products [3].

In order to obtain a stable emulsion, the oil must be present as droplets stabilised by emulsifier or a stabilising agent. Several different emulsifiers are available and some of the most commonly used are protein based and phospholipids based together with mono- and di-acylglycerols. The type of emulsifier used will significantly affect the characteristics of the resulting emulsion e.g. the droplet size, the droplet charge and the viscosity of the emulsion. Moreover, several studies have demonstrated that the emulsifier can protect the oil against lipid oxidation [3, 4]. It has been suggested that the protecting effect may be due to several reasons; First of all, it is hypothesised that oxidation is initiated at the interface between oil and water phases. Since the emulsifier is located at the interface it constitutes a physical barrier around the oil droplet, which may prevent contact between prooxidants in the water phase and the oil in the emulsion droplets [2]. The charge of the droplets may also affect the oxidative stability [5, 6]. For example protein based emulsifiers, and thus the interface of emulsion droplets produced with these, may be positively or negatively charged depending on the pI of the emulsifier and the pH of the emulsion. A positively charged interface will repel positively charged

metal ions, thereby reducing oxidation, whereas a negatively charged interface may attract the positively charged ions and thereby increase oxidation. Some emulsifiers are able to chelate transition metal ions that would otherwise catalyse oxidation, and thereby they can act as antioxidants. Thus, emulsifier at the interfase and especially surplus of emulsifier in the water phase of the o/w emulsion may exert antioxidative effects. Surplus emulsifier in the water phase, may also increase the viscosity of the emulsion, and could in this way reduce the mobility of compounds in the water phase and thereby reduce oxidation.

In addition to the above mentioned characteristics of the emulsion droplet interface, the droplet size and the droplet size distribution affects the physical stability of the emulsion and in some cases it may also influence the oxidative stability and interaction with other ingredients such as pro and antioxidants. In some cases it has been observed that smaller droplets are less oxidatively stable than larger droplets. This has been explained by the fact that emulsions containing smaller droplets have a larger total interfacial area than those having larger droplets and, a larger total interfacial area means a larger contact area for oxidative reactions between prooxidants and the oil [7]. However, this correlation is not always observed [8], and other factors may be more important for the oxidative stability.

Milk proteins are naturally occurring emulsifying proteins, which are widely used in the industry. Milk proteins constitute mainly two types: caseins (the major part) and whey proteins. Clusters of hydrophobic amino acids in the milk proteins are responsible for their ability to adsorb to the surface of oil droplets. Due to the different characteristics of the caseins and the whey proteins their adsorption to the oil droplet surface will result in different thickness of the interfacial layer [9]. As previously mentioned, the thickness of the interfacial layer may have significant impact on lipid oxidation.

The different protein composition of caseins and whey proteins may also affect the oxidative stability due to the antioxidative effect of different amino acid residues. Thus, casein molecules have many phosphorylated serine residues, which are able to chelate prooxidative transition metal ions, whereas whey proteins have many sulfhydryl groups which are able to scavenge free radicals [10, 11].

Like proteins, phospholipids are also commonly used as emulsifiers. Characteristic for phospholipids is their amphilic nature with a hydrophilic head group and lipophilic tail that will orientate them towards the water and oil phase, respectively. Phospholipids spontaneously form micelles, when present above CMC (Critical Micelle Concentration) and

in case of higher concentration of phospholipid than needed to cover the oil droplet surface this will result in micelle formation in the continuous phase [12]. Such micelles may bind lipid hydroperoxides and metal ions, resulting in lower concentration of these in or near emulsion droplets possibly resulting in a reduction of oxidation [13].

The effect on lipid oxidation of using different emulsifiers based on milk protein (casein or whey), soy phospholipids or a mixture of milk phospholipids and milk proteins in o/w emulsions with 70% oil has recently been investigated [14, 15]. It was demonstrated that emulsions prepared with proteins at different pH values oxidised differently and that protein based emulsions tended to oxidise less at high pH, independent of iron addition, compared to lower pH (4.5). At both pH values casein emulsions were more stable than those with whey protein. For phospholipid based emulsions the effect of pH was not consistent. For emulsions prepared with phospholipids, differences in oxidation stability between emulsifiers were observed. In these studies the emulsifier to oil ratio was 0.04 and for casein emulsions a ratio of 0.02 was also evaluated.

It could be hypothesised that similar effects of emulsifier type (milk protein (casein or whey), soy phospholipids or a mixture of phospholipids and milk proteins), pH and addition of iron can be observed in 5% emulsions. This study was performed to investigate whether this hypothesis could be confirmed or whether the oil concentration would lead to other effects of emulsifiers, pH and iron addition. Such knowledge would also be important for the understanding of possible differences in lipid oxidation mechanisms between high fat and low fat food matrices and thus the effect of dilution of a high fat delivery system.

Therefore, the aim of this study was to investigate the protective effects of the same five emulsifiers on lipid oxidation in 5% fish oil-in-water emulsions at different conditions, with an emulsifier to oil ratio of 0.04 or 0.15. The phospholipid based emulsifiers were thus soy lecithin and two milk phospholipid concentrates (with either 20% or 75% phospholipids), and the protein based emulsifiers were whey protein isolate and sodium caseinate containing solely protein and no phospholipid. The phospholipid based emulsifier with 20% phospholipid was an intermediate between phospholipids and protein based emulsifiers as it contained 54% protein and 23 % phospholipid, but for simplicity it is categorized as a phospholipid based emulsifier throughout this paper. Throughout the paper, the term "no iron" means no iron added to the emulsions, thus only endogenous iron present in the oil and emulsifier is present in these emulsions.

2 Materials and methods

2.1 Materials

Refined non-deodorized fish oil without added antioxidants (product nr.: 43-10) was donated by Maritex A/S (Sortland, Norway), subsidiary of TINE BA. The fatty acid composition was (in mol%): 16:0 8.8, 16:1(n-7) 8.2, 18:1(n-9) 15.5, 18:2(n-6) 1.9, 21:1(n-9) 11.3, 20:5(n-3) 9.6, 22:1(n-11) 6.0 and 22:6(n-3) 11.8; and the tocopherol content was roughly 200 mg/kg for α-tocopherol. The peroxide value (PV) was < 0.1 meq/kg. Emulsifiers sodium caseinate (Miprodan ® 30 fra Arla, FF), whey protein isolate (Lacprodan ® DI-9224) and milk phospholipid based emulsifiers (MPL) (Lacprodan ® PL-20 and Lacprodan ® PL-75) was kindly donated by Arla Foods Ingredients amba (Viby J, Denmark). The protein content was: sodium caseinate: 93.5%, whey protein isolate: 92%, MPL20: 53.8% and MPL75: 3.1%, as reported in the data sheets from Arla Foods Ingredients amba. MPL20 and MPL75 were reported to contain 22.6% and 76% phospholipids, respectively; mainly sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in both. Emulsifier based on soy lecithin (Solec ^{TM} E-40-B) was donated by The Solae Company (Århus, Danmark) and had a phospholipid content of min. 56% (as acetone insolubles), as reported by the manufacturer. For details on emulsifier composition please refer to Table 1. All other chemicals used were of analytical grade.

2.2 Preparation of emulsions (incl. storage and sampling)

Emulsions (1200 g) were prepared with 5% oil and 0.2% or 0.75% of one of five different emulsifiers (Table 2). Sodium caseinate and whey protein isolate were dispersed in the buffer and pH was adjusted to 2 (for emulsions with final pH 3) or pH 7, before emulsification. Soy lecithin and MPL75 emulsifier were dispersed in the oil before emulsification.

The emulsions were prepared in a two step process. Primary homogenisation was performed using an Ultra-Turrax (IKA T25, Janke & Kunkel IKA-Labortechnik, Staufen, Germany) at 13,500 rpm. The buffer (and emulsifier, when sodium caseinate, whey protein isolate and MPL20 were used) was stirred for a few seconds, where after the oil (and emulsifier, when lecithin and MPL75 were used) was added during 1 min followed by 2 min of mixing. pH was measured and adjusted to 3.0 or 7.0. Subsequently, a secondary emulsification was performed at room temperature using a two valve high pressure Panda homogenizer (GEA Niro Soavi Spa, Parma, Italy) with pressures of 800 and 80 bar in the first and second valve, respectively. pH was measured and adjusted if necessary.

Each of the obtained emulsions was distributed in separate sterile 100 ml Pyrex bottles (65 g in each bottle). Sodium azide was added to all bottles to inhibit microbial growth. Iron, (Fe^{2+} 100 μM) was

added to one set of bottles (Table 2). Emulsions were stored at room temperature (20°C) in the dark. Chemical analyses (PV measurements and dynamic headspace GC-MS analyses) were performed at study start and after 2, 5 and 7 days of storage for emulsions with iron or after 14, 28 and 42 days of storage for emulsions without iron. Droplet size distributions were measured at day 1 and at day 7 or 42 for emulsions with or without iron added, respectively. Viscosity and zeta potential measurements were performed after 4 days of storage. Droplet size, zeta potential and viscosity were measured at the sampling day on non-frozen samples. Samples for analysis of oxidation products, primary (PV) and secondary (volatiles), were flushed with nitrogen and stored at –40°C until use. For chemical determinations and droplet size measurements, duplicate or triplicate samples were withdrawn from the same bottle.

2.3 Viscosity

Viscosity was measured using a Brookfield DV-II+ rotary viscometer, RV spindle 1 (Brookfield Laboratories Inc., Stoughton, MA, USA). Emulsion (400 ml) was filled into a 600 ml beaker and measured at a speed of 100 rpm, at room temperature. Initial viscosity was read after 60 s.

2.4 Droplet size

The droplet size distribution of the emulsions was determined by laser diffraction with a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). Drops of emulsion were added to circulating water (2800 rpm) to give 14-17 % obscuration. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Results are given as surface area mean diameter D[3,2].

2.5 pH and Zeta potential

The surface charge was determined by measurement of the zeta potential. Twenty µl of emulsion was dispersed in 10 ml buffer (same pH as the sample) and the mixture transferred to a DTS-1060C cell for measurement at 25°C in a Zeta-Sizer (Malvern Instruments Ltd, Worcestershire, UK). The zeta potential range was set to –100 to 50 mV, and the experiment duration was 20 s.

2.6 Peroxide value (PV)

Lipids from the emulsions were extracted using chloroform/methanol (1:1, v/v) according to a method described by Bligh and Dyer [16], modified to use a reduced amount of solvents (30 ml chloroform and 30 ml methanol). Peroxide values were determined in duplicate on the extracts using the ferro-thiocyanate method described by Shanta and Decker [17].

2.7 Secondary oxidation products (volatiles)

For determination of volatile secondary oxidation products, 4 g of emulsion was weighed into a flask together with 5 g water and added approximately 30 mg 4-methyl-1-pentanol as internal standard and 1 ml antifoam solution (400 µl synperonic/ 500ml H_2O). The volatiles were released by bubbling nitrogen gas through the emulsions for 30 min, at 150 ml/min and 45°C and subsequently trapped on Tenax GR® tubes. Water was removed from the trap by purging the tube with nitrogen for 20 min at 50 ml/min. The volatiles were desorbed from the Tenax tubes on an automatic thermal desorber at 200°C for 3 minutes, collected on a cold-trap at –30°C and released at 220°C for 3 min (ATD-400, Perkin Elmer, Norwalk, CN). Subsequently the volatiles were separated by gas chromatography (HP 5890 IIA, Hewlett Packard, Palo Alto, CA, USA). The oven temperature programme was: 45°C held for 5 min, 1.5 °C/min to 55 °C, 2.5°C/min to 90°C, 12°C/min to 220°C and finally held at 220°C for 4 min. The individual compounds were analysed by mass spectrometry (HP 5972 mass-selective detector, Hewlett Packard, Palo Alto, CA, USA). The MS was operating in the electron ionisation mode at 70eV and mass to charge ratios between 29 and 200 were scanned. The compounds were identified by both MS-library searches and quantified through calibration curves. Results from the analyses are given in µg/ kg emulsion. Eight external standards were used for identification, namely: 1penten-3-one, t-2-pentenal, 1-penten-3-ol, 4-hexenal, 2-hexenal, nonanal, and two 2,4-heptadienals, which were the only compounds found in detectable amounts in the samples.

2.8 Statistics

Results of analysis of volatile oxidation products were subjected to principal component analysis (PCA) using Unscrambler (Version 9.0, Camo, Oslo, Norway). Values were autoscaled by 1/SD and full cross validation was used to validate the model. Results were compared statistically by two-way analysis of variance (ANOVA) using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). The individual samples were compared on a 0.05-level of significance by the Bonferroni multiple comparison test.

3 Results

3.1 Physical measurements

The measured pH values in the final emulsions were 2.9 - 3.1 and 7.1 - 7.2 for pH 3 and pH 7 emulsions, respectively. The low pH is lower than the lowest pH used in the 70% emulsions (pH 4.5). This is due to the fact that emulsions were prepared with buffer pH 2 and adjusted to exact pH 3 after production to keep the pH at the aimed value. This was chosen, to be able to see more clearly the effect of pH. The viscosities were almost similar for all samples. Average viscosities were 12.1 $\pm$ 0.6 cp and 11.7 $\pm$ 0.3 cp at pH 3 and 7, respectively, except for one sample MPL20H which had a slightly higher viscosity (16.0 cp) at pH 7.

Mean droplet sizes, D[3,2], for all emulsions at all time points ranged between 0.13 – 2.86 µm (Table 3). Only few emulsions had droplet sizes above 2 µm at pH 3 (MPL20L and MPL75L) and above 1 µm at pH 7 (LecL and MPL75L). For all emulsions, it was observed that the droplet sizes were larger when a low amount of emulsifier was used, both initially and at the end of storage. Droplet sizes in pH 3 emulsions with low emulsifier concentration increased significantly after 7 or 42 days compared to initial sizes, with and without iron added, respectively – except MPL20L3 which did not increase under any conditions. In addition, emulsions produced with Cas, MPL20 and MPL75 were physically unstable as observed by creaming.

At pH 3 and at high emulsifier concentration droplet sizes were smaller, initially around 0.5 µm except for Whey (0.16 µm). For MPL20 and MPL75 without iron added, the droplet size increased during storage, whereas droplets were stable in Cas, Whey and Lec emulsions.

After 7 days of storage in emulsions at pH 3 with iron added the droplet sizes increased as shown in Table 3.

At pH 7, in contrast to pH 3, droplet sizes were more similar and in general did not change as much as at pH 7. Thus, at pH 7 there were almost no changes in droplet size during storage in the samples with the high concentration of emulsifier, except in MPL75, for which the droplet size increased significantly during storage (Table 3). Cas, Whey and MPL20 emulsions had the smallest droplets where as Lec and MPL75 had slightly larger droplets. At low emulsifier concentration none of the emulsions separated, but droplet sizes increased significantly in all emulsions from day 1 to day 7 or 42, except for Lec. Cas and Whey emulsions initially had the smallest droplet sizes, whereas MPL20 and especially MPL75 had larger droplet sizes. In Lec emulsion droplet size was initially largest, but as the only emulsion, droplets did not increase in size during storage. Thus, at pH 7 emulsions produced with protein based emulsifiers (Cas and Whey) generally had smaller droplet sizes compared to those produced with phospholipid based emulsifiers (MPL20, MPL75 and Lec).

As expected the zeta potential differed amongst the emulsions depending on the type of emulsifier used. This was especially pronounced at pH 3 where zeta potential was negative for Lec and MPL75 emulsions and positive for Cas, Whey and MPL20 emulsions (Table 3). At pH 3 varying the emulsifier concentration resulted in Cas, Lec and MPL75 emulsions, with or without iron and Whey without iron, having significantly more positive (Cas) or less negative (Lec and MPL75) zeta potentials at low emulsifier concentration compared to higher emulsifier concentration. Addition of iron slightly reduced the positive zeta potential for CasL and WheyL whereas it resulted in significantly less negative zeta potentials for LecL and MPL75 (both L and H).

At pH 7, all emulsions had negative zeta potential (-70 to -35 mV)(Table 3), with MPL75 and Lec having the most negative zeta potentials followed by Whey and Cas and finally MPL20 . There was only a slight effect of emulsifier concentration on zeta potential and mainly in the Cas emulsion, where a higher emulsifier concentration resulted in less negative zeta potential (-42 vs. -48 mV). Addition of iron did not seem to affect the zeta potential of the droplets at any conditions. The emulsifier type, in contrast, had largest effect on zeta potential at pH 3. At this pH, changing the emulsifier concentration mainly affected Lec emulsions and to a lower extent Cas and MPL75, whereas addition of iron mainly affected MPL75 followed by Cas.

3.2 Primary oxidation products – Peroxide values (PV)

In general, it was observed that emulsions with high emulsifier concentration had lower or similar PV compared with the same emulsions with lower emulsifier concentration (Figure 1). However, there were some exceptions for Cas and Whey emulsions, which will be discussed below. At pH 3, irrespective of emulsifier concentration and addition of iron, PV was highest for MPL75 followed by Lec and then MPL20, Whey and lowest for Cas (Figure 1a and b). MPL75 based emulsions were physically unstable at low emulsifier concentrations and low pH and therefore PV was only determined right after production in these samples. At pH 7, the picture was less unambiguous as will be further discussed below.

3.2.1 No iron addition: In pH 3 emulsions, PV was initially below 5 meq/kg oil for all emulsions except MPL75 (Figure 1a). Changing emulsifier concentration did not affect the rank order between the different emulsions. However, the emulsifier concentration affected the relative differences within samples with the same type of emulsifier. Thus, the emulsion stabilised by lecithin reached a higher PV (95 meq/kg) after 42 days of storage when using a low concentration of emulsifier compared to the use of a high concentration of emulsifier (70 meq/kg). The reverse was observed for casein and

whey stabilised emulsions (9 and 12 meq/kg vs. 17 and 22 meq/kg, respectively). MPL20 samples had similar PV after 28 days of storage independent of emulsifier concentration. This also meant that PV was slightly higher in Whey and Cas emulsions with high emulsifier concentration than in the similar emulsions with low emulsifier concentration.

In emulsions without iron added and prepared at pH 7, PV were initially higher than in pH 3 emulsions, and for all emulsions except LecL and MPL75H, PV developed faster in pH 7 emulsions than in pH 3 emulsions. In pH 7 emulsions, development of PV clearly divided emulsifiers into two groups: Group 1 (Lec>MPL75>MPL20) had higher PV than group 2 (Cas and Whey). At the end of the storage period, in emulsions with low emulsifier concentration it was observed that Cas had lower PV than Whey, whereas the opposite was the case at high emulsifier concentration. This meant that a higher PV was found in Whey emulsions with low concentrations of emulsifier whereas for Cas emulsions PV was approximately the same in emulsions with low and high concentrations of emulsifier.

3.2.2. Iron addition: In pH 3 emulsions, similar to emulsions without iron PV were initially below 5 meq/kg oil for all emulsions except MPL75 (Figure 1b). Also similar to samples without iron added, higher initial PV at pH 7 was observed compared to at pH 3 and in contrast to emulsions without iron, all pH 7 emulsions developed higher PV than the corresponding pH 3 emulsions. Moreover, the order of the emulsions according to PV was similar to that at pH 3 at both emulsifier concentrations (i.e. MPL75>Lec>MPL20>Whey≥Cas) except in one case namely for Whey emulsions. Thus, at high emulsifier concentration Whey had the lowest PV, but at low emulsifier concentration Whey had as high PV as MPL75 after 7 days of storage. The PV in emulsions with low emulsifier concentrations ranged between 39 meq/kg and 68 meq/kg in the order Whey=MPL75>Lec>MPL20>Cas. Interestingly, whereas low concentration of emulsifier at both pH values resulted in higher PV than high concentration of emulsifier for the Whey emulsion, a similar effect of emulsifier concentration was not observed for Cas emulsions as PV were similar in these emulsions irrespective of the emulsifier concentration.

It is not possible to compare emulsions prepared with and without iron at the end of their storage periods, as the storage times were different. However, comparison of emulsions prepared with iron after storage at 7 days with their corresponding emulsions prepared without iron after 14 days generally showed that iron addition as expected increased lipid oxidation as observed from PV.

3.3 Volatile secondary oxidation products

From the raw data it was observed that for all conditions (pH, iron addition and emulsifier concentration) emulsions prepared with MPL75 had a significantly higher content of the volatile oxidation products detected and quantified and generally the emulsions prepared with caseinate had the lowest concentration (data not shown). Due to the high number of samples and additional high number of volatile compounds PCA analyses were performed to get an overview of the effect of the experimental design conditions on the oxidative stability. PCA performed with results from all emulsions only showed that MPL75 emulsions were different from the other samples (result not shown). Therefore, two PCA analyses were performed without data from these emulsions (Figure 2a-d), for data from experiments with and without iron added, respectively. In both analyses PC1 explained 50 % of the variation in the data and PC2 explained 17%. For both analyses (with and without iron), the loadings plot showed that the volatiles were located to the right in the 1st and 4th quadrant (Figure 2b and 2d).

3.3.1 No iron addition: In the loadings plot, volatiles from samples stored longer were located below samples stored for a shorter time (Figure 2b) indicating that PC2 explained differences in volatiles concentrations due to storage time. Samples at pH 7 were in general located higher in the scores plot compared to samples at pH 3 prepared with the same emulsifier (Figure 2a), indicating positive correlation between low pH and development of volatiles during storage. This interpretation was confirmed by raw data for five of the eight volatiles, namely 1-penten-3-one, hexanal, 2-pentenal, nonanal and 1-penten-3-ol, whereas the remaining three volatiles (2-hexenal, 4-heptenal and 2,4-heptadienal) showed the opposite. Higher oxidation at lower pH was opposite to the findings from PV. Samples with high concentrations of emulsifier were located higher and slightly further to the right compared to samples with lower concentration of emulsifier. This suggested that emulsions with high concentration of emulsifier had slightly higher concentrations of volatiles. Raw data of four volatiles (1-penten-3-ol, 2-hexenal, 4-heptenal and nonanal) confirmed, in general for all emulsifiers, that the concentration of volatiles increased with increasing emulsifier concentration (data not shown).

All Cas and Whey samples were located in the left side of the scores plot, indicating that they had a low concentration of volatiles. However, all Whey samples were located higher and slightly further to the right than the corresponding Cas samples, which suggested that Whey samples had slightly higher concentration of volatiles than Cas samples. As the only type of emulsion, all Lec samples were located in the right side of the scores plot, indicating that Lec emulsions had the highest concentration of volatiles. Interestingly, Lec emulsions at pH 3 were in general located both higher and further to

the right than pH 7 emulsions. This suggested that the effect of a low pH on development of volatiles was stronger in Lec samples than in the other samples. Likewise, MPL20H3 was located further to the right than the other MPL20 samples. Thus, in both Lec and MPL20 with high emulsifier concentration and low pH a higher concentration of volatiles seemed to be present compared to the other samples with the same emulsifier. Raw data of volatiles in general confirmed this interpretation (data not shown). It was observed from the PCA that MPL20 followed the same pattern as Cas and Whey samples with H7- located highest followed by L7-, H3- and L3- lowest. Since a lower position indicates a less oxidatively stable emulsion, this finding indicates decreasing oxidative stability in the order just mentioned.

3.3.2 Iron addition: When iron was added, the data analysis of emulsions showed a different picture. The PCA scores plots showed that in emulsions with iron added Whey and Lec samples with low concentration of emulsifier were located further to the right compared to samples with higher concentration and that this was most pronounced at pH 7 (Figure 2c). Moreover, Whey and Lec samples with pH 7 were located further to the right compared to samples at pH 3 prepared with the same emulsifier (Figure 2c). This finding showed that both a neutral pH and/or low concentration of emulsifier in Whey and Lec emulsions resulted in higher concentration of volatiles during storage. The raw data confirmed that neutral pH emulsions oxidized faster and that a low concentration of emulsifier resulted in higher concentrations of most volatiles at pH 7, whereas there was no clear effect of the emulsifier concentration at pH 3 (data not shown). These findings were in general in agreement with the PV data. Cas samples were distributed in the lower half of the plot, with CasL7 in the right side and the other samples to the left. Thus, a low concentration of emulsifier and a high pH also increased oxidation in Cas emulsions. The raw data confirmed that there did not seem to be any clear effect of the emulsifier concentration at low pH, whereas at neutral pH Cas emulsions with low concentration of emulsifier developed higher concentration of all volatiles than emulsions with high concentration of emulsifier. One sample with MPL20 was located to the right (MPL20L3), whereas the other MPL20 samples were located in the left-middle side of the plot, except MPL20H7 which was located to the far left, indicating very low concentration of volatiles in this sample. Interestingly, this suggested that for MPL20 emulsions a neutral pH decreased oxidation when a high concentration of emulsifier was used, as confirmed by six of eight volatiles at pH 7 and by seven of eight volatiles at pH 3. This was opposite to the other emulsions with iron added. The location of the protein based emulsions (Cas and Whey) in the lower half of the plot indicated that they were correlated, and different from the phospholipid based emulsions (MPL20 and Lec), which were located in the upper half of the plot. Since the volatiles from samples stored longer were located higher in the plot, MPL20 and Lec had higher concentrations of volatiles than Cas and Whey later in the storage period.

Interestingly, the plot showed that irrespective of emulsifier type, low emulsifier concentration resulted in more oxidised samples than high emulsifier concentration at neutral pH, as confirmed by raw data, except for MPL75 where the opposite was observed. At low pH the opposite was the case, high emulsifier concentration resulted in more oxidised samples, at least for some of the volatiles.

4. Discussion

4.1 Physical properties

The physical properties of an emulsion may influence its oxidative stability. Therefore, changes in the physical properties related to the emulsifier type were measured. Indeed, the differences in surface charge, may affect the physical stability of the emulsion droplets. The zeta potential was negative at pH 7 for all emulsions. This was expected for Cas and Whey emulsions, as pH 7 is above the pI of casein and whey proteins and in accordance with results obtained in studies performed with 70% emulsion[14,15].

At pH 3 the zeta potential was positive for Cas and Whey as well as for MPL20, which also contained protein. On the contrary at pH 3, Lec and MPL75 were negative due to the negative charge of the polar headgroup. However, the zeta potential was less negative at pH 3 than at pH 7, probably due to the presence of proteins, which are positively charged at pH 3. Similar differences in surface charge was observed with 70% emulsions, however in these studies emulsions with MPL75 were less affected by difference in pH and emulsions with lecithin was either very little affected (with iron) or unaffected (without iron) [14,15]. This could indicate different partitioning of the emulsifier in the 70 % emulsions compared to the 5 % emulsions. The differences between 5% and 70% emulsions at low pH could also result from the differences in pH (3 vs. 4.5, respectively).

At pH 7 and pH 3 Cas is expected to be negatively and positively charged, respectively. Surprisingly, increasing emulsifier concentration resulted in less negative and less positive zeta potential at pH 7 and 3, respectively. This could be due to the stretching of casein proteins over the surface at low concentration, exposing more charged groups [18].

The stable droplet size of the emulsions with protein based emulsifiers (Cas and Whey) at high concentration (0.75 %, corresponding to 1:7 ratio of emulsifier to oil) indicated that at this concentration there was enough emulsifier to stabilise the droplets. This suggests that there was plenty emulsifier for coverage of the droplets and in addition some unadsorbed protein in the water

phase. Fang and Dalgleish (1993) have reported that a ratio of 1:29 (emulsifier:oil) was necessary for full coverage of soy oil droplets by casein in their study, however the necessary amount of protein depends on the droplet size. At the low emulsifier concentration in the present study (0.2 % emulsifier, corresponding to 1:25 ratio of emulsifier to oil), the droplet sizes were smaller in Cas than in Whey emulsions at pH 7. This observation was in accordance with a study by Hunt and Dalgleish (1994), who observed that less casein was needed, compared to whey protein, to fully cover the surface of droplets. This is probably due to the fact that casein proteins are unstructured and flexible, whereas whey proteins are globular. Thus, casein may be expected to have higher emulsification capacity. Smaller droplets in Cas compared to Whey emulsions were also observed in 70% emulsions with similar oil:emulsifier ratio [14]. In contrast, at low emulsifier concentration at pH 3 the increase in droplet size during storage was much higher for Cas than for Whey. The decreased stability of droplets in CasL3 compared to WheyL3 could not be explained by differences in zeta potential as these were similar but it could indicate a better emulsifying capacity of Whey at low pH, which could also explain the smaller droplet size of Whey vs. Cas droplets at pH 3, as also observed by Hu *et al.* [8].

Emulsions produced with MPL20 had droplet sizes slightly larger than Cas and Whey. Differences in pH and iron addition as well as storage affected these emulsions similarly even though at pH 7 the zeta potential was less negative for MPL20. Thus, for the emulsifiers with high protein content, the differences in zeta potential did not seem to affect physical stability. MPL75 resulted in physically unstable emulsions only at pH 3 when present in low concentration. This could be due to differences in conformation and interaction with water at the low pH, affecting packing of the phospholipids molecules on the surface of the droplets. This is in accordance with observations made in 70% o/w emulsions with MPL75, where smaller droplets were measured at neutral pH compared to low pH, indicating better emulsification capacity at neutral pH [14]. Zeta potentials did not seem to affect the physical stability of the droplets of MPL75 emulsions, since at pH 7 the very negative zeta potential in all types of MPL75 emulsions compared to MPL20 emulsions did not confer additional stability. In addition, at pH 3 the zeta potentials of MPL75H3+ and MPL75L3- were similar but the former was much more physically stable.

Lec had larger average droplet sizes than Cas and Whey at both pH 3 and at pH 7 but changes in droplet sizes during storage were more or less similar to those of Cas and Whey. In contrast, in 70% emulsions at neutral pH, lecithin resulted in smaller and stable droplets compared to whey, but larger than casein[14]. Thus, lecithin was not as efficient an emulsifier as caseinate and whey protein due to the composition with less protein and a lower amount of phospholipid than MPL75. At pH 7 the physical stability of Lec emulsions seemed similar to that of MPL75 emulsions, but at pH 3 Lec

seemed to work more efficiently as emulsifier despite the lower amount of PL. A possible explanation could be a more favourable PL composition. The less negative zeta potential of Lec at pH 3 compared to pH 7 did not seem to affect the physical stability of the droplets.

Viscosities were similar for all emulsions independent of the type or concentration of emulsifier used and the pH (except for an unexplainable slightly higher viscosity for emulsions stabilised with a low amount of MPL20). Thus, the emulsifier content was so low that viscosity was not affected and any differences in oxidative stability could thus not be caused by this.

4.2 Lipid oxidation

It is well known that the solubility of iron ions is higher at low pH. At higher pH, iron ions may precipitate in the emulsions or on the emulsion droplets, resulting in lower or higher concentration of iron in the water phase, compared to the concentration expected from the amount added [19]. In addition, the charge of proteins in the food emulsions differs depending on their pI relative to the pH of the emulsion and this may affect lipid oxidation.

The following discussion is therefore divided to discuss emulsions at low and neutral pH separately.

4.2.1 Effect of emulsifier type- low pH

Results showed, that Cas and Whey emulsions were more oxidatively stable than MPL20 followed by Lec, whereas MPL75 was the least oxidatively stable samples.

The finding that Cas emulsions were more stable than Whey emulsions was in accordance with the literature [20-23] and similar to results observed in 70% emulsions [14, 15]. In the present study, Cas emulsions had larger droplets than Whey. Thus, the total interfacial area in Cas was smaller than in Whey emulsions and this could partly explain the better oxidative stability of the Cas emulsions. Hunt and Dalgleish (1994) showed that under their experimental conditions, casein covered the surface better than whey protein and this could contribute to the improved oxidative stability in Cas emulsions. Hu et al. (2003) suggested that the better stability of casein over whey emulsions was due to the amino acid composition of casein and the fact that the phosphoseryl groups remain anionic at pH3, whereby casein in the water phase can chelate the iron ions. Effect of excess emulsifier in the water phase will be further discussed later.

MPL20 had much less physically stable droplets at high emulsifier concentration. Thus, the lower oxidative stability as observed from PV and volatile oxidation products of MPL20 vs. Cas and Whey could be due to less efficient coverage of the droplets as well as a higher possible content of free fatty acids originating from the emulsifier. Indeed, coverage of oil droplets has been suggested to have large impact on the oxidative stability of o/w emulsions [24]. Lec and MPL75, which contained only or almost only PL, were the least oxidatively stable emulsions. This was in contrast to observations in 70% emulsions at low pH, where Lec emulsions were as stable as or more oxidatively stable compared to Cas and Whey [14]. These observations were also in contrast to observations in 10% n-3 PUFA emulsions with iron added, where lecithin stabilised emulsions were more oxidatively stable than Cas emulsions at low pH [25]. Thus, in the present study, the proteins seemed to offer more oxidative stability compared to the phospholipids. A plausible explanation for this could be the differences in the charge of the emulsion droplets. Thus, whereas protein based emulsions had positively charged droplets, which could result in repulsion of positively charged metal ions known to initiate oxidation, the droplets in phospholipid based emulsions were negatively charged at pH 3, which could result in attraction of metal ions [19]. In addition, excess protein in the water phase may chelate metal ions, as discussed later.

The higher stability of MPL20 compared to Lec seemed to indicate that the protein content positively influenced the oxidative stability, which could be due to either better covering of the surface or more efficient metal/radical scavenging effect of proteins in the aqueous phase. The difference in oxidative stability between MPL20 and Lec could also be due to the difference in composition of PL, since the individual PLs have been demonstrated to have different antioxidative efficiencies due to the head groups [26-28]. However, the higher content of PL and possibly more beneficial composition of PLs in Lec compared to MPL20 were not sufficient to compensate for the lower protein content. In addition to the head groups of the PLs, also the fatty acid composition may affect stability. Since Lec compared to MPL20 had a higher content of PUFA (56% vs. 13%) and a higher PL content (56% vs. 23%), this could have reduced the stability of Lec emulsions compared to MPL20 emulsions. Interestingly, in 70% o/w emulsions at low pH (4.5) and without iron added, emulsions with lecithin or MPL20 were more stable or as stable as emulsions with milk protein (whey or casein) [14]. The emulsifier:oil ratio was the same in the two studies, but the droplet size was much larger in the 70% o/w emulsions. Thus, the total surface area was smaller and it is thus likely that excess phospholipid formed micelles in the aqueous phase to a larger extent in the 70 % o/w emulsions compared to the 5 % o/w emulsions. Such micelles have been suggested to entrap lipid hydroperoxides and thereby reduce oxidation[29]. The fact that MPL75 emulsions were the least stable, and even more oxidatively unstable than Lec despite a higher protein content, was most likely due to MPL75 having a high PV

already when used for production of the emulsion. This was previously observed in similar emulsions with 70% fish oil [14,15].

The differences in oxidative stability among emulsions produced with different emulsifiers can most likely also be ascribed to differences in the antioxidative (free radical scavenging and metal chelating) properties as will be further discussed later. The general lower oxidative stability of 5% vs. 70% emulsions, independent of emulsifier type is puzzling, especially as more oxygen can be solubilized in oil than in water. However, the total interfacial area was higher in the 5 % emulsions than in the 70 % emulsion and this could have played a role.

4.2.2 Effect of emulsifier type- neutral pH

At neutral pH, Cas and Whey emulsions were also more oxidatively stable than MPL20, Lec and MPL75, but at this pH differences in oxidative stability were less pronounced, compared to emulsions at pH 3. Interestingly, in contrast to the findings at low pH, the effect of emulsifier type on oxidative stability was similar in 10 and 70% oil emulsions at neutral pH, where protein based emulsifier resulted in more oxidatively stable emulsions compared to PL-based emulsions [14,15]. This may be due to the fact that at pH 7 differences in droplet sizes were smaller than at pH 3 and all droplets were negatively charged and thereby these parameters influenced oxidative stability to a lower extent in pH 7 emulsions than in pH 3 emulsions. Nevertheless, the effect of the emulsifier type could most likely be attributed to the same factors as those discussed for pH 3 emulsions. Again, these observations were in contrast to observations in 10% n-3 PUFA emulsions with iron added, where lecithin stabilised emulsions were more oxidatively stable than Cas emulsions also at neutral pH [25]. However, in this study it was also observed that differences in oxidative stability between emulsions at pH 7 produced with different emulsifiers were less obvious than at low pH.

4.2.3 Effect of emulsifier concentration – low pH

At low pH, the effect of increasing emulsifier concentration was not the same for all emulsifiers and PV and volatile oxidation products did not show the same trend. For emulsions prepared with Lec, MPL20 and MPL75 and with iron added a higher emulsifier concentration resulted in less oxidation as evaluated from both PV and volatiles and a similar trend was also observed for PV in similar emulsions without iron added. However, higher concentration of volatile oxidation products was observed with higher emulsifier concentration in emulsions without iron. This finding suggested that

when iron was not present in Lec, MPL20 and MPL75 increasing the emulsifier concentration resulted in increased decomposition of lipid hydroperoxides, perhaps due to the presence of metal ion in these emulsifiers. For Cas and Whey emulsions without iron both PV and volatiles showed that a higher concentration of emulsifier increased oxidation, but it has to be emphasized that the effect of increasing the emulsifier concentration was small and will therefore not be discussed further. However, in Cas and Whey emulsions with iron a higher concentration of emulsifier decreased formation of PV particularly in the Whey emulsions, whereas emulsifier concentration did not show any clear effect on the formation of the volatile oxidation products. Similarly, it was previously observed in 70% o/w emulsions with iron added, that increasing the concentration of protein emulsifier, increased the oxidative stability of the emulsion [15].

A higher concentration of emulsifier may result in thicker interface and this may result in an emulsion with a better oxidative stability. If emulsifier is present in excess, emulsifier not contributing to emulsifying the added oil will be present in the water phase. Here they may cause antioxidative effects, e.g. by chelating metal ions or by scavenging free radicals present in the aqueous phase [10,20,24]. Actually, Berton et al (2011) concluded that proteins used as emulsifiers in emulsion, themselves did not efficiently protect against lipid oxidation and less so than surfactants. However, when excess emulsifier was present in the water phase, emulsions were stabilised against oxidation. The better oxidative stability observed for Lec, MPL75, MPL20 emulsions with high concentration of emulsifier in emulsions with iron was most likely due to a thicker interfacial layer and the ability of phospholipids to form micelles in the water phase, which can entrap hydro-peroxides or metal ions, as previously described. For MPL20 emulsions the ability of the proteins to scavenge free radicals or metal ions present in the water phase may also have played a role. For casein emulsions with iron there was only a small difference in oxidative stability between emulsions with low and high emulsifier concentration as determined by PV. This difference was larger, as determined by PV, in Whey emulsions. These differences may be due to differences in distributions of emulsifier between the oil-water interface and the aqueous phase and due to differences in antioxidant properties of the emulsifier when present in these two compartments.

4.2.4 Effect of emulsifier concentration – neutral pH

When iron was added, in pH 7 emulsions, an increase in emulsifier concentration increased the oxidative stability as measured by PV, whereas a similar effect could not be observed for all emulsions when no iron was added. A higher concentration of emulsifier also reduced formation of volatiles, when iron was present, but a lower or even slightly opposite effect was observed from the volatiles without iron, as will be discussed later.

Interestingly, in pH 7 emulsions with high emulsifier concentration, Whey and Cas emulsions had similar droplet size and zeta potential, but Whey was more stable than Cas, independent of iron addition whereas the opposite was the case at other conditions. Moreover, the effect of increasing the emulsifier concentration was more pronounced for Whey emulsions than for Cas emulsions. A possible explanation could be that at pH 7 the metal chelating properties of excess casein in the water phase is less important than at pH 3, because iron is less soluble at neutral pH and it may therefore precipitate at the droplet interface rather than being present in the water phase. In contrast, a thicker interface may have a positive effect on the resistance towards oxidation [9].

4.2.5 Effect of iron addition

Addition of iron resulted in increased oxidation in all samples. However, as oxidation is accelerated when iron is added, it is not relevant to compare the oxidative stability of similar samples with and without iron added. Instead, this discussion will focus on the differences in oxidative stability between the different emulsifiers with and without iron.

The differences between Cas, Whey and MPL20 seemed similar in samples with and without iron added. Thus, either the endogenous iron ions in emulsions without iron added was sufficient to give similar difference as in samples with iron added, or the effect of zeta potential was not pronounced.

Similar to the pH 3 emulsions, it was expected, and observed from peroxide values and volatile secondary oxidation products, that the addition of iron to the emulsions at pH 7 resulted in increased lipid oxidation. This was observed even though the solubility of iron ions is lower at pH 7 than at pH 3, thus the concentration of soluble iron ions was high enough to still cause oxidation. Moreover, precipitation of iron may occur at the surface of the droplets and this could potentially catalyze oxidation [19]. Addition of iron resulted in Cas and Whey emulsions being affected to the highest degree by neutral pH when emulsifier was present in low concentration. This may be due to the fact that, at neutral pH the droplets were negatively charged and at low emulsifier concentration less excess protein was present in the water phase to chelate the high concentration of transition metal ions. Especially whey emulsions showed larger increase in oxidation under these conditions than other emulsions.

In both pH 3 and pH 7 emulsions, there was a difference in effect of emulsifier concentration as observed from the content of volatiles, depending on the presence or absence of iron ions. In the presence of iron, as expected, a higher emulsifier concentration resulted in more stable emulsions. However, a less clear trend was observed, without iron added, since emulsions with high emulsifier content had a similar or slightly lower content of volatiles compared to emulsions with lower emulsifier content. The lack of protective effect of increased emulsifier concentration was thus not as pronounced as in samples with iron.

4.2.6 Interactions between pH and iron

Importantly, iron addition changed the effect of pH for some of the emulsions as will be discussed in the following. Particularly from PV it was observed that in the presence of iron, pH 7 emulsions oxidized faster than the corresponding pH 3 emulsions irrespective of the emulsifier type and emulsifier concentration, except for MPL20 in which higher concentrations of volatiles were formed at pH 3 than at pH 7. The observation that emulsions were less stable at pH 7 compared to pH 3 is in contrast to observations in high fat 70% emulsions, but in accordance with observations from other 5% fish oil emulsions [15,22]. In the absence of iron, pH 7 emulsions also oxidized faster for MPL20, Whey and Cas emulsions irrespective of the emulsifier concentration. However, at low emulsifier concentrations Lec emulsions at pH 7 and pH 3 oxidized equally fast. Moreover, Lec emulsions generally had higher concentrations of volatiles at pH 3 than at pH 7, probably due to increased decomposition of lipid hydroperoxides. This is in accordance with previous observations in 10% n-3 PUFA o/w emulsions, where oxidation was observed to be faster at pH 3 than at pH 7 irrespective of iron addition [25]. It was suggested that iron added after homogenisation will not precipitate onto the droplet surface, but will at high pH values precipitate out of solution. At high emulsifier concentrations MPL75 emulsions oxidized faster at pH 3 than at pH 7. Further studies are needed to be able to explain these differences.

5. Conclusion

Lipid oxidation in 5 % fish oil-in-water emulsions were significantly affected by emulsifier type, emulsifier concentration, pH and iron addition. Generally, Cas emulsions were the most oxidatively stable, followed by Whey, MPL20, Lec and MPL75 in increasing order of oxidative instability. Moreover, a high emulsifier concentration in general resulted in more physically and oxidatively stable emulsions at pH 7 particularly in emulsions with iron. At pH 3 the effect of increasing the emulsifier concentration depended on both the presence of iron and the emulsifier type. In the

presence of iron a high emulsifier concentration generally decreased PV, whereas the effect on volatile oxidation products was less pronounced. In the absence of iron, oxidation was more pronounced at high concentrations of emulsifier for Whey and Cas when evaluated from both PV and volatiles, whereas PV data suggested a protecting effect of increasing emulsifier concentration for Lec, MPL20 and MPL75, but the volatiles data showed the opposite. The effect of pH depended on both emulsifier concentration and iron addition. Thus, in emulsions with iron added, pH 7 emulsions always oxidized faster irrespective of emulsifier concentration. In emulsions without iron and with low emulsifier concentration, pH 7 emulsions oxidized faster for MPL20, Whey and Cas emulsions whereas there was no effect of pH for Lec emulsions. When high emulsifier concentrations were used, oxidation was most pronounced at pH 7 in Lec, Cas, Whey and MPL20 emulsions, whereas for MPL75 emulsions oxidation was more pronounced in pH 3 emulsions. To sum up, this study demonstrated that the stability of emulsions is dependent on emulsifier type and concentration as well as physical conditions (pH and iron content). In addition, when comparing with high fat emulsions, it was observed thatthe above mentioned differences affect emulsions differently depending on oil content.

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Tables and figures

Figure 1 PV during storage of emulsions with 0.2 or 0.75% emulsifier at pH 3 and pH 7, without (a) and with (b) iron. Letters indicate significant differences $p<0.05$.

Figure 2 Plot from PCA analysis of volatile oxidation products in samples without iron (a: scores and b: loadings) and with iron added (c: scores and d: loadings). For code names refer to Table 2.MPL 75 is not included in the analysis.

Table1 Composition of emulsifiers

	Whey	CAS	MPL20	MPL75	Lec
PV [meq peroxides/kg oil]	4.3 ± 0.4	2.3 ± 1.5	1.5 ± 0.3	21.9 ± 1.0	1.3 ± 0.0
Oil content [%]	0.3 ± 0.1	0.8 ± 0.1	24.1 ± 0.7	82.6 ± 0.9	85.8 ± 0.6
Fatty acid composition [%]	---	---			
Total SFA			45.2 ± 0.5	60.6 ± 0.4	19.4 ± 0.1
14:0			6.1 ± 0.1	5.6 ± 0.0	0.1 ± 0.0
16:0			24.4 ± 0.1	29.3 ± 0.2	15.7 ± 0.1
18:0			13.1 ± 0.2	19.9 ± 0.0	3.2 ± 0.0
20:0			0.4 ± 0.0	3.3 ± 0.0	0.3 ± 0.0
Total MUFA			35.9 ± 0.1	26.7 ± 0.1	23.6 ± 0.0
18:1n-9			32.5 ± 0.2	23.2 ± 0.1	21.8 ± 0.0
Total PUFA			12.9 ± 0.4	4.0 ± 0.0	56.0 ± 0.0
18:2n-6			8.0 ± 0.5	1.7 ± 0.0	50.4 ± 0.1
18:3n-3			1.1 ± 0.0	0.1 ± 0.0	5.3 ± 0.0
Protein content [%]†	92.0	93.5	53.8	3.1	---
PL content [%]†	---	---	22.6	76.0	> 56
PL class composition [%]‡	---	---			
GluCer			2.00 ± 0.01	3.57 ± 0.01	---
LacCer			6.76 ± 0.02	10.47 ± 0.02	---
PC			27.70 ± 0.05	21.03 ± 0.16	29.70 ± 0.40
PE			25.55 ± 0.14	13.08 ± 0.02	15.31 ± 0.06
PI			8.80 ± 0.06	6.62 ± 0.04	28.50 ± 0.10
PS			8.60 ± 0.05	6.59 ± 0.10	3.48 ± 0.03
SM			20.59 ± 0.13	38.64 ± 0.11	---
PG					1.08 ± 0.02
PA					9.71 ± 0.09
LysoPC					12.23 ± 0.28

WPI: Whey protein isolate; CAS: Sodium caseinate; MPL20: Milk phospholipid 20%; MPL75: Milk phospholipid 75%; LEC: Soy lecithin; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; PL: Phospholipids; GluCer: Glucosylceramide; LacCer: Lactosylceramide; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PS: Phosphatidylserine; SM: Sphingomyelin; PG:Phosphatidylglycerol; PA: Phosphatidic acid ; LysoPC: Lysophosphatidylcholine

Individual fatty acids are given when they constitute more than 2.0%.

†As reported on the data sheets provided by the manufacturers; ‡As determined by the Laboratory of Food Technology and Engineering, Department of Food Safety and Quality, Ghent University, Belgium

Table 2 Study design

For each of the five emulsifiers, sodium caseinate (Cas), whey protein (Whey), soy lecithin (Lec) and milk phospholipid 20 (MPL20) and milk phospholipid 75 (MPL75), eight emulsions, as shown in the table, were prepared. Sample names were composed by prefix, as stated, indicating emulsifier type followed by postfix as indicated in the table eg. CasL3+ for an emulsion stabilised by sodium caseinate in lowest emulsifier concentration at pH 3 and added iron.

Postfix	Emulsifier conc (%)	pH	$FeSO_4$ (100 µM)
L 3	0.2	3	-
L 3+	0.2	3	+
L 7	0.2	7	-
L 7+	0.2	7	+
H 3	0.75	3	-
H 3+	0.75	3	+
H 7	0.75	7	-
H 7+	0.75	7	+

Tabel 3 Zeta potential and droplet sizes. Different letters in rows shows statistically differences between samples ($p<0.05$).

Sample Name	Zeta potential (mV)		Droplet size D[3,2] (µm)		
	+ iron	- iron	Day 1	Day 7 (+ iron)	Day 42 (- iron)
CasL3	37.5± 2.7	42.8±1.2	0.58±0.08^{cd}	1.37±0.08^{e}	nd
CasH3	30.0±0.3	30.4±0.1	0.50±0.02^{cd}	0.65±0.02^{d}	0.42±0.04^{b}
WheyL3	36.5±0.2	39.6±0.5	0.24±0.00^{ab}	0.38±0.02^{bc}	0.32±0.02^{a}
WheyH3	36.1±0.2	36.8±0.6	0.16±0.00^{a}	0.18±0.00^{ab}	0.19±0.01^{a}
LecL3	-14.7±0.7	-17.8±0.3	0.89±0.01^{e}	1.35±0.38^{e}	1.38±0.59^{d}
LecH3	-30.7±1.5	-28.8±0.7	0.60±0.07^{d}	0.41±0.06^{c}	0.43±0.07^{b}
MPL20L3	30.7±0.6	29.4±1.0	2.86±0.11^{g}	2.12±0.1^{f}	nd
MPL20H3	30.8±0.5	30.3±0	0.44±0.04^{b}	0.72±0.21^{e}	0.84±0.11^{c}
MPL75L3	-11.3±0.5	-20.5±0.8	2.12±0.03^{f}	nd	nd
MPL75H3	-19.7±0.5	-28.2±1.0	0.44±0.03^{bcd}	0.41±0.04^{c}	1.14±0.53^{c}
CasL7	-48.4±0.6	-48.9±1.3	0.17±0.01^{abc}	0.32±0.02^{b}	0.48±0.04^{e}
CasH7	-42.6±1.3	-42.8±1.0	0.15±0.00^{ab}	0.16±0.00^{a}	0.17±0.00^{ab}
WheyL7	-48.3±2.0	-48.8±1.1	0.23±0.01^{c}	0.64±0.05^{d}	0.74±0.02^{g}
WheyH7	-51.8±1.3	-50.6±1.0	0.13±0.00^{a}	0.13±0.00^{a}	0.14±0.00^{a}
LecL7	-62.2±2.3	-61.2±2.8	0.91±0.08^{f}	1.03±0.03^{f}	1.03±0.06^{h}
LecH7	-59.6±2.4	-60.1±2.0	0.35±0.00^{d}	0.36±0.00^{c}	0.38±0.00^{d}
MPL20L7	-36.2±0.8	-36.6±1.5	0.41±0.02^{d}	0.83±0.04^{e}	0.63±0.10^{f}
MPL20H7	-34.9±1.0	-35.3±0.5	0.18±0.00^{c}	0.19±0.00^{c}	0.20±0.00^{b}
MPL75L7	-67.3±1.1	-63.6±0.9	0.71±0.01^{e}	0.85±0.08^{e}	1.44±0.03^{i}
MPL75H7	-67.3±1.5	-67.4±2.1	0.2±0.01^{bc}	0.35±0.00^{c}	0.30±0.00^{c}

Figure 1a

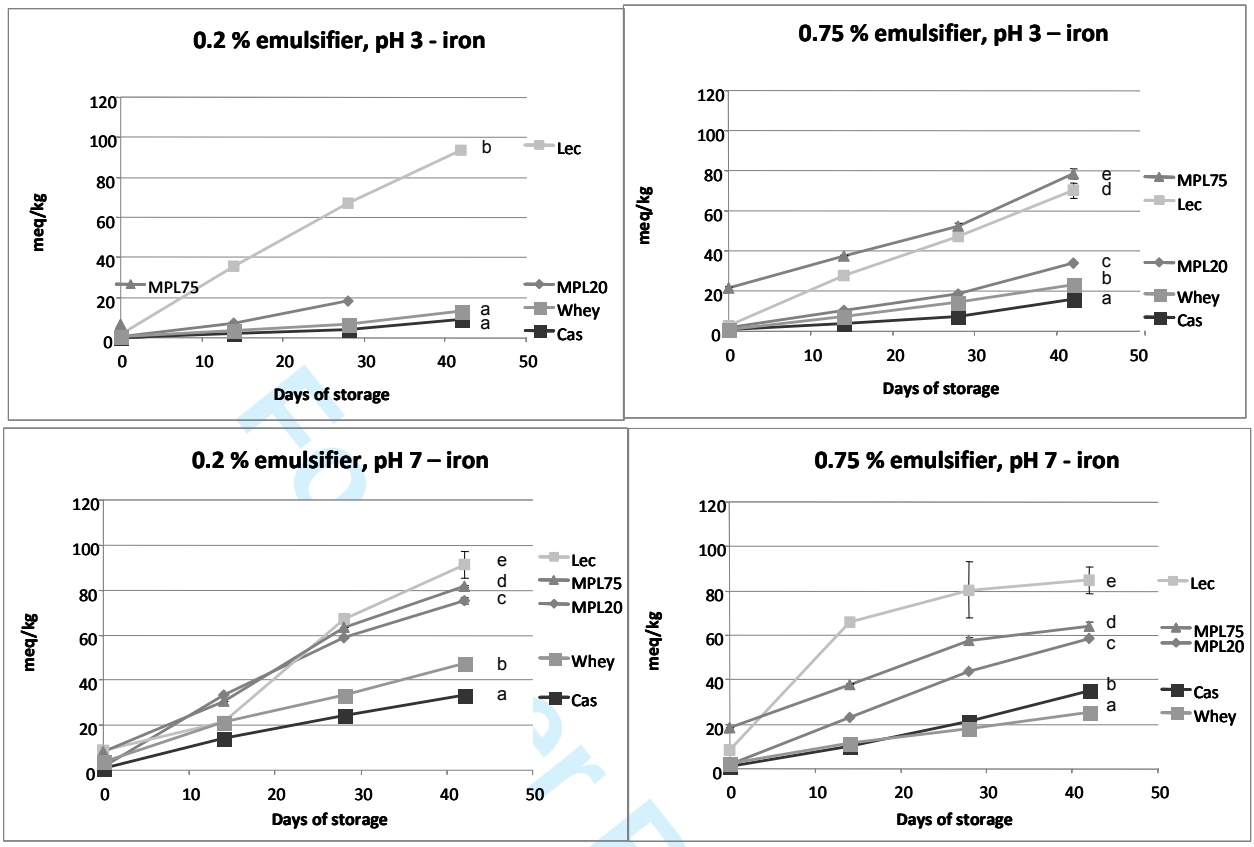


Figure 1b

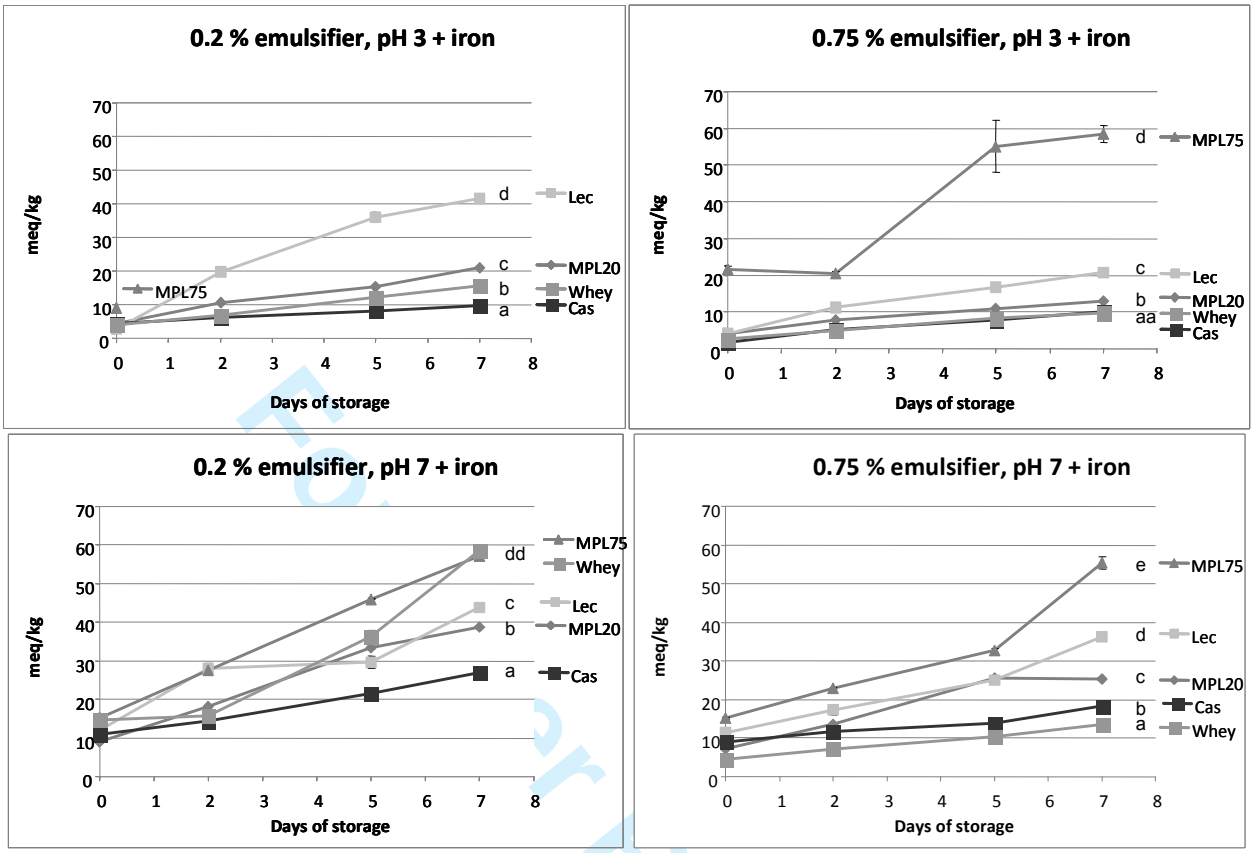
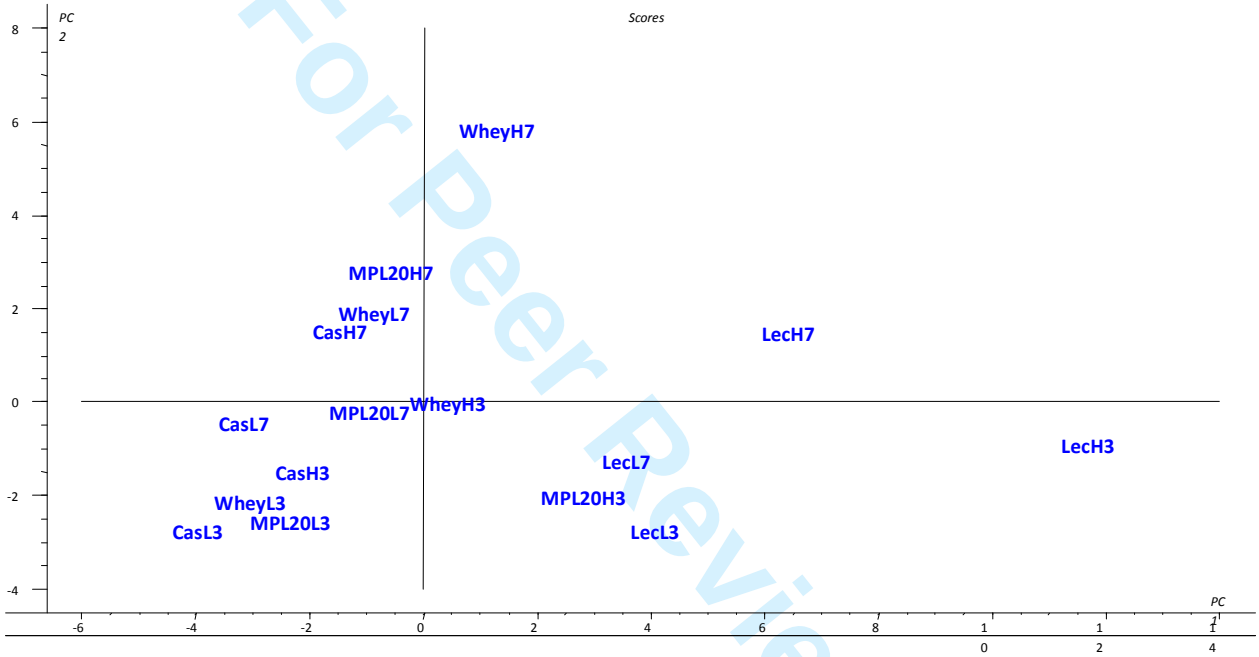


Figure 2a. Scores plot from PCA on emulsions except MPL75 without iron

Figure 2b. Loadings plot from PCA on emulsions except MPL75without iron



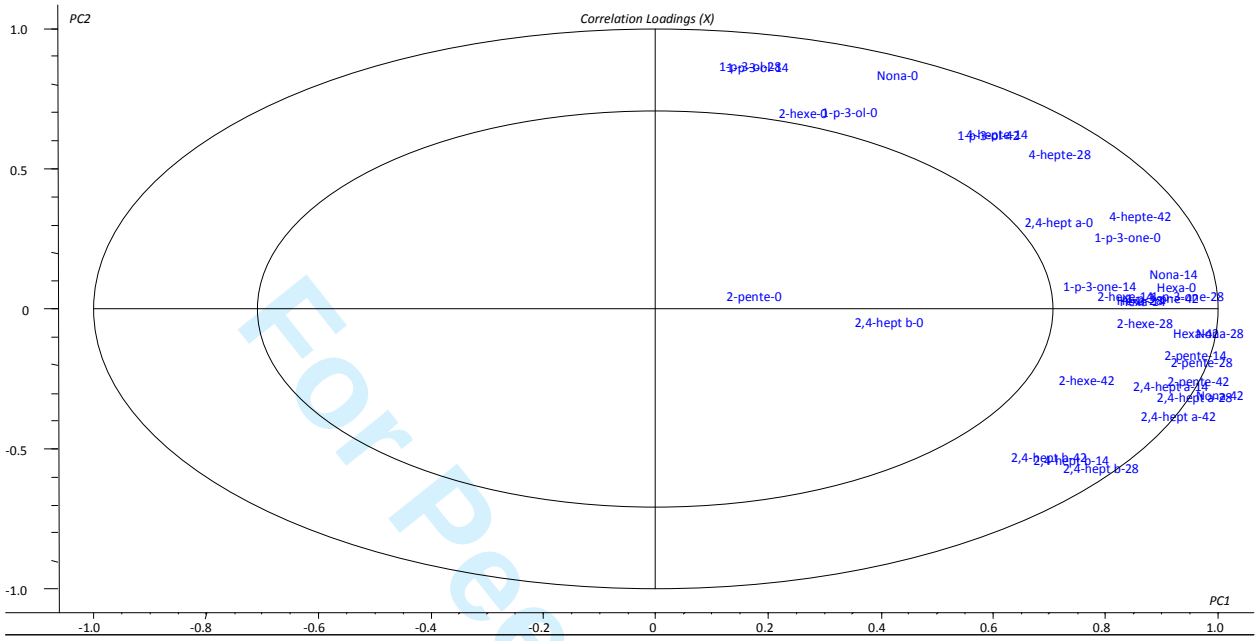
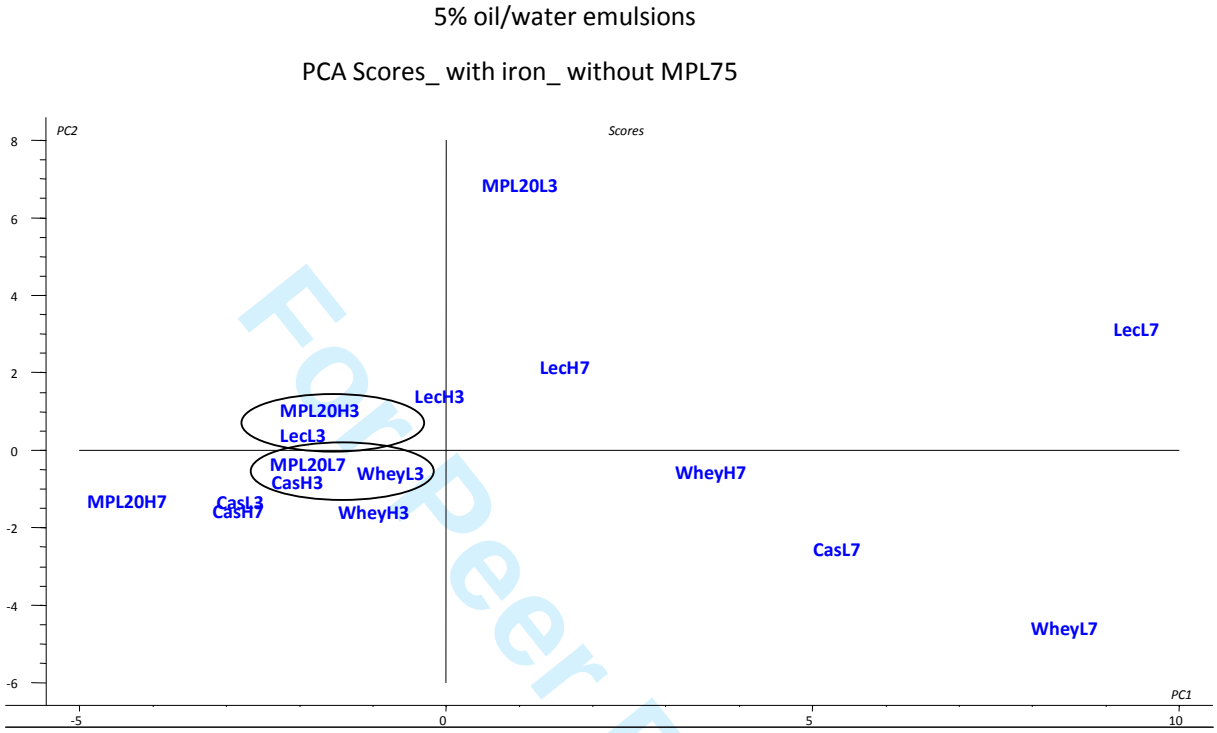


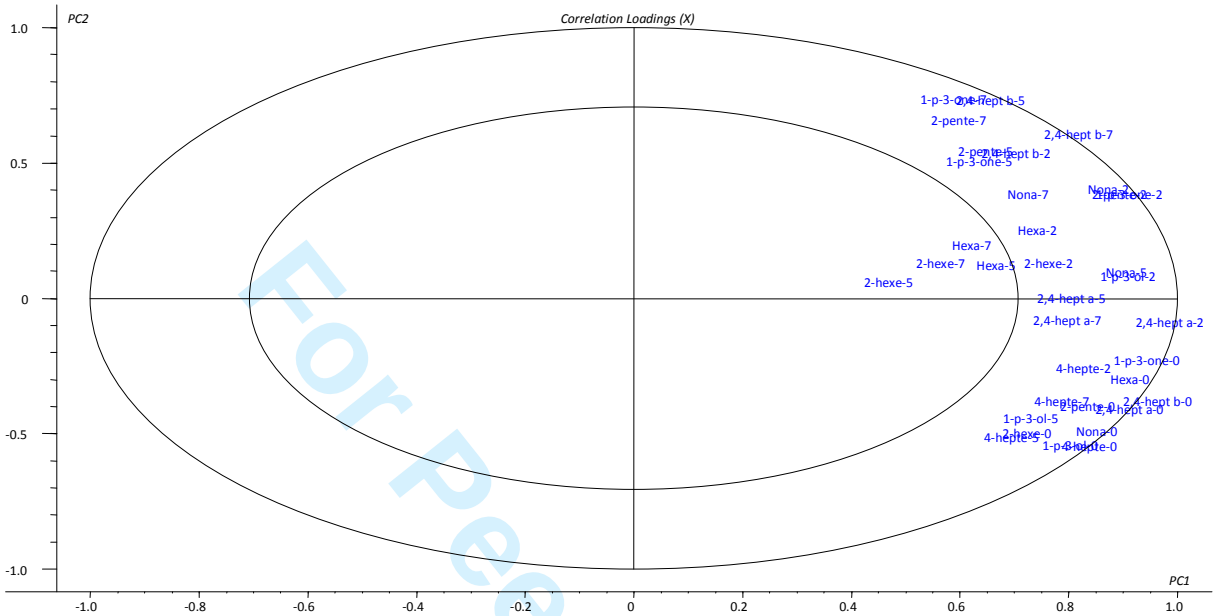
Figure 2c. Scores plot from PCA on emulsions (except MPL75) with iron

Figure 2d. Loadings plot from PCA on emulsions (except MPL75) with iron



5% oil/water emulsions

PCA Loadings_ with iron_ without MPL75



PAPER VII

Lipid oxidation in milk enriched with neat fish oil or pre-emulsified fish oil

Horn AF, Nielsen NS & Jacobsen C

Draft intended for Food Chemistry

Lipid oxidation in milk enriched with neat fish oil or pre-emulsified fish oil

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Abstract: The objective of the study was to investigate the differences in the oxidative stability of fish oil enriched milk when fish oil was added as either neat oil or pre-emulsified oil with sodium caseinate, a combination of the whey proteins α-lactalbumin and β-lactoglobulin, or purified β-lactoglobulin as emulsifier. Results showed that the addition of fish oil as neat oil increased the oxidative stability as compared to the addition of fish oil in a delivery emulsion. During storage the milk samples added various delivery emulsions developed differently with regards to peroxide values. At day 14 the order of oxidative stability was whey protein isolate > β-lactoglobulin > sodium caseinate. However, no clear pattern for differences between samples was observed for the secondary volatiles oxidation products quantified.

Keywords: Omega-3 fatty acids, delivery emulsion, whey protein isolate, sodium caseinate, β-lactoglobulin

1. Introduction

Long chain polyunsaturated omega-3 fatty acids have in both epidemiological and interventional studies been shown to possess a wide range of health beneficial effects, e.g. decrease the risk of heart-diseases and increase mental health (Riediger et al., 2009). For this reason, an increasing interest in substituting the original fat in some food products with fish oil that is rich in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) has developed. However, due to the unsaturated nature of these fatty acids are at risk of oxidizing. Lipid oxidation results in development of volatile oxidation products which gives undesirable off-flavours to the fish oil enriched food product.

A possible strategy for protecting the fatty acids against oxidation could be to incorporate them in an emulsion (a delivery emulsion) prior to their addition to the food product. However, studies have shown that the possible advantage of using a delivery emulsion is food system dependent. In yoghurt and salad dressing the addition of fish oil as neat oil resulted in products with a higher oxidative stability than when omega-3 PUFA was added as an emulsion, whereas in milk, cheese, fish paté and energy bars the use of a delivery emulsion was preferential (Let et al., 2007;Nielsen and Jacobsen, 2009;Ye et al., 2009). Nevertheless, the delivery emulsions could not completely inhibit lipid oxidation in these fish oil enriched food products.

Milk is considered a healthy, natural product, with a high content of caseins and whey proteins that could potentially have antioxidative effects (Faraji et al., 2004;Ries et al., 2010;Tong et al., 2000). Moreover, milk has been shown to be an efficient carrier for omega-3 fatty acids and to facilitate the biological actions of omega-3 fatty acids even at low doses (Visioli et al., 2000). Milk is therefore considered a good system for incorporation of fish oil.

Due to the contradicting results obtained on the effect of using delivery systems in different food products there is a need for a better understanding of the mechanisms behind these results. This includes a better understanding of the effect of the composition of the delivery system including the choice of emulsifier. On this background it was chosen to investigate the oxidative stabilizing effect of three different delivery emulsions when added to milk.

The aim of this study was to compare lipid oxidation in fish oil-enriched milk, when fish oil was added as an oil-in-water delivery emulsion or as neat oil. Delivery emulsions were prepared with either i) a combination of commercial whey protein isolates (to have almost equal concentrations of α-lactalbumin and β-lactoglobulin), ii) non-commercial purified β-lactoglobulin or iii) sodium caseinate. Fish oil enriched milk was compared to milk without fish oil.

2. Materials and methods

2.1 Materials

Milk with 0.5 and 1.5% fat were purchased locally. Sodium caseinate (Miprodan® 30), whey protein isolate (Lacprodan® DI-9224), whey protein isolate enhanced with α-lactalbumin (Lacprodan® ALPHA-20), and a non-commercial purified β-lactoglobulin were kindly donated by Arla Foods Ingredients amba (Viby J, Denmark). Cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway). The oil was stored at -40°C until use. PV and tocopherols were determined in the fish oil as described in section 2.5.1 and 2.4.2, respectively and results are given in Table 1. The fatty acid composition of the fish oil is given in Table 3, and determined as described in section 2.4.2. Data provided by Arla Foods Ingredients on the emulsifiers stated the following compositions. Sodium caseinate: 93.5% protein with a composition as casein in bovine milk, whey protein isolate: 92% protein; 22-24% α-lactalbumin and 48-52% β-lactoglobulin, whey protein isolate enhanced with α-lactalbumin: 88-94% protein; 60% α-lactalbumin and 20-25% β-lactoglobulin and non-commercial β-lactoglobulin: 92.5% protein; 7.3% α-lactalbumin and 76.4% β-lactoglobulin. All other chemicals and solvents used were of analytical grade.

2.2 Preparation of fish oil-in-water emulsions

Three emulsions were prepared (200 g of each) with 10.0% fish oil, 89.0% distilled water and 1% emulsifier. The emulsifiers were sodium caseinate, β-lactoglobulin, or a combination of whey protein isolate and whey protein isolate enhanced with α-lactalbumin (1:1). A premix was made by adding the fish oil slowly to the water during the first minute of mixing at 16,000 rpm (Ystral mixer, Ballrechten-Dottingen, Germany). Total mixing time was 3 minutes. The premix was afterwards homogenized on a microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75 μm). Homogenization was carried out at a pressure of 10,000 psi, running 3 passes.

2.3 Production of fish-oil-enriched milk, storage and sampling

Milk (2 kg) was pasteurized by heating to 72°C and homogenized at a total pressure of 22.5MPa on a two-stage valve homogenizer (Rannie, APV, Albertslund, Denmark). Neat fish oil (10.0 g) was added prior to homogenization, whereas delivery emulsions (100.0 g) were added after homogenization and stirred by hand. A combination of two types of commercial milk with 0.5% and 1.5% milk fat respectively was used to give final concentrations of 0.5% (w/w) fish oil and 1.0% (w/w) milk fat in the fish oil-enriched milks. A reference milk (1.5% milk fat) was prepared by a similar heating and homogenization procedure, but without additional oil.

Both the fish oil-enriched milks and the reference milk were stored in 100 mL bottles at 1.7 ± 0.3°C in the dark for 11 days. Samples were taken at day 0, 4, 7 and 11 for lipid oxidation analyses. Furthermore, samples were taken of the three delivery emulsions and the neat oil for PV determination at day 0. Both the three delivery emulsions and the five milk samples were characterized by pH at day 0, and droplet size distributions at day 1. Droplet size distributions were measured again at day 11 in milk samples. The neat oil and the five milk samples were furthermore characterized by fatty acid compositions and contents of tocopherols.

2.4 Characterization of the emulsions and milks

2.4.1 Droplet size and pH

Droplet size distributions of both milk samples and delivery emulsions were determined in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) by laser diffraction. All samples were diluted in recirculating water (2800 rpm), reaching an obscuration of 14-17%. For milk samples the Fraunhofer method was used, which assumes that all sizes of particles scatter with equal efficiencies and that the particles are opaque and transmit no light (Rawle, 1996). For measurements on delivery emulsions, the refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively.

Determination of pH was done during stirring of the emulsion/milk samples.

2.4.2 Lipid extractions, fatty acid composition and tocopherol

Lipids were extracted from all samples using a modified form of the method described by Bligh and Dyer (1959) with a reduced amount of solvent (30.0 ml methanol and chloroform, 1:1). For lipid extractions on the delivery emulsions 10 g sample was used, and on the fish oil-enriched milks 15 g sample was used.

Fatty acid compositions were determined on the lipid extract or directly on the neat oil by fatty acid methylation (AOCS, 1998a) followed by separation through gas chromatography (HP 7890 A, Hewlett Packard, Palo Alto, CA, USA; Column: DB-WAX, 10 m x 0.1 mm x 0.1 µm) (AOCS, 1998b).

The contents of tocopherols were determined by HPLC (Agilent 1100 Series; Column: Waters Spherisorb 3 µm Silica; 4.6 x150 mm), by injecting each lipid extract twice. Tocopherols were analyzed according to the official AOCS method (AOCS, 1998c).

2.5 Measurements of lipid oxidation

2.5.1 Primary oxidation products – peroxide values

Peroxide values were determined on the lipid extracts (prepared as described in section 2.4.2), or directly on the neat oil, by colorimetric determination of iron thiocyanate at 500 nm as described by Shantha and Decker (1994).

2.5.2 Secondary oxidation products – Dynamic headspace GC-MS

Milk samples (8 g) was added 0.5 mL Synperonic antifoam and purged with nitrogen (150 mL/min) for 30 min at 45°C and trapped on Tenax GR tubes. Volatiles were analyzed by gas chromatography (HP 5890 Series, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m x 0.25 mm x 1.0 µm; J&W Scientific, Folsom, CA, USA) with mass spectrometrical detection (HP 5972 inert mass-selective detector, Agilent Technologies, Palo Alto, CA, USA). The oven program in the gas chromatograph had an initial temperature of 45°C for 5 min, increasing with 1.5°C/min until 55°C, with 2.5°C/min until 90°C and with 12.0°C/min until 220°C, where the temperature was kept steady for 4 min. The individual compounds were analyzed by mass-spectrometry. From a comparison of chromatograms from non-oxidised and oxidised samples, the following volatiles were selected for quantification: 1-penten-3-one, pentanal, 1-pentan-3-ol, 2-pentenal, hexanal, 2-hexenal and 2,4-heptadienal. In the chromatograms two peaks were identified as 2,4-heptadienal. From previous studies of these two peaks (not published) it is anticipated that they represent the two isomers *t,c*-2,4-heptadienal and *t,t*-2,4-heptadienal, and the one reported here is therefore expected to be *t,t*-2,4-heptadienal. Calibration curves were made by dissolving the compounds in ethanol (96 %) followed by the addition of an amount of this ethanol corresponding to 3-1000 ng of the compounds to 8 g of the reference milk. Volatiles were collected and analysed similarly as in the milk samples. Measurements were made in triplicate on each sample.

2.6 Statistical analyses

All data were analysed by one- or two-way analysis of variance with Bonferronis multiple comparison test as post test (GraphPad Prism, version 4.03, GraphPad Software Inc., La Jolla, CA, USA). All references to significant differences ($p < 0.05$) between samples or between sampling times, are based on this statistical analysis of data.

3 Results

3.1 Characterization of the neat oil and delivery emulsions

The fish oil used had an initial PV of 0.1 meq peroxides/kg oil (Table 1). When delivery emulsions were prepared, PV was increased to 0.3, 0.8 and 1.8 meq peroxides/kg oil for Em_WP, Em_Lg and Em_CAS, respectively. Droplet size distributions were similar in the three delivery emulsions prepared (Figure 1). In addition, mean droplet sizes were not significantly differing between the two delivery emulsions prepared with whey proteins. However the emulsion with CAS had significantly smaller droplets (Table 1). The pH ranged from 6.5-6.7 for the three delivery emulsions (Table 1).

3.2 Characterization of the milk samples

The milk emulsions had pH values of 6.6-6.7 at day 0 and all had pH values of 6.8 at day 11 (Table 2). Droplet size distributions were bimodal in all milk samples, and there was a tendency towards smaller droplets in FO and REF than the three milks added delivery emulsions (Figure 1).

The fatty acid composition showed that the content of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were similar in the three milk samples with FO added in a delivery emulsion (Table 3). The FO samples had a slightly higher content of SFA and correspondingly the content of MUFA and PUFA were lower. This tendency was even more pronounced in the reference milk sample.

3.3 Lipid oxidation in the milk samples

3.3.1 Tocopherols

The reference milk only contained α-tocopherol, whereas the other samples had both α-, γ- and δ-tocopherols. During storage, contents of α-tocopherol decreased significantly in all samples, and γ- and δ-tocopherol furthermore decreased significantly in all milk emulsions with fish oil. The three tocopherol compounds were present in very different concentrations initially as stated for α- and γ-tocopherol in Table 2. However, for all three tocopherol compounds the initial rank order was REF^{a} < FO^{b} < CAS^{c} = Lg^{c} = WP^{c}. The concentrations of α-tocopherol decreased the most during storage by 92-96% in milk samples with fish oil and 41% in the reference sample. The concentrations of γ-tocopherol decreased by 42-67% in milk samples with fish oil (Table 2), and concentrations of δ-tocopherol decreased only by 18-38% (data not shown). Among the milk samples added delivery emulsions WP decreased the least in the concentration of tocopherols during storage indicating the least oxidation.

3.3.2 Peroxide values (PV)

PV in all samples containing fish oil significantly increased during storage. PV in the reference sample did not increase significantly during storage (Figure 2). Furthermore, this sample had significantly lower PV at all sampling time points. The other four milk samples did not differ significantly at day 0. However, at day 14 the PV increased in the order $REF^{a} < FO^{b} < WP^{c} < Lg^{d} < CAS^{e}$. Thus, all milk samples with delivery emulsions were more oxidized than the milk added neat fish oil. Moreover, although PV differed significantly between the three milk samples containing delivery emulsions differences between these three samples were much smaller than the difference between the milk samples with delivery emulsions and the milk containing neat fish oil.

3.3.3 Secondary volatile oxidation products

Seven secondary volatile oxidation products (1-penten-3-one, pentanal, 1-pentan-3-ol, 2-pentenal, hexanal, 2-hexenal and 2,4-heptadienal) were quantified, of which two are shown in Figure 3. The volatile 1-penten-3-ol stems from oxidation of n-3 fatty acids, whereas hexanal stems from oxidation of n-6 fatty acids. During storage all milk samples with fish oil had increasing concentrations of the seven secondary volatile oxidation products quantified. However, the reference sample only increased in the concentration of hexanal during storage. At day 0 it was furthermore only the concentration of the two volatiles stemming from oxidation of n-6 fatty acids, pentanal and hexanal that differed significantly between samples at day 0. In general, the concentration of hexanal was significantly higher in the three samples with delivery emulsions added, and a similar pattern was observed for the concentrations of pentanal. At day 11 the milk with neat fish oil (FO) had a significantly lower concentration of two of the seven volatiles (1-penten-3-ol and 2-pentenal) than the milk samples with fish oil added as a delivery emulsion as illustrated by 1-penten-3-ol in Figure 3. In addition FO had a significantly lower concentration of pentanal, 2-hexenal and 2,4-heptadienal than two out of the three samples with fish oil added as a delivery emulsion (data not shown). Thus, in general the addition of neat oil resulted in fish oil enriched milk with better oxidative stability than when the fish oil was added in a delivery emulsion. With regards to differences between the three milks containing different delivery emulsions, there was no clear pattern. The concentration of 1-penten-3-ol, 2-hexenal and 2,4-heptadienal were significantly lower in Lg than WP and CAS as exemplified by 1-penten-3-ol in Figure 3. On the other hand Lg had a significantly higher concentration of pentanal than the other two, whereas 2-pentenal did not differ significantly between the three samples (data not shown).

4 Conclusions

Overall conclusions were, that the fish oil enriched milk with fish oil added as neat oil was more oxidatively stable than the milks with fish oil added as a delivery emulsion. Furthermore, no differences were observed in the oxidative stabilities of the milks with different delivery emulsions added.

5 Acknowledgements

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Table 1. Data on the fish oil and the delivery emulsions used for production of the milk samples.

	Fish oil	Em_WP	Em_Lg	Em_CAS
pH	---	6.5	6.6	6.7
D[3,2] [μm]	---	0.122 ± 0.001^{b}	0.124 ± 0.001^{b}	0.118 ± 0.000^{a}
PV [meq peroxides/kg oil]	0.1 ± 0.0	0.3 ± 0.2	0.8 ± 0.0	1.8 ± 0.1
α-tocopherol [μg/g oil]	207 ± 16	---	---	---
γ-tocopherol [μg/g oil]	100 ± 1	---	---	---

---: Not determined

Table 2. pH and tocopherol contents in the milk samples at day 0 and day 11 of storage. Letters indicate significant differences ($P < 0.05$).

Milk sample	pH		α-tocopherol [µg/g oil]		γ-tocopherol [µg/g oil]	
	Day 0	Day 11	Day 0	Day 11	Day 0	Day 11
WP	6.7	6.8	73 ± 0^{c}	5 ± 1^{a}	30 ± 0^{c}	17 ± 0^{c}
Lg	6.7	6.8	73 ± 0^{c}	3 ± 0^{a}	30 ± 1^{c}	10 ± 1^{b}
CAS	6.6	6.8	70 ± 2^{c}	3 ± 0^{a}	29 ± 1^{c}	12 ± 0^{ab}
FO	6.6	6.8	50 ± 3^{b}	4 ± 1^{a}	19 ± 1^{b}	11 ± 1^{a}
REF	6.6	6.8	17 ± 2^{a}	10 ± 4^{b}	0 ± 0^{a}	0 ± 0^{a}

Table 3. Fatty acid compositions of the fish oil and the five milk samples at day 0 given in area %.

		Fish oil	REF	FO	CAS	Lg	WP
Fatty acid							
14:0		3.0	10.8	9.1	8.0	8.0	8.2
15:0		0.3	1.2	1.0	0.8	0.8	0.9
16:0		8.9	31.5	25.9	23.1	23.1	23.0
18:0		1.9	13.4	10.4	9.0	9.0	8.9
	Σ SFA	14.1	57.4	46.8	41.3	41.4	41.4
16:1(n-7)		8.2	2.0	3.8	4.6	4.6	4.6
18:1(n-9)		16.0	25.7	23.2	22.1	22.0	21.9
18:1(n-7)		5.2	3.0	3.4	3.6	3.7	3.7
20:1(n-9+n-11)		11.6	0.1	3.5	5.0	5.0	5.1
22:1(n-11)		6.1	-	1.6	2.3	2.3	2.3
22:1 (n-9)		0.8	-	0.2	0.3	0.3	0.3
	Σ MUFA	48.3	31.4	36.2	38.4	38.5	38.5
16:2(n-4)		0.4	0.5	0.5	0.4	0.4	0.5
16:3(n-4)		-	0.6	0.5	0.5	0.5	0.5
18:2(n-6)		1.8	2.1	2.2	2.2	2.1	2.1
18:3(n-3)		0.8	0.7	0.7	0.8	0.8	9.8
18:4(n-3)		2.5	0.8	1.3	1.5	1.5	1.5
20:4(n-3)		0.7	-	0.2	0.3	0.3	0.3
20:5(n-3)		9.3	0.1	2.5	3.6	3.7	3.7
22:5(n-3)		1.1	0.1	0.4	0.5	0.5	0.5
22:6(n-3)		11.6	-	3.1	4.5	4.5	4.5
	Σ PUFA	29.8	5.4	12.2	15.3	15.2	15.4
	Σ n-3	26.6	1.7	8.3	11.3	11.4	11.4

Only fatty acids present in a concentration of > 0.5% in one of the samples are given by name. -: not detected

All standard deviations < 0.3% (absolute %)

Figure 1. Droplet size distributions in milk samples and delivery emulsions.

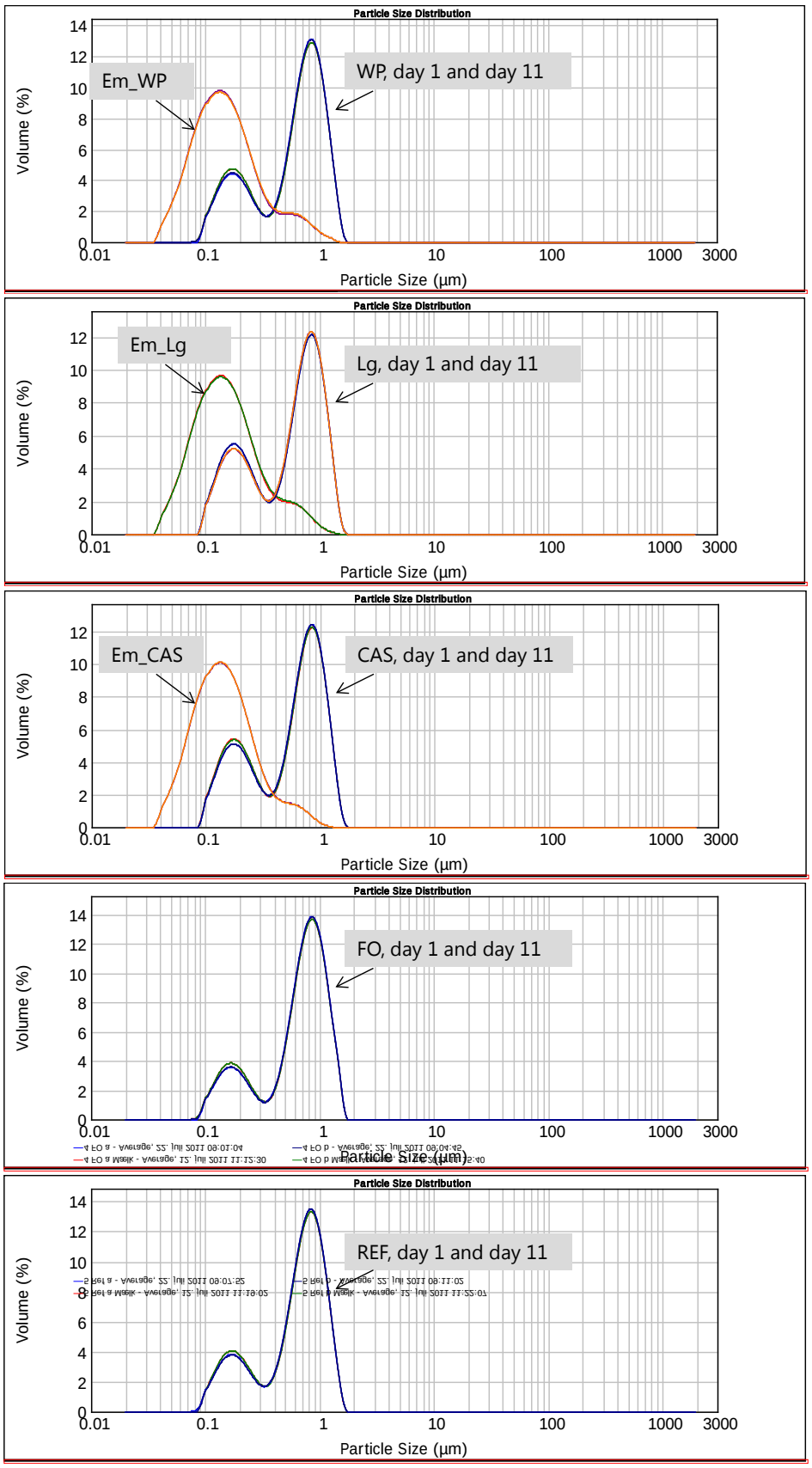


Figure 2. Peroxide values in milk samples during storage for 11 days. REF: Milk without fish oil; FO: Milk added neat fish oil; WP, Lg, CAS: Milk with fish oil added in a delivery emulsion prepared with whey protein isolate, β-lactoglobulin or sodium caseinate, respectively.

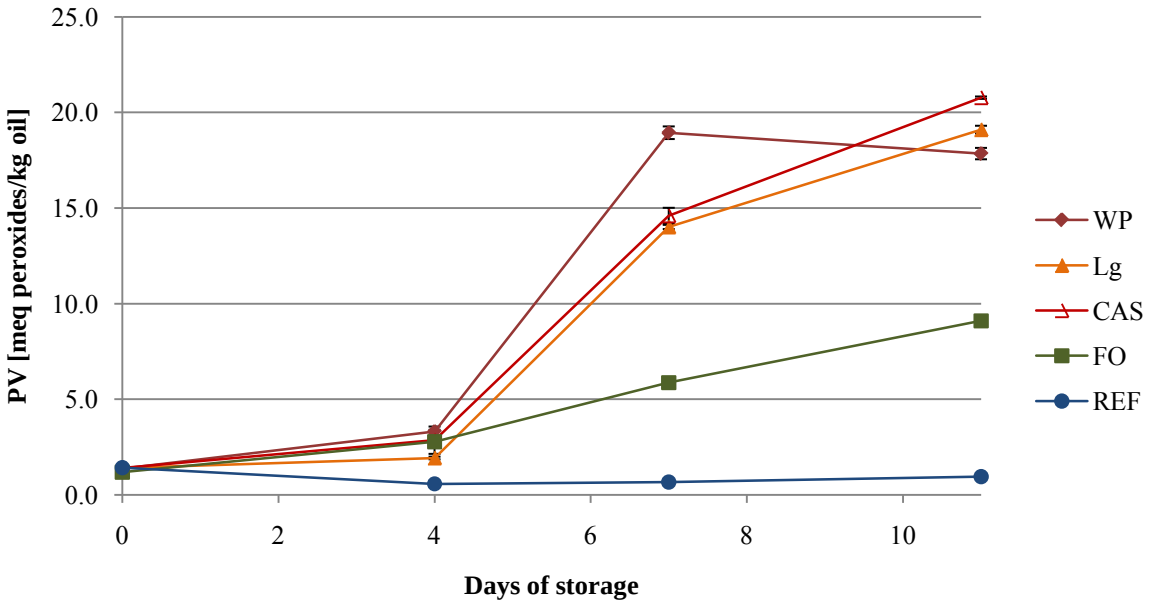
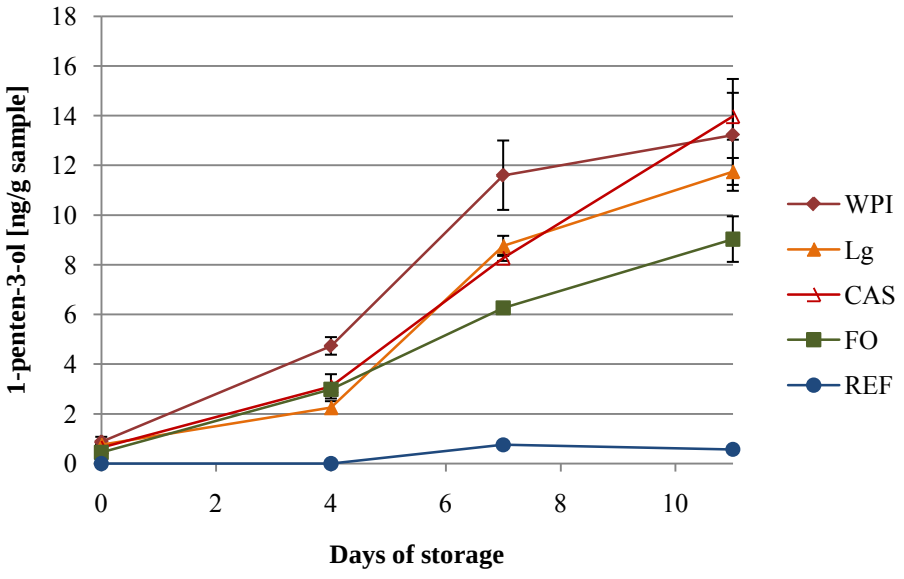
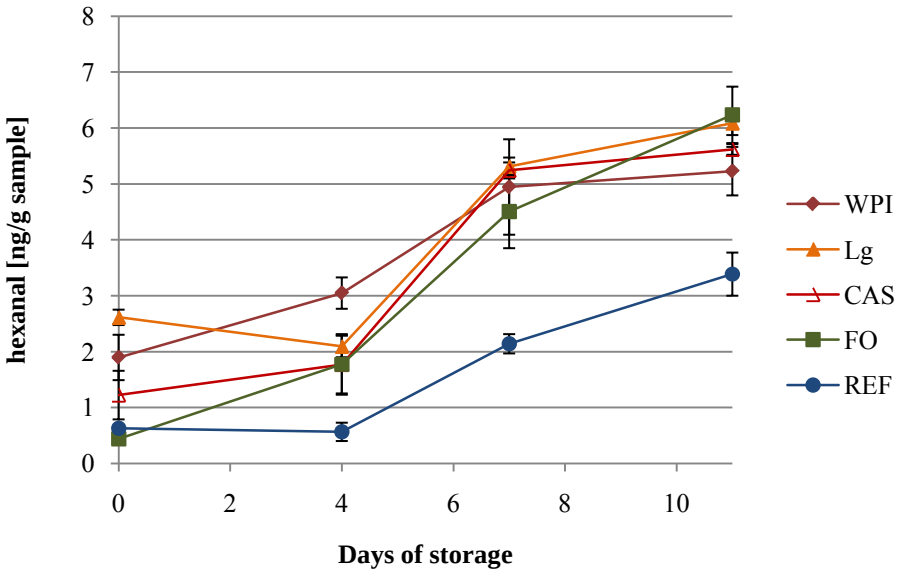


Figure 3. Development of 1-penten-3-ol (A) and hexanal (B) during storage for 11 days. REF: Milk without fish oil; FO: Milk added neat fish oil; WP, Lg, CAS: Milk with fish oil added in a delivery emulsion prepared with whey protein isolate, β-lactoglobulin or sodium caseinate, respectively.



A



B

PAPER VIII

Addition of fish oil to cream cheese affects lipid oxidation, sensory stability and microstructure

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Article

Addition of Fish Oil to Cream Cheese Affects Lipid Oxidation, Sensory Stability and Microstructure

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Abstract: The objective of this study was to investigate the differences in the oxidative stability during storage of fish oil enriched cream cheeses when fish oil was added either as neat oil or pre-emulsified oil with sodium caseinate, whey protein isolate, or a combination of milk proteins and phospholipids as emulsifier. Results showed that the addition of fish oil decreased the oxidative stability of cream cheeses regardless of the addition method, especially when the cheese was stored longer than five weeks. The oxidative stability of fish oil enriched cream cheeses was highest when fish oil was added as neat oil or in a delivery emulsion prepared with a combination of milk proteins and phospholipids. Adding the fish oil in a delivery emulsion prepared with whey protein or caseinate resulted in a less oxidative stable product. It was furthermore shown that the microstructure of the cream cheeses was affected by fish oil addition, and it was suggested that the change in microstructure was partly responsible for the oxidative stability of the cream cheeses.

Keywords: omega-3 fatty acids; delivery emulsion; whey protein isolate; sodium caseinate; milk phospholipids; peroxide value; volatile oxidation products; sensory evaluation; confocal laser scanning microscopy

1. Introduction

Both epidemiological and intervention studies have shown that long chain polyunsaturated omega-3 fatty acids possess a wide range of health beneficial effects [1]. For this reason, an increasing interest in substituting the original fat in some food products with fish oil that is rich in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) has developed. However, a major concern in fish oil enriched foods is lipid oxidation and the development of volatile oxidation products resulting in undesirable off-flavors in the fish oil enriched food product.

Dairy products are in general considered healthy food products. Moreover, they have a natural content of the potentially antioxidative milk proteins and are thus considered good vehicles for fish oil addition. However, studies have shown that various fish oil enriched dairy products might differ to a great extent in their oxidative stability [2,3]. To the authors' knowledge, only one study has previously been reported on fish oil enriched cream cheese, and this study only evaluated the sensory performance of the cheeses [4]. It was observed that it was possible to add 15 g fish oil per kg spreadable fresh cheese (Philadelphia type) before reaching a level where the sensory quality was significantly impacted. In comparison, it was possible to add 3 g fish oil per kg non-flavored semi-solid processed fresh low-fat cheese (110 g fat/kg) and 40 g fish oil per kg non-flavored processed cheese (320 g fat/kg). The spreadable fresh cheese with 15 g fish oil/kg could be stored for up to 5 weeks before the sensory quality decreased. Since the shelf life of a cream cheese is usually more than 20 weeks the above-mentioned results indicate that the addition of fish oil has to be improved in order to avoid lipid oxidation and increase shelf life. One strategy to protect the unsaturated fatty acids could be to incorporate them in an emulsion (a delivery emulsion) prior to their addition to the food product.

This approach was evaluated in processed cheese, and it was observed that the use of a delivery emulsion prepared with a milk protein complex (casein and whey protein) as emulsifier increased the oxidative stability throughout storage as compared to the addition of neat oil [3]. However, despite the better oxidative stability, the sensory perception of the cheese was still different from the control upon storage when fish oil was added in a concentration of 30 g or more per kg cheese (corresponding to approximately 89 mg fish oil in a serving size of 30 g cheese). Thus, improvements of the delivery emulsion used could be advantageous, as could a better understanding of lipid oxidation in these types of food products.

The aim of this study was to compare lipid oxidation in fish oil enriched cream cheese upon storage, when 1.3% fish oil was added as a 70% fish oil-in-water delivery emulsion or as neat oil. This amount of fish oil makes it possible to claim "High in omega-3" according to EU Commission Regulation No 116/2010 [5]. Delivery emulsions were prepared with either sodium caseinate, whey protein isolate or an emulsifier with a combination of milk proteins and milk phospholipids. Fish oil enriched cream cheese was compared to cream cheese without fish oil. Lipid oxidation was followed by the

development in peroxide value, concentration of volatile secondary oxidation products and sensory performance over 20 weeks of storage. The microstructure of the cream cheeses was furthermore imaged by confocal laser scanning microscopy.

2. Materials and Methods

2.1. Materials

Raw cream cheese was provided by Arla Foods Holstebro Flødeost (Holstebro, Denmark). Sodium caseinate (Miprodan® 30), whey protein isolate (Lacprodan® DI-9224), and milk phospholipid/milk protein (Lacprodan® PL-20) were kindly donated by Arla Foods Ingredients amba (Viby J, Denmark). Cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway). The oil was stored at −40 °C until use. The initial PV in the fish oil and the content of tocopherol compounds were determined as described in section 2.7.1 and 2.6, respectively, and results are given in Table 2.

2.2. Preparation of 70% Fish Oil-in-Water Emulsions

Delivery emulsions were prepared as described by Horn *et al.* [6] in a Stephan Universal mixer (Stephan, UMC5, 1995, Hameln, Germany) with water instead of buffer as the continuous phase. Three emulsions were prepared (1000 g of each) with 70.0% fish oil, 27.2% distilled water and 2.8% emulsifier. The emulsifiers were sodium caseinate, whey protein isolate or an emulsifier with a combination of milk phospholipids (approximately 20%) and milk proteins (approximately 50%). The pressure was reduced in the mixer during production to minimize air bubbles in the emulsions. Emulsions were kept at approximately 5 °C until production of the fish oil enriched cream cheese. The emulsions are named Em_CAS (emulsion with sodium caseinate), Em_WPI (emulsion with whey protein isolate) and Em_MPL20 (emulsion with a combination of milk phospholipids and milk proteins) according to the emulsifier used for their production.

2.3. Production of Fish Oil Enriched Cream Cheese

Enriched cream cheeses (batches of 25 kg) were prepared in a pilot plant at Arla Foods amba, Arla Strategic Innovation Centre (Brabrand, Denmark). Raw cheese was taken from an intermediate step of the production of cream cheese (50+). Raw cheese was weighted off and poured into a 50 L Stephan Universal mixer (Stephan, UMMISK 40E-GNI, 1979, Hameln, Germany), and held under vacuum for 30 s before being pasteurized by heating to 72 °C at 1500 rpm. Hereafter, either neat oil or one of the delivery emulsions was added to the heated cheese (1.3% fish oil w/w), and the cheese was stirred for 5 min. Then the cheese was held under vacuum once more for 60 s, before it was moved to a high pressure homogenizer (Invensys APV, R18-38, Silkeborg, Denmark), where it was homogenized at 65 °C/175 bar. A reference cheese was prepared by a similar heating and homogenization procedure, but without addition of fish oil. All cream cheeses were tapped directly into 300 g white plastic containers while still warm, and sealed before cooling to approximately 5 °C.

2.4. Storage and Sampling

Both the fish oil enriched cream cheeses and the reference cheese were stored at 4.6 ± 0.4 °C in white plastic containers for 20 weeks in the dark. Samples were taken at week 0, 2, 5, 10, 15 and 20 for lipid oxidation analyses. Furthermore, samples of the three delivery emulsions and the neat oil were taken for PV determination at the day of production. The pH value was determined in both the three delivery emulsions and the five cream cheese samples at day 0 or 1 and week 20, respectively. The neat oil and the five cream cheese samples were furthermore characterized by fatty acid compositions and their contents of tocopherols.

2.5. Characterization of the Delivery Emulsions and Cream Cheeses

2.5.1. Droplet Size and pH

Droplet size distributions were determined in the delivery emulsions by laser diffraction on a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). The emulsions were pretreated according to the method described by Let *et al.* (2007) [2]. Emulsion (1 g) was dissolved in 5 g SDS buffer (10 mM NaH_2PO_4, 5 mM SDS), mixed for 30 seconds and then sonicated for 15 min in a water bath at 0 °C. Droplets of the pretreated emulsions were diluted in recirculating water (3000 rpm), reaching an obscuration of 12%–15%. The refractive indices of sunflower (1.469) and water (1.330) were used as particle and dispersant, respectively.

In both delivery emulsions and cream cheeses, the pH was determined in a suspension of the emulsion in distilled water (1:1).

2.5.2. Confocal Laser Scanning Microscopy

The cream cheeses were subjected to imaging by confocal microscopy. Prior to that, samples were stained with Fluorescein isothiocyanate for the proteins and Nile red for the oil. Microscopy was performed on a Leica TCS SP II (Leica Microsystems GmbH, Heidelberg, Germany) inverted vertically, at room temperature with a 100x oil immersion objective.

2.6. Lipid Extraction, and Analyses of Fatty Acid Compositions and Tocopherols

Lipids were extracted from all samples using a modified form of the method described by Bligh and Dyer (1959) [7] with a reduced amount of solvent (30.0 mL methanol and chloroform, 1:1). For lipid extractions on the delivery emulsions 5 g sample was used, and on the fish oil enriched cream cheeses 10 g sample was used.

Fatty acid compositions were determined directly on the neat oil by fatty acid methylation [8] followed by separation through gas chromatography (HP 7890 A, Hewlett Packard, Palo Alto, CA, USA; Column: DB-WAX, 10 m × 0.1 mm × 0.1 μm) according to the official AOCS method [9].

The content of tocopherol was determined by HPLC (Agilent 1100 Series; Column: Waters Spherisorb 3 μm Silica; 4.6 × 150 mm), by injecting each lipid extract twice. Tocopherols were analyzed according to the official AOCS method [10].

2.7. Measurements of Lipid Oxidation

2.7.1. Primary Oxidation Products—Peroxide Values

Peroxide values (PV) were determined on the lipid extracts (prepared as described in section 2.6), or directly on the neat oil, by colorimetric determination of iron thiocyanate at 500 nm as described by Shantha and Decker (1994) [11].

2.7.2. Secondary Oxidation Products—Dynamic Headspace GC-MS

Cream cheese (5–8 g) was added 10 mL distilled water and purged with nitrogen (150 mL/min) for 30 min at 45 °C and trapped on Tenax GR tubes. Subsequently, water was removed by purging the tube in the opposite direction with nitrogen for 20 min (50 mL/min). The volatiles were desorbed again by heat (200 °C) in an Automatic Thermal Desorber (ATD-400, Perkin Elmer, Norwalk, CN), cryofocused on a cold trap (−28 °C), released again (220 °C), and led to a gas chromatograph (HP 5890 Series, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m × 0.25 mm × 1.0 μm; J&W Scientific, CA, USA) with mass spectrometrical detection (HP 5972 inert mass-selective detector, Agilent Technologies, USA). The oven program in the gas chromatograph had an initial temperature of 45 °C for 5 min, increasing with 1.5 °C/min until 55 °C, with 2.5 °C/min until 90 °C and with 12.0 °C/min until 220 °C, where the temperature was kept steady for 4 min. The individual compounds were analyzed by mass-spectrometry (Electron ionization mode, 70 eV; mass to charge ratios between 30 and 250). From a comparison of chromatograms from non-oxidized and oxidized samples, the following volatiles were selected for quantification: butanal, 2-ethylfuran, *t*-2-butenal, pentanal, 1-penten-3-ol, *t*-2-pentenal, 1-pentanol, hexanal, *t*-2-hexenal, heptanal, 2-pentylfuran, *t*-2-heptenal, *t*,*t*-2,4-heptadienal. Calibration curves were made by dissolving the compounds in ethanol (96%) followed by the addition of an amount of this mix corresponding to 3–1000 ng of the compounds to 8 g of the reference cheese. Volatiles were collected and analyzed similarly as in the cream cheese samples. Measurements were made in triplicate on each sample. Three of the volatiles could not be extracted completely from the chromatograms (1-penten-3-ol, *t*-2-pentenal and heptanal) due to overlapping compounds and were thus later taken out. Calibration curves were parallel shifted in order to obtain positive values, thus the concentrations of volatiles are not given as exact values.

2.8. Sensory Evaluation

Sensory evaluation was conducted by a panel consisting of twelve assessors, eight females and four males. The assessors were tested and trained in descriptive sensory analysis according to ISO standards [12,13]. Before sensory profiling, a vocabulary of descriptors for odor, appearance, flavor and texture was developed. The descriptors were evaluated on an unstructured 15 cm scale anchored 1.5 cm from both ends.

Sensory evaluation was performed five times during the storage period of 20 weeks. The evaluations were performed after 2, 5, 10, 15 and 20 weeks of storage. Due to the long time duration in between evaluations a two-hour training session was held two days before each evaluation was conducted.

All samples were served individually in closed Petri dishes and the lids were marked with a three-digit code. Each Petri dish contained two separately placed portions of the same cream cheese sample. One of the portions was used for evaluating flavor and texture as perceived in the mouth (pasty, melt down and fatty), while the other portion was used for evaluating the texture (firmness and spreadability) and appearance directly in the Petri dish. All samples were evaluated in duplicates and in randomized order. Evaluations were performed according to ISO standard 8589 [14] in separated booths under normal daylight. To clean the mouth between samples, the assessors used water and peeled cucumber, where the soft center with the pips was removed. Between eight and twelve assessors participated in each evaluation. Data were collected using the computer system FIZZ (Network Version 2.0, Biosystems, Couternon, France).

2.9. Statistical Analyses

All chemical data were analyzed by one- or two-way analysis of variance with Bonferronis multiple comparison test as posttest (GraphPad Prism, version 4.03, GraphPad Software Inc., La Jolla, CA, USA). All references to significant differences ($p < 0.05$) between samples or between sampling time points, are based on this statistical analysis of data.

Results from sensory profiling were corrected for level effect by the method of Thybo and Martens (2000) [15]. After level correction a principal component analysis (PCA) on mean values was performed to study the differences between the different types of cream cheese samples and storage times. The correction for level effect and PCA were calculated using The Unscrambler® 9.1 (CAMO, Trondheim, Norway).

3. Results

3.1. Characterization of the Neat Oil and Delivery Emulsions

The fatty acid composition of the fish oil used for the production of delivery emulsions and fish oil enriched cream cheese, showed that it contained approximately 14% 48% and 29% saturated fatty acids, monounsaturated and polyunsaturated fatty acids, respectively (Table 1). Approximately 27% of the total fatty acids were n-3 fatty acids. The fish oil had an initial PV of 0.1 meq peroxides/kg oil (Table 2). The contents of tocopherols were 207 μg/g oil and 100 μg/g oil of α- and γ-tocopherol, respectively (Table 2).

The PV in the delivery emulsions were all ≤0.3 meq lipid hydroperoxides/kg oil (Table 2). The pH values of the delivery emulsions were 6.8-7.3. The droplet size distributions for all three delivery emulsions were monomodal, but Em_MPL20 had a wider distribution than Em_CAS and Em_WPI (data not shown). The mean oil droplet sizes of the delivery emulsions are given in Table 2. All delivery emulsions were highly viscous with a consistency as yoghurt, and similar emulsions have previously been shown to be physically stable for up to 42 days [6].

Table 1. Fatty acid composition of the fish oil determined by fatty acid methylation followed by separation through gas chromatography. Fatty acids are given in area%.

SFA		**MUFA**		**PUFA**	
Fatty acid	**Av [%]**	**Fatty acid**	**Av [%]**	**Fatty acid**	**Av [%]**
14:0	3.0	16:1 (n-6)	8.2	18:2 (n-6)	1.8
16:0	8.9	18:1 (n-9)	16.0	18:3 (n-3)	0.8
18:0	1.9	18:1 (n-7)	5.2	18:4 (n-3)	3.5
		20:1 (n-9+n-11)	11.6	20:4 (n-3)	0.7
		22:1 (n-11)	6.1	20:5 (n-3)	9.3
		22:1 (n-9)	0.8	22:5 (n-3)	1.1
				22:6 (n-3)	11.6
Total	**13.8**	**Total**	**48.3**	**Total**	**29.4**
				Total n-3	**26.6**

Only fatty acids in a concentration >0.5% are given by name; All standard deviations are <0.1%; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; Av: Average.

Table 2. Data on fish oil and delivery emulsions used for production of the cream cheese samples.

	Fish oil	**Em_CAS**	**Em_WPI**	**Em_MPL20**
Emulsifier type	--	CAS	WPI	MPL20
pH	--	7.1	6.8	7.3
PV [meq peroxides/kg oil]	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0
Droplet size, D3,2 [µm]	--	8.3	21.2	12.6
α-tocopherol [µg/g oil]	207 ± 16	--	--	--
γ-tocopherol [µg/ g oil]	100 ± 1	--	--	--

--: Not determined. CAS: Sodium caseinate; WPI: Whey protein isolate; MPL20: A commercially available emulsifier with a combination of milk proteins and milk phospholipids.

3.2. Characterization of the Cream Cheeses

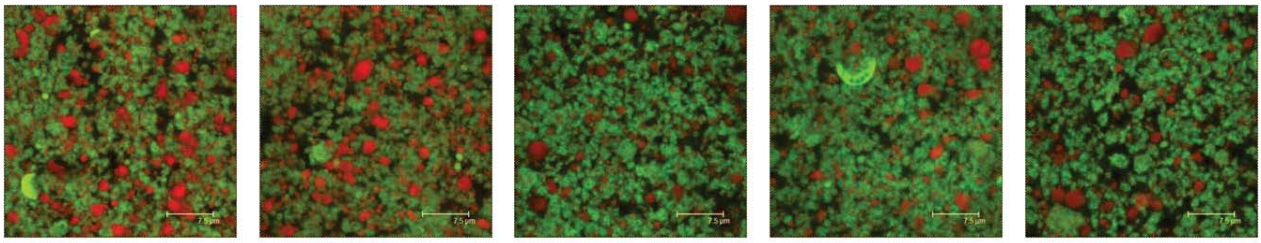
The pH of the cream cheeses was 4.7–4.8 throughout the 20 weeks of storage (Table 3). From the microscopic imaging it was observed that the reference cheese and the cheese added fish oil as neat oil had relatively large unprotected lipid droplets (colored red in Figure 1), whereas the three cream cheeses with added fish oil through delivery emulsions had far fewer unprotected oil droplets. Particularly MPL20 could be distinguished from the other samples since more of the lipid (including the milk lipid) was hidden within the protein structure. This observation was confirmed by freeze-fracture cryo-scanning electron microscopy (included as supplementary material).

Table 3. pH and tocopherol contents in cream cheeses at week 0 and week 20 of storage. Letters indicate significant differences ($P < 0.05$).

Cream cheese sample	pH		α-tocopherol [µg/g oil]		γ-tocopherol [µg/g oil]	
	Week 0	Week 20	Week 0	Week 20	Week 0	Week 20
CAS	4.8	4.7	26 ± 5b	12 ± 0ab	8 ± 1b	3 ± 0b
WPI	4.8	4.7	35 ± 1c	11 ± 0a	10 ± 0d	4 ± 0c
MPL20	4.8	4.7	30 ± 3bc	8 ± 0a	9 ± 0c	4 ± 0c
FO	4.8	4.7	31 ± 1bc	8 ± 1a	9 ± 0c	4 ± 0c
REF	4.8	4.7	16 ± 1a	17 ± 0b	1 ± 0a	0 ± 0a

CAS, WPI and MPL20: Cream cheeses with fish oil added as delivery emulsion prepared with sodium caseinate, whey protein isolate and an emulsifier with a combination of milk proteins and phospholipids, respectively. FO: Cream cheese with fish oil added as neat oil. REF: Cream cheese without fish oil added.

Figure 1. Confocal micrographs of the cream cheeses prepared without fish oil added (REF), with fish oil added as neat oil (FO) or fish oil added in a delivery emulsion emulsified by different emulsifiers (MPL20, WPI and CAS). Lipids are stained red, proteins green.



3.2.1. Contents of Tocopherol

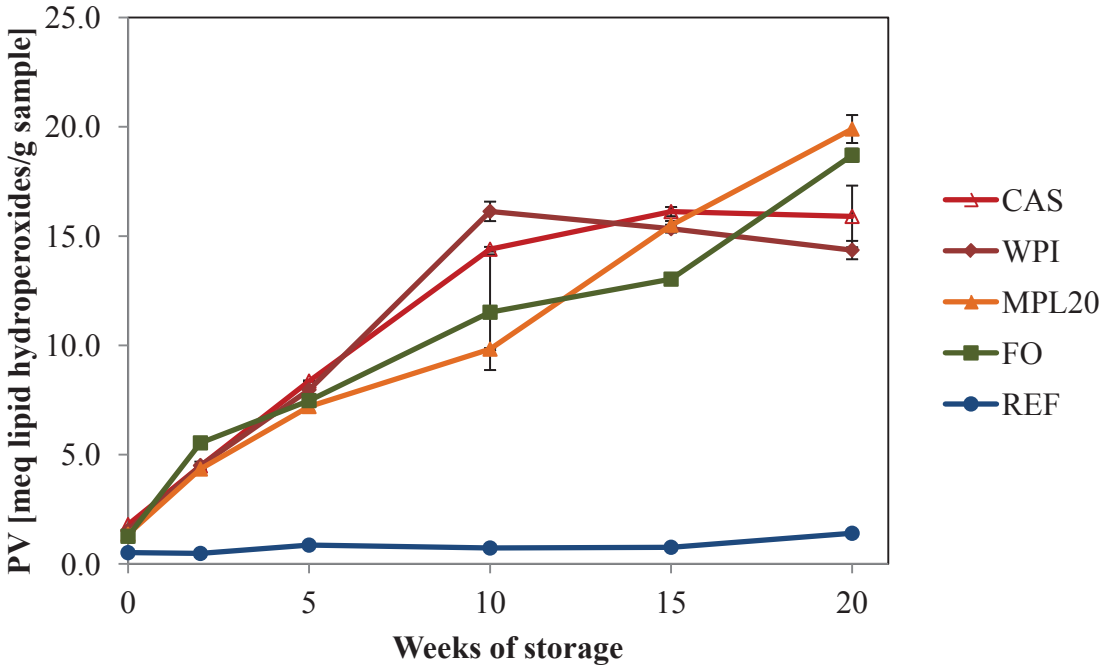
The content of α-tocopherol was 16 µg/g oil in the reference cream cheese at day 0 as compared to 26–31 µg/g oil in the cream cheeses with fish oil (Table 3). Similarly, the content of γ-tocopherol was significantly lower in the reference cream cheese than in the other cream cheeses. The higher content of tocopherols in the cream cheeses with fish oil was due to the endogenous tocopherols in the fish oil itself (Table 2). The content of both α- and γ-tocopherol were stable in the reference sample during the 20 weeks of storage (Table 3). However, a significant decrease was observed in the contents of both tocopherols for the four samples enriched with fish oil. The content of α-tocopherol decreased to 8–12 µg/g oil at week 20 and the content of γ-tocopherol to 3–4 µg/g oil. The decrease in α-tocopherols was lowest in CAS, 14 µg/g oil, compared to 22–24 µg/g oil in the other three cream cheeses with fish oil.

3.3. Lipid Oxidation in Emulsions

3.3.1. Peroxide Values

PV were not significantly different between samples at week 0. However, PV in all samples except the reference increased significantly during storage, and a significant increase was already observed between week 0 and 2 (Figure 2). When comparing samples with fish oil, it was found that these samples did not differ significantly until week 10. At week 10 the sample with MPL20 had a significantly lower PV than the other samples, and WPI a significantly higher PV. However, between week 10 and 20, PV in CAS and WPI did not increase whereas it increased significantly in MPL20 and FO. Thus, at week 20, the rank order was $REF^{a} < WPI^{b} = CAS^{b} < FO^{c} = MPL20^{c}$.

Figure 2. Peroxide values in cream cheeses with fish oil added as neat oil (FO), or in a delivery emulsion prepared with different emulsifiers (CAS, WPI or MPL20) compared to a reference cheese without fish oil (REF) during storage for 20 weeks.



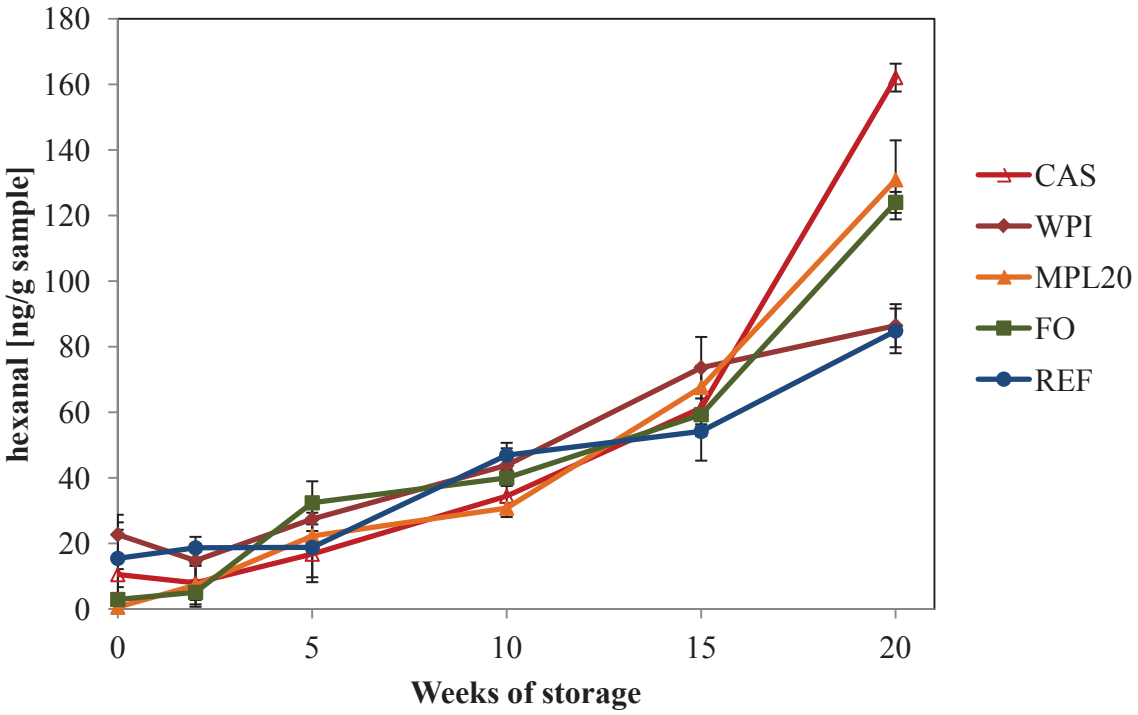
3.3.2. Secondary Volatile Oxidation Products

The volatiles pentanal and hexanal stemming from oxidation of n-6, and, most important in this context, a wide range of volatiles stemming from oxidation of n-3 fatty acids, such as 2-ethylfurane, *t*-2-butenal, *t*-2-hexenal, and *t*,*t*-2,4-heptadienal were quantified. The volatiles could be grouped in volatiles that increased significantly in all cream cheese samples during storage (Group 1: pentanal, 1-pentanol, hexanal and 2-pentylfurane, represented by hexanal in Figure 3) and volatiles that only increased in the samples with fish oil added (Group 2: butanal, 2-ethylfurane, *t*-2-butenal, *t*-2-hexenal, *t*-2-heptenal and *t*,*t*-2,4-heptadienal, represented by *t*-2-hexenal in Figure 4).

As an example of group 1 volatiles, hexanal is presented in Figure 3. It was observed that the concentration of hexanal as well as concentrations of pentanal, 1-pentanol and 2-pentylfurane

increased significantly in the reference cream cheese between week 0 and 20. The presence of fish oil in the other cream cheeses increased the rate at which these volatiles were produced, and, at week 20, all four volatiles were present in significantly higher concentrations in the cream cheeses with fish oil compared to the reference cream cheese. Interestingly, concentrations of these volatiles did not increase significantly in the MPL20 sample until between week 10 and 15 or between week 15 and 20. In comparison, CAS had significantly increased concentration of pentanal already between week 2 and 5 and increased concentrations of hexanal and 2-pentylfurane between week 5 and 10. Concentrations of both hexanal and 2-pentylfuran increased significantly in the sample with fish oil added as neat oil (FO) between week 2 and 5.

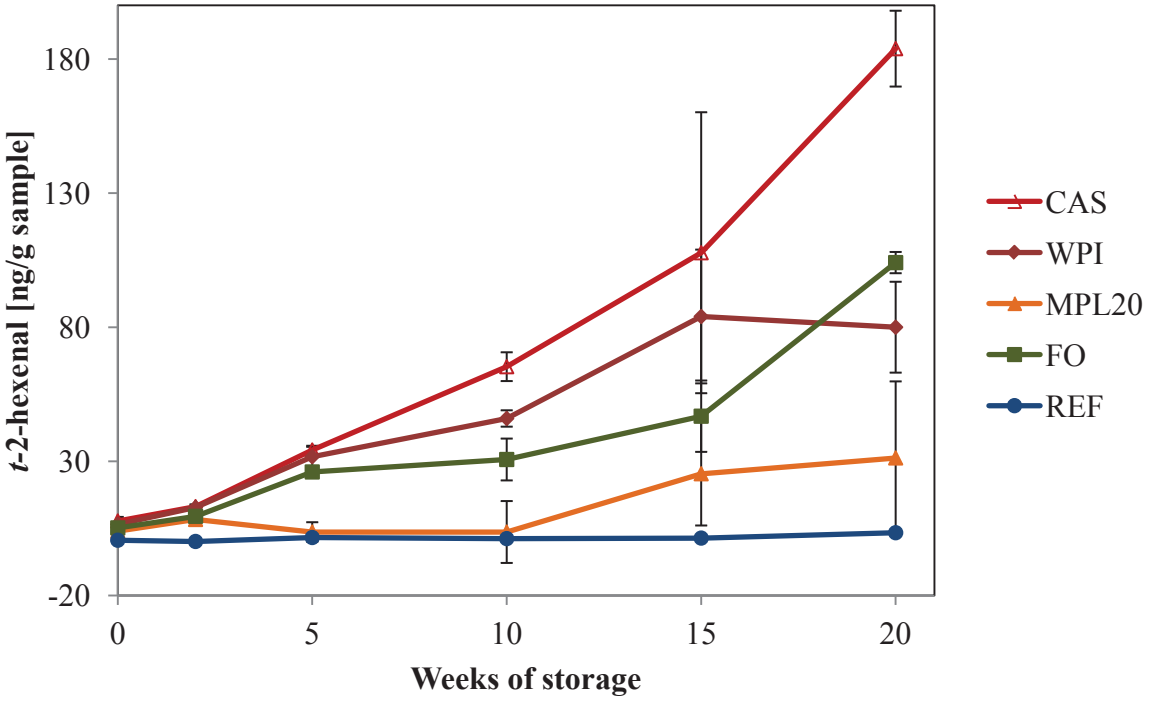
Figure 3. The development in hexanal concentrations (ng/g sample) in cream cheeses with fish oil added as neat oil (FO), or in a delivery emulsion prepared with different emulsifiers (CAS, WPI or MPL20) compared to a reference cheese without fish oil (REF) during storage for 20 weeks.



Comparison of individual samples at the different sampling time points showed that until the very last part of the storage period the main differences observed existed between the reference cream cheese and the cheeses added fish oil. However, it was observed that hexanal was the only volatile for which concentrations differed between the samples already at week 0, with WPI having a higher concentration than all other samples. Concentrations of pentanal and 1-pentanol differed significantly between samples at week 5, and the concentrations of these volatiles were also higher in WPI than any other sample. Concentrations of 2-pentylfurane did not differ significantly between samples until week 10, where the samples were ranked as follows $REF^{a} \leq MPL20^{ab} \leq FO^{bc} = CAS^{bc} \leq WPI^{c}$. Interestingly, at week 20, WPI had in general the lowest concentration of these four volatiles, whereas CAS had the highest.

The concentrations of the other six group 2 volatiles (butanal, 2-ethylfurane, *t*-2-butenal, *t*-2-hexenal, *t*-2-heptenal and *t*,*t*-2,4-heptadienal) did not increase significantly between week 0 and 20 in the reference cream cheese, but all increased significantly during storage in the four samples with fish oil added (*t*-2-hexenal is shown in Figure 4).

Figure 4. The development in *t*-2-hexenal (ng/g sample) in cream cheeses with fish oil added as neat oil (FO), or in a delivery emulsion prepared with different emulsifiers (CAS, WPI or MPL20) compared to a reference cheese without fish oil (REF) during storage for 20 weeks.



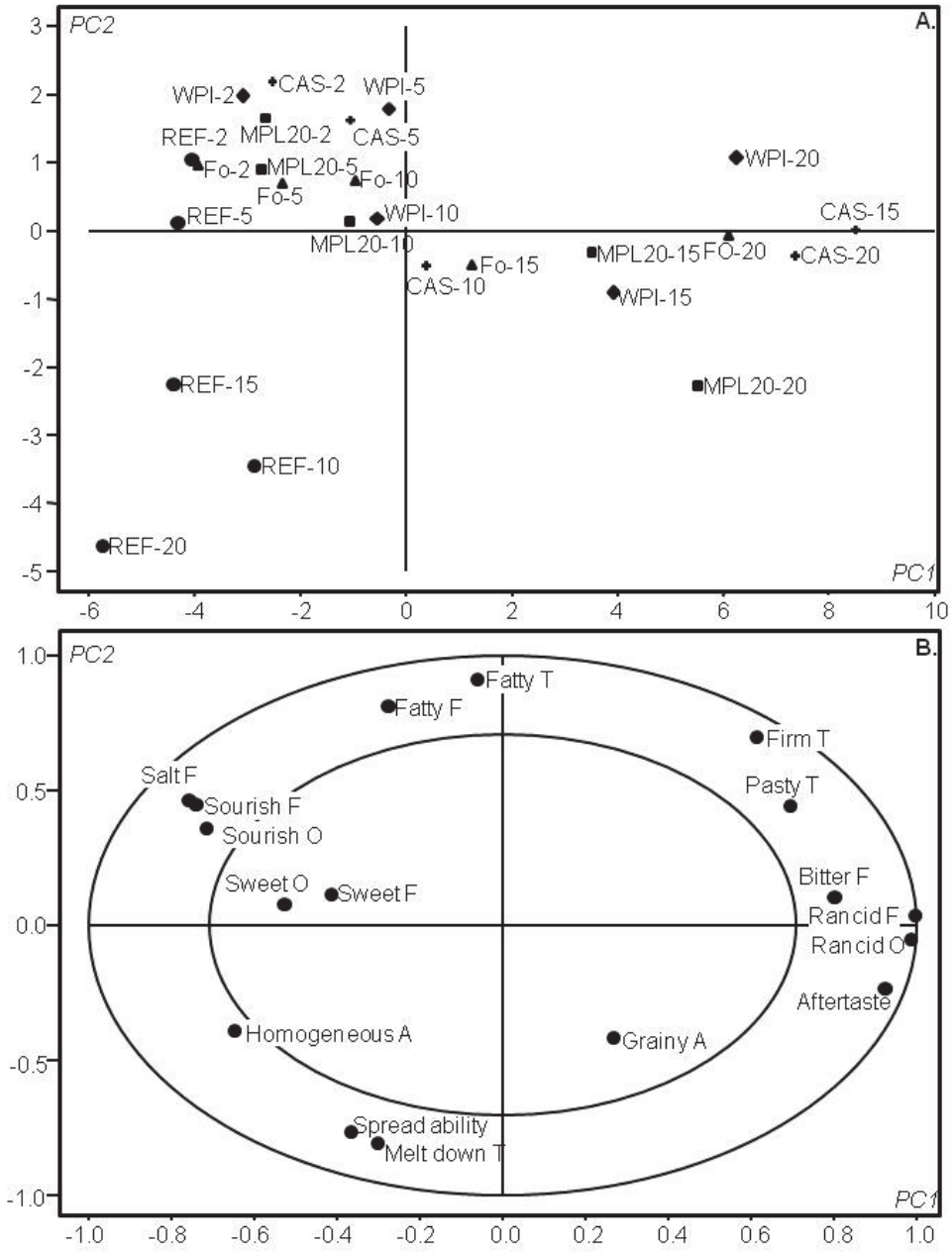
The volatile 2-ethylfuran was one of the first to increase significantly in the fish oil enriched cream cheeses. The concentration increased significantly already between week 2 and 5 in CAS, WPI and FO, and between week 5 and 10 in MPL20. Butanal increased significantly in CAS, WPI and MPL20 between week 5 and 10 whereas FO followed between week 10 and 15. For the concentrations of the other four volatiles (*t*-2-butenal, *t*-2-hexenal, *t*-2-heptenal and *t*,*t*-2,4-heptadienal) a significant increase was not observed in MPL20 until between week 15 and 20. In comparison, a significant increase in *t*-2-butenal and *t*-2-hexenal was observed between week 5 and 10 in CAS and between 10 and 15 in WPI. The FO sample showed more or less a similar pattern to MPL20.

The overall picture from volatiles data was that the reference sample oxidized the least followed by a group of MPL20 and FO and then a group of WPI and CAS. Differences between WPI and CAS were small, but generally CAS oxidized slightly more than WPI.

3.3.3. Sensory Profiling of the Cream Cheeses

Figure 5 shows a PCA model of the results from the sensory profiling. Cream cheeses stored for a short time (all samples at week 2 and 5 and WPI, MPL20 and FO at week 10) had negative scores for PC1 and positive scores for PC2. These cream cheeses were described by salty, sour, sweet and fatty flavors, sour and sweet odors and fatty texture as these descriptors were found at the corresponding location in the correlation loadings plot.

Figure 5. A PCA plot on the data from the sensory profiling of the cream cheeses. The upper figure (**A**) is the scores plot, and the lower figure (**B**) is the correlation loadings plot. The first and second principal component explains 76% and 12% of the variation, respectively. The numbers given as postfix to the sample names indicate the storage time in weeks.



Hence, this finding indicates that the sensory characteristics were generally stable during the first part of the storage period. However, it should be pointed out that especially WPI-5 and CAS-5 had higher PC1 scores than REF-1 indicating that some bitter flavor, aftertaste, rancid odor and flavor had developed in WPI-5 and CAS-5. The intensity of these sensory descriptors increased with storage time for all samples with fish oil, and after 20 weeks of storage all fish oil containing samples were dominated by these characteristics. However, the intensity of these descriptors did not develop equally fast in all the samples. Thus, after 15 weeks, CAS had a PC1 score which was as high as that of the CAS sample that have been stored for 20 weeks. In contrast, none of the other fish oil enriched cream cheeses obtained as high PC1 scores as the CAS samples, even after 20 weeks. This indicates that CAS samples became more rancid than any of the other samples. For the cream cheeses with fish oil-in-water emulsions MPL20, had a lower PC1 score than both WPI and CAS from week 5 and throughout the storage period. Hence, MPL20 developed less bitter flavor and rancid flavor and odor than the other two.

The second principal component mainly explained the variation in the texture and appearance samples. A positive PC2 score, which was seen for most of the samples, was correlated to fatty, firm and pasty texture. In contrast a negative PC2 score was correlated to spreadability, melt down, homogeneous and grainy appearance. Only the reference cream cheese had a negative PC2 score at all sampling points after 5 weeks of storage, whereas the fish oil enriched samples maintained a positive PC2 score throughout storage. Hence, the texture for the reference cream cheese changed from fatty, firm and pasty to a higher degree of homogeneous, grainy, better spreadability and faster melt down but also a more grainy appearance during storage. Apparently, the same changes were not observed in fish oil enriched samples.

4. Discussion

Overall, the addition of 1.3% (w/w) fish oil to cream cheese increased lipid oxidation during storage as compared to the reference cheese with no fish oil added. In accordance with the study by Kolanowski and Weißrodt (2007) [4], we observed a decrease in the sensory quality after five weeks of storage. Specifically the six volatiles butanal, 2-ethylfurane, *t*-2-butenal, *t*-2-hexenal, *t*-2-heptenal and *t*,*t*-2,4-heptadienal were observed to increase significantly during storage in the samples with fish oil. Hence, some of these volatiles may have contributed to the development of bitter and rancid off-flavors in the cream cheeses.

In contrast to previous studies where the addition of neat fish oil has been compared to the addition of only one type of delivery emulsion prepared with denatured whey protein in one study or a milk protein complex (casein and whey protein) in another study [2,3], this study investigated three types of delivery emulsions. Hence, despite the unacceptable sensory perception of the fish oil enriched cream cheeses in the later part of the storage period, some interesting observations were obtained on the differences between cheeses. First of all, results showed that the effect of using a delivery emulsion is not straight forward, as the type of delivery emulsion used clearly affected the stability of cream cheeses. Hence, cream cheeses with neat oil or MPL20 emulsion added were found to be similarly oxidized, but less oxidized than cream cheeses with CAS or WPI emulsions added.

The lack of protecting effect of caseinate was surprising, as casein in a previous study carried out by our research group has been shown to provide the best protection against oxidation on lipid oxidation in simple 70% fish oil-in-water emulsions (prepared almost similarly to the delivery emulsions added to the cream cheeses but with a 10 mM sodium acetate imidazole buffer instead of water as the aqueous phase) [6]. In addition, the difference between 70% fish oil-in-water emulsions with sodium caseinate and whey protein isolate were increased when additional iron was added, confirming a metal chelating effect of casein [6,16]. In the simple 70% fish oil-in-water emulsion the antioxidative effect of casein present in the aqueous phase was suggested to be responsible for the good oxidative stability, as also suggested in other studies [17,18]. However, this protective effect of casein in the aqueous phase seems to be lacking in the cream cheeses. Hence, metal ions may be chelated by casein surrounding the oil droplets instead whereby metal ions are coming into closer proximity of the oil, than otherwise expected.

With regard to the whey proteins, Let *et al.* (2007) [2] observed that the oxidative stability of fish oil enriched milk could be increased by adding fish oil as an emulsion after homogenization, and with denatured whey protein as emulsifier. The authors suggested that the advantage of using a delivery emulsion in this system, in contrast to neat oil, was due to a better protection of the fish oil droplets when emulsified by denatured whey protein (in the delivery emulsion) than when emulsified by the protein material present in the milk during homogenization (when neat oil was added). In contrast to milk, the cream cheese undergoes homogenization after addition of fish oil both when it is added as neat oil, and when added as an emulsion, and it is therefore not known exactly which proteins are responsible for emulsifying the fish oil in the cream cheese with the WPI delivery emulsion. However, the protection against oxidation is lower than the protection provided by the protein material present in the cream cheese that emulsifies the fish oil, when it is added as neat oil.

Whereas fish oil enriched milk was shown to be more stable when the oil was added as an emulsion, results from volatiles and sensory data showed that in dressing and yoghurt it was preferable to add the fish oil as neat oil [2]. This finding was explained by the increase in temperature during emulsion production, which led to some oxidation in the emulsion before addition to the food product. This was not compensated for by a better oxidation protection by the emulsion after addition to dressing and yoghurt whereas the opposite was the case for milk. Both milk and cream cheeses are heated during production, but as results on the method of fish oil addition are contradictory for these two food systems, the effect of heating is not unambiguous. In fish oil enriched energy bars, the use of a delivery emulsion prepared with sodium caseinate was observed to increase the oxidative stability of the bars in comparison to the addition of neat fish oil [19]. The increased oxidative stability of energy bars with emulsions added were explained by the ability of caseinate to provide a protective layer around the oil droplets to increase the distance between pro-oxidants in the surrounding matrix. However, a full understanding of why delivery emulsions are successful in some food applications and not in others is still lacking. More knowledge is needed in order to understand the physical structure of the emulsions after addition as well as to understand the interactions between the emulsifier in the delivery emulsion and other ingredients in the food product.

Interestingly, differences in the microstructure of the cream cheeses were also observed between cheeses with fish oil delivery emulsions (WPI, CAS and MPL20) and cheeses without/with neat oil added (REF and FO). These differences may be ascribed to excess protein present in the aqueous phase

of the 70% fish oil-in-water delivery emulsions. Hence, when added to the cream cheese, the emulsifier present in the aqueous phase of the delivery emulsion may emulsify the milk fat present in the cream cheese, whereby fewer unprotected milk fat droplets are observed in the micrographs (Figure 1). The emulsification of milk fat by excess casein in the aqueous phase, might therefore also partly explain the lack of protective effect of casein mentioned above.

Differences in the microstructure among cream cheeses with different delivery emulsions was also observed, as even less lipid was visible in the cream cheese with the MPL20 delivery emulsion added than when the other two emulsions (Em_WPI and Em_CAS) were used. The improved incorporation of lipids in the protein structure of the cream cheese with the MPL20 emulsion could help to explain the better oxidative stability of this cream cheese compared to the other two cream cheeses with delivery emulsions added. However, it cannot be the sole explanation as the cream cheese with neat fish oil, where the fish oil is the least incorporated in the protein structure, oxidizes similarly.

More studies are needed, in order to fully understand lipid oxidation in a complex matrix as the cream cheese.

5. Conclusions and Perspectives

Overall, the approach of using delivery emulsions for fish oil addition to cream cheese did not improve the oxidative stability of the product during storage, compared to the addition of neat fish oil. Interestingly, the microstructure of the cream cheeses changed substantially when fish oil was added through delivery emulsions. Furthermore, it was observed that the choice of emulsifier for preparing delivery emulsions significantly affects the oxidative stability of the fish oil enriched food product.

To be able to add fish oil to this type of product in the future, and to be able to store the product for more than five weeks, a combination of different approaches for protecting the oxidatively labile fish oil, could be an advantage. This could be the use of a delivery emulsion prepared with a combination of proteins and phospholipids, an optimized protection of the oil during production of the delivery emulsion and the cream cheeses, such as an oxygen-free environment, or the inclusion of antioxidants. Hence, more studies are needed to explore which approach or combination of approaches has the best effect in order to prepare fish oil enriched cream cheese of a good quality with regard to lipid oxidation and the development of off-flavors.

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Supplementary Material

Five freeze-fracture cryo-scanning electron micrographs are included as supplementary material. One of each cream cheese.

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