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DTU Food
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Technical University of Denmark

National Food Institute, Division of Seafood Research

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PREFACE

The present thesis entitled ”The influence of ingredients or lipophilized antioxidants on the oxidative stability of fish oil enriched food systems” concludes my PhD project carried out at the National Food Institute (DTU Food), Technical University of Denmark (former National Institute of Aquatic Resources (DTU Aqua) and Danish Institute for Fisheries Research). My PhD was under supervision of Senior Researcher Charlotte Jacobsen (main supervisor), Section leader for Aquatic lipids and Oxidation and Senior Researcher Nina Skall Nielsen (co-supervisor).

The project began August 1, 2006 and continued until March 31, 2010, but was interrupted by a 2 months leave due to another project assignment. Additional 5 months extension was due to project delay from a collaboration partner at Aarhus University (Denmark). In these 5 months I worked on other project assignments.

This PhD project constituted part of the project “Nutritious and tasty n-3 PUFA rich food products for a slim and healthy population (Nu3Health)”, which was financed by the Danish Council for Strategic Research (Programme committee for food, nutrition and health) and the Directorate for Food, Fisheries and Agri Business. A travel grant awarded from LMC (Centre for Advanced Food Studies) supported my research stay abroad at the Department of Food Science, University of Massachusetts, Amherst, USA. The duration of the research stay was 3 months (September 10, 2008 until December 7, 2008). An Young scientist award supported participation in the 25th Nordic Lipid Symposium 2009 (Elsinore, Denmark) and an European student travel award supported participation in the 2010 American Oil Chemists’ Society (AOCS) annual meeting and expo in Phoenix, Arizona, USA.

March 31, 2010
Kgs. Lyngby, Denmark

Ann-Dorit Moltke Sørensen

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SUMMARY

The relatively low intake of fish and the health beneficial n-3 polyunsaturated fatty acids (PUFAs) in the Western countries has created a growing market for n-3 PUFA enriched food products e.g. products enriched with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil. EPA and DHA are more susceptible to lipid oxidation than PUFAs from vegetable oils due to their highly polyunsaturated nature and it is therefore necessary to develop methods to protect these PUFAs.

The main objective of this PhD work was to expand current knowledge on oxidative stabilization of fish oil enriched food systems. This included an evaluation of the effects of adding modified antioxidants to the food products and the influence of ingredients on the oxidative stability of emulsions and food products.

Vegetables in fish oil enriched mayonnaise based shrimp and tuna salads did not improve the overall oxidative stability compared to pure fish oil enriched mayonnaise. The findings indicated that asparagus in shrimp salad had an antioxidative effect, but this effect was neutralized by the prooxidative effect caused by the shrimps. In tuna salads, the high level of volatiles was suggested to be due to the natural volatiles from the different vegetables. Replacing egg yolk with a milk protein based emulsifier did not reduce the lipid oxidation in fish oil enriched light mayonnaise and this finding was explained by the initial low quality of the milk protein based emulsifier.

The results obtained from fish oil enriched mayonnaise salads and light mayonnaise showed that fish oil can be added without further addition of antioxidants as long as the storage temperature and time were controlled. Thus, fish oil enrichment of mayonnaise based shrimp and tuna salads results in acceptable products when the storage at 2°C is maximum 6 weeks. It was possible to enrich the light mayonnaise with 4% fish oil when stored at 2°C for 113 days as concluded from the concentration of secondary oxidation products.

Studies on protection of o/w and w/o emulsions by ascorbic acid derivatives with different polarities indicated that ascorbic acid acted as a better antioxidant in w/o emulsion than o/w emulsion. This was explained by the higher concentration of ascorbic acid in the aqueous phase in a w/o emulsion and thereby closer location of ascorbic acid to the oil-water interface.

Evaluation of the effect of lipophilization of selected phenolic antioxidants revealed that in general, the lipophilized dihydrocaffeic acid and rutin increased the oxidative stability of o/w emulsions and fish oil enriched milk compared with their parent hydrophilic compound. The results supported a cut-off effect in relation to the acyl chain length of the alcohol esterified to the phenolic compound. Octyl dihydrocaffeate (C8 acyl chain) was a stronger antioxidant than oleyl dihydrocaffeate (C18 acyl chain) and rutin laurate (C12 acyl chain) was a stronger antioxidant than rutin palmitate (C16 acyl chain). Interestingly, it seemed that the cut-off effect was not only specific for the individual lipophilized phenolic compounds, but that it also depended on the emulsion system, i.e. the optimal chain length seems to vary between different emulsion systems.

Based on the results it was concluded that addition of dry spices (oregano, thyme or rosemary) or spice extracts (rosemary or green tea) improved the oxidative stability of fish oil enriched tuna salads and milk. However, these spices and extracts are not appropriate due to their contribution to an off-flavor of the spices or green tea in the final products. A tasteless oregano-based extract did not improve the oxidative stability of fish oil enriched tuna salads. It was concluded that the changes in the type of oil used for the mayonnaise from soy oil to rapeseed oil reduced the lipid oxidation and thereby resulted in the unclear effect of origanox.

Overall, the PhD work has contributed with interesting findings regarding oxidative stabilization of fish oil enriched food systems. Amongst the alternatives investigated, the most promising way to reduce lipid oxidation of fish oil enriched food emulsions seems to be addition of lipophilized antioxidants and spice extracts, as long as they do not add additional flavor to the product.

SAMMENFATNING

Et lavt indtag af de gavnlige n-3 langkædede polyumættede fedtsyrer (PUFA) i de vestlige lande, har skabt et marked for n-3 berigede fødevarer dvs. produkter beriget med eicosapentaensyre (EPA) og docosahexaensyre (DHA) fra eksempelvis fiskeolie. EPA og DHA er mere følsomme overfor oxidation sammenlignet med PUFA fra vegetabilske olier på grund af deres højere grad af umættethed. Det er derfor nødvendigt at finde metoder til at beskytte disse fedtsyrer mod oxidation, således at der ikke udvikles en fiskesmag i produkter beriget med fiskeolie.

Det overordnede formål med dette Ph.d. projekt var at forøge den viden der findes omkring oxidativ stabilitet af fiskeolieberigede fødevare produkter. Dette inkluderede undersøgelse af ingrediensernes indflydelse på den oxidative stabilitet af fødevareprodukter samt effekten af at tilsætte modificerede antioxidanter til simple model emulsioner såvel som mere komplekse emulsionssystemer som eksempelvis mælk beriget med fiskeolie.

De undersøgte grøntsager var ikke i stand til at forbedre den overordnede oxidative stabilitet af hverken reje- eller tunsalat sammenlignet med den fiskeolieberigede mayonnaise, som salaterne er fremstillet med. På trods af at asparges i rejesalat havde en antioxidative effekt, blev denne effekt udlignet af den prooxidative effekt af rejerne. I tunsalat forekom der en høj koncentration af flygtige oxidationsprodukter. Dette skyldes sandsynligvis den naturlige aroma profil af de forskellige grøntsager i tunsalaten. Derudover viste resultaterne at udskiftning af æggeblomme med en mælkeprotein-baseret emulgator i light mayonnaise reducerede den oxidative stabilitet. Dette modsagde den opstillede hypotese, men skyldes formentlig den lave kvalitet af den mælkeprotein-baserede emulgator. Resultaterne opnået med fiskeolieberigede mayonnaisesalater og light mayonnaise indikerer at det er muligt at berige disse produkter med fiskeolie uden tilsætning af antioxidanter. Berigelse af reje- og tunsalat er mulig hvis salaterne opbevares ved 2°C og en holdbarhed på maximum 6 uger. Indenfor de første 6 uger var der ikke signifikant forskel mellem salaterne med og uden fiskeolie mht. lipid oxidation. Berigelse af light mayonnaise (40% fedt) med 4% fiskeolie er mulig såfremt holdbarheden er maksimalt 113 dage ved en opbevaringstemperatur på 2°C. Denne light mayonnaise var ikke signifikant forskellig fra light mayonnaise uden fiskeolie målt på flygtige oxidationsprodukter.

Undersøgelser af oxidationsbeskyttelse af olie-i-vand (o/v) og vand-i-olie (v/o) emulsioner med askorbinsyrederivater med forskellig polaritet viste, at askorbinsyre (hydrofil) var en bedre

antioxidant i v/o end i o/v emulsioner. Dette blev tilskrevet af den højere koncentration af askorbinsyre i den vandige fase af v/o emulsionen og derved en tætter placering på grænsefladen mellem olie og vand.

Effekten af udvalgte antioxidant derivater viste at lipofilisering af dihydrokaffesyre og rutin øgede den oxidative stabilitet af o/v emulsioner og fiskeolieberiget mælk sammenlignet med dihydrokaffesyre og rutin. De opnåede resulter kan forklares med en så kaldt "cut-off" effekt i relation til kædelængden af alkoholen esterifiseret til phenolerne. Octyl dihydrokaffeester (C8 kæde) resulterede i en mere oxidativ stabil o/v emulsion og mælk sammenlignet med oleyl dihydrokaffeester (C18 kæde), og rutin laurat (C12 kæde) resulterede i en mere oxidativ stabil mælk sammenlignet med rutin palmitat (C16 kæde). Det er derudover særdeles interessant, at se at den så kaldte "cut-off" effekt ikke kun er specifik for den enkelte phenol, men ligeledes er afhængig af emulsionssystemet dvs. den optimale kædelængde for den forestrede fedtsyre varier fra emulsionssystem til emulsionssystem.

Det blev konkluderet at tørrede krydderier som oregano, timian og rosmarin kunne forbedre den oxidative stabilitet af fiskeolieberiget tunsalat, og at ekstrakter af rosemarin eller grøn te kunne forbedre den oxidative stabilitet af fiskeolieberiget mælk. Disse krydderier og ekstrakter er dog ikke anvendelige i tunsalat og mælk, da de gav afsmag i produkterne. Derfor blev et smagsløst oregano ekstrakt, origanox, evalueret i fiskeolieberiget tunsalat, men der blev ikke fundet nogen klar antioxidativ effekt af ekstraktet. Dette blev forklaret med, at denne tunsalat var baseret på rapsolie i modsætning til tunsalat med krydderier, som var baseret på sojaolie. Fedtsyrer i rapsolie er mere mættede og derfor mere oxidationsstabile, hvilket menes at have resulteret i den uklare effekt af origanox.

Dette PhD arbejde har bidraget til interessante resultater omkring stabilisering af fiskeolieberigede fødevareemulsioner. Den mest lovende metode til at øge holdbarheden af fiskeolieberiegede fødevareemulsioner på antages at være ved tilsætningen af lipofiliserede antioxidanter eller krydderiekstrakter.

ABBREVATIONS

A·	Antioxidant radical
AA	Antioxidant dimer
AH	Antioxidant
ANOVA	Analysis of variance
BHT	Butylated hydroquinone
CLA	Conjugated linoleic acid
CMC	Critical micelle concentration
DHA	Docosahexaenoic acid
DS	Dry spice
EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
GC-MS	Gas chromatograph – mass spectrometer
L·	Lipid alkyl radical
LA	Lipid conjugate with antioxidant
LC	Long chain
LDL	Low density lipoprotein
LH	Lipid
LL	Lipid dimer
LO·	Lipid alkoxyl radical
LOA	Lipid alkoxyl conjugate with antioxidant
LOH	Lipid alcohol
LOL	Lipid dimer
LOO·	Lipid peroxyl radical
LOOA	Lipid peroxyl conjugate with antioxidant
LOOH	Lipid hydroperoxide
LOOL	Lipid dimer
M	Metal
o/w	Oil-in-water
PCA	Principal component análisis
PGPR	Polyglycerol polyricinoleat
PLSR	Partial least squares regression
PUFA	Polyunsaturated fatty acid
PV	Peroxide value
SE	Spice extract
w/o	Water-in-oil

LIST OF PUBLICATIONS

Papers included in the thesis:

I **Sørensen, A.-D.M.**, Nielsen, N.S. & Jacobsen, C. Oxidative stability of fish oil enriched mayonnaise based salads. *European Journal of Lipid Science and Technology,* 2010, 112, 476-487.

II **Sørensen, A.-D.M.**, Nielsen, N.S., Hyldig, G. & Jacobsen, C. The influence of emulsifier type on lipid oxidation in fish-oil-enriched light mayonnaise. *European Journal of Lipid Science and Technology,* 2010, 112, 1012-1023.

III **Sørensen, A.-D.M.**, Nielsen, N.S., Decker, E.A., Let, M.B, Xu, X. & Jacobsen, C. The efficacy of compounds with different polarities as antioxidant in emulsions with fish oil. *Journal of American Oil Chemists Society, Accepted 2010 (DOI: 10.1007/s11746-010-1696-5).*

IV **Sørensen, A.-D.M.**, Nielsen, N.S., Yang, Z., Xu, X. & Jacobsen, C. The effect of lipophilization of dihydrocaffeic acid on its antioxidative properties in fish oil enriched emulsion. *European Journal of Lipid Science and Technology, Submitted 2011.*

V **Sørensen, A.-D.M.**, de Diego, S., Petersen, L.K., Nielsen, N.S., Lue, B.-M., Yang, Z., Xu, X. & Jacobsen, C. The antioxidative effect of lipophilized dihydrocaffeic acid and rutin in fish oil enriched milk. *In preparation*.

Other publications

Sørensen, A.-D.M., Haahr, A.-M., Becker, E.M., Skibsted, L.H., Bergenståhl, B., Nilsson, L. & Jacobsen, C. Interactions between iron, phenolic compounds, emulsifiers, and pH in omega-3 enriched oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 2008, 56, 1740-1750.

Jacobsen, C., Let, M.B., **Sørensen, A.-D.M**., Horn, A.F., Timm-Heinrich, M., Nielsen, N.S. Applications of natural antioxidants in omega-3 enriched foods. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, 2008, 7, 3288-3295.

Sørensen, A.-D.M., Xu, X., Zhang, L., Kristensen, J.B. & Jacobsen, C. Human milk fat substitute from butter fat: Production by enzyme interestification and evaluation of oxidative stability. *Journal of the American Oil Chemists' society,* 2010, 87, 85-194*.*

Jacobsen, C., Horn, A.F., **Sørensen, A.-D.M.**, Farvin, K.H.S. & Nielsen, N.S. Antioxidative strategies to minimize oxidation in formulated food systems containing fish oils and omega 3 fatty acids in "Antioxidants and Functional Components in Aquatic Foods", Ed. Kristinsson, H.G., Wiley Blackwell, *Accepted 2009.*

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CHAPTER 1 INTRODUCTION

Long chain (LC) n-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to have several beneficial health effects [Riediger et al., 2009;Psota et al., 2006].

The first indications of the beneficial health effects of n-3 PUFAs were observed back in the 1970s when Bang and Dyerberg (1972) discovered that high intake of marine lipids resulted in a lower incidence of ischaemic heart disease in Greenlandic Eskimos. Since then, interest in the health benefits of marine n-3 PUFAs has grown into a large research area. Some of the proposed beneficial health effects include reduced morbidity and mortality from cardiovascular diseases, reduced incidence of cancer and treatment of inflammatory disease. Furthermore, DHA is especially important for brain development in infants. The beneficial health effects of n-3 PUFA have recently been reviewed by Ruxton et al. (2007).

Even though EPA and DHA can be synthesized in the human body, these PUFAs are needed in the diet since in most cases they are not synthesized up to a sufficient level [Ruxton et al., 2007]. The major source of EPA and DHA is fish and fish products. However, several investigations indicate that the average intake of fish and fish products in Western countries is too low to ensure adequate amounts of EPA and DHA in the human body. Different recommendations exist regarding EPA and DHA intake. The Danish Veterinary and Food Administration recommend eating fatty fish at least twice a week [FVST, 2006], whereas other countries such as Canada, Sweden, the United Kingdom and Japan recommend a daily intake of EPA and DHA at a level around 0.3-0.5 g [Kris-Etherton et al., 2002]. In 2009 the European Food Safety Authority (EFSA) recommended a daily reference intake value for EPA and DHA at 0.25 g [Heller, 2009].

1.1 EPA AND DHA ENRICHED FOOD PRODUCTS

Due to the low intake of fish in Western countries, there is a growing market for n-3 PUFA enriched food products. However, incorporation of fish oil into foods might

introduce stability problems due to oxidation of EPA and DHA, which will lead to an unpleasant fishy off-flavor [Jacobsen et al., 2000;Jacobsen et al., 2001b;Let et al., 2003;Let et al., 2007a]. EPA and DHA are more susceptible to lipid oxidation than PUFAs from vegetable oils due to their highly polyunsaturated nature. However, a way to protect these PUFAs could be by the addition of antioxidants.

Several food products are either oil-in-water (o/w) emulsions e.g. milk, mayonnaise and dressing or water-in-oil (w/o) emulsions e.g. margarine and are complex matrices where several factors in these food emulsions are able to affect initiation and the progress of lipid oxidation. An emulsion can be defined as two immiscible liquids, where one of the liquids is dispersed as small spherical droplets in the other. Thus, an o/w emulsion occurs when oil droplets are dispersed in water, whereas a w/o emulsion occurs when water droplets are dispersed in oil [McClements, 2005].

Complexity of real food emulsions makes it very difficult to predict the specific oxidation mechanisms and efficacy of antioxidants. The majority of studies on improvement of oxidative stability by antioxidant addition have been carried out in bulk oils or model emulsion systems due to the more simple interactions in these systems compared with real food products. However, the National Food Institute, Division of Seafood Research (formerly the National Institute of Aquatic Resources and Danish Institute for Fisheries Research) has contributed with evaluating antioxidant efficacy in several studies in real food products enriched with fish oil e.g. mayonnaise, milk, drinking yoghurt, dressing, energy bar and fish paté [Jacobsen et al., 2008]. Results have shown that antioxidants with metal chelating properties are important for the oxidative stability of fish oil enriched mayonnaise [Jacobsen et al., 2001a], whereas metal induced oxidation seemed less important in fish oil enriched milk [Let et al., 2005b].

1.2 OBJECTIVES

The main objective of this PhD work was to expand the knowledge about oxidative stabilization of fish oil enriched food systems, including the impact of antioxidant polarity on efficacy in different food systems. Mechanisms of lipid oxidation and antioxidative protection of n-3 PUFAs in complex food systems are naturally related. Thus, knowledge

on the oxidation mechanisms in each particular food product intended for fish oil enrichment may lead to a possible way to overcome the oxidation problem. Adding antioxidants with appropriate properties for the respective food product may be strategies to overcome lipid oxidation, or by substituting prooxidative ingredients with other ingredients.

In order to achieve the main objective, the work in this PhD project was divided in two parts: A) Influence of ingredients and oil content on the oxidative stability and B) Efficacy of natural and modified antioxidants. In our research group, at lot of research has been performed on fish oil enriched mayonnaise and milk. Therefore, these food systems were chosen as model food systems in this PhD work. In addition, a simple emulsion system was included to be able to conclude on the efficacy of antioxidants without impact from other ingredients.

The hypotheses behind the work in each of the two areas are described in detail below.

Influence of ingredients and oil content on the oxidative stability

Vegetables contain a variety of phenolic compounds which are known to have antioxidant activity. Until now, the influence of such ingredients on lipid oxidation in complex food systems has not received much attention. Some antioxidative effects have been observed previously for vegetables such as sweet pepper and asparagus. This PhD work aimed to test the following hypothesis:

- *Vegetables present in fish oil enriched mayonnaise based salads can improve the oxidative stability compared to that of fish oil enriched mayonnaise*

Based on results obtained from the fish oil enriched mayonnaise based salads and the general consumer demand for healthier products, a new approach to improve the oxidative stability of fish oil enriched light mayonnaises was changing the emulsifier. The most common emulsifier used for mayonnaise production is egg yolk. However, egg yolk has a high content of iron and has been shown to be an important factor in the low

oxidative stability of fish oil enriched mayonnaise. A second aim was therefore to test the following hypothesis:

- *Milk proteins improve the oxidative stability of fish oil enriched light mayonnaise compared to an egg yolk emulsifier*

In addition, oxidative stability is expected to decrease with increasing fish oil concentration based on the high susceptibility of EPA and DHA to oxidation. Therefore this study aimed to investigate the hypothesis:

- *Higher fish oil concentration will increase the lipid oxidation in light mayonnaise*

Efficacy of antioxidants

The second part of the PhD work had three objectives, namely to evaluate 1) the efficacy of antioxidants in o/w versus w/o emulsions, 2) the efficacy of modified antioxidants, and 3) the efficacy of spices or spice extracts as antioxidants in emulsified food.

The first two objectives were based on the polar paradox phenomenon which states that lipophilic antioxidants are more efficient in emulsions compared to hydrophilic antioxidants. Based on the polar paradox, the study aimed to investigate the following hypotheses:

- *Hydrophilic antioxidants will offer better protection in w/o than o/w emulsions*
- *Lipophilization of a phenolic compound will change its partitioning into different phases in o/w emulsions compared with its parent compound*
- *Lipophilization of a phenolic compound will increase its antioxidant efficacy in o/w emulsions compared with its parent compound*
- *Lipophilization of phenolics can increase their antioxidant efficacy in a complex food matrix (fish oil enriched milk)*

Dry spices, spice and green tea extracts contain a variety of phenolic compounds with antioxidative properties. Therefore, this PhD work also aimed to test the following hypotheses:

- *Rosemary or green tea extracts can improve the oxidative stability of fish oil enriched milk*
- *Dry spices can improve the oxidative stability of fish oil enriched tuna salads*
- *Oregano extracts can improve the oxidative stability of fish oil enriched tuna salads*

1.3 THESIS OUTLINE

The present thesis is divided into three main parts. The first part consists of the introduction and background theory (Chapter 1-4). In Chapter 1, the beneficial health effects of marine EPA and DHA, enrichment of food products with those fatty acids and the objectives of the present PhD work are presented. Chapters 2-4 summarize current knowledge regarding lipid oxidation, mechanisms of antioxidant action and their efficacy due to location. Moreover, factors influencing lipid oxidation are briefly described according to the model and food system under evaluation. The second part of the thesis describes the experimental approach, results and discussion (Chapter 5-6). Conclusions based on the results obtained are drawn in Chapter 7, where future perspectives also are discussed. Finally, the third part contains the four submitted scientific papers, one paper in preparation (preliminary paper draft) together with other appendices with results not included in any of the already written scientific papers.

CHAPTER 2 LIPID OXIDATION AND SENSORY IMPACT

Oxidation of lipids in different food products leads to unpleasant odors and flavors that make the food products unacceptable or unpalatable. Lipid oxidation can be categorized as autoxidation, photooxidation or enzymatic oxidation. This chapter will deal exclusively with aspects of autoxidation. Hence, the contents of this chapter give a brief overview about the formation of different primary and secondary volatile oxidation products during the autoxidation of unsaturated lipids, primarily EPA and DHA.

2.1 AUTOXIDATION

Autoxidation occurs when unsaturated fatty acids are exposed to oxygen and is an autocatalytic chain reaction. Autoxidation of unsaturated fatty acids involves 3 steps; initiation, propagation and termination [Frankel, 2005]. The main initiation and propagation reaction steps, plus further decomposition of lipid hydroperoxides to volatiles are illustrated in Figure 1.

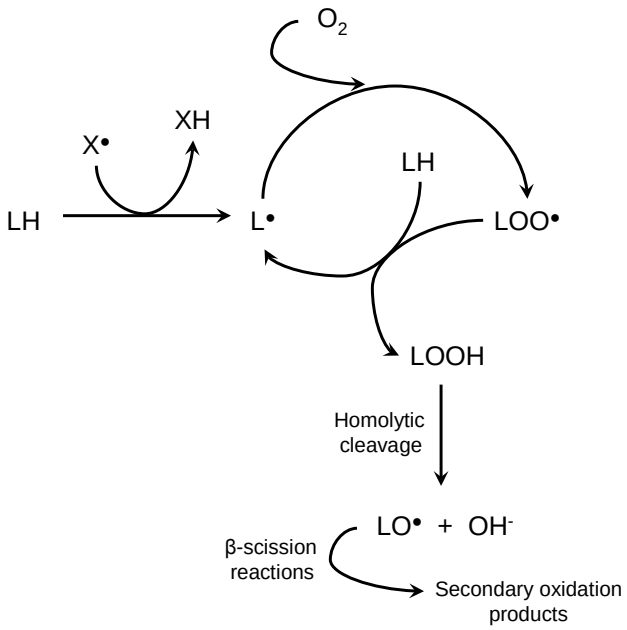

Figure 1 Oxidation mechanism of polyunsaturated lipids. LH: Unsaturated lipid; $X^{\bullet}$: Radical initiator; $L^{\bullet}$: Lipid alkyl radical; $LO^{\bullet}$: Lipid alkoxyl radical; $LOO^{\bullet}$: Lipid peroxyl radical; LOOH: Lipid hydroperoxide.

The reactions in Figure 1, Scheme 1 and Scheme 2 illustrate lipid oxidation of free fatty acids. However, oil contains primarily triacylglycerides. Oxidation of triacylglycerides results in the formation of monohydroperoxides located on the sn-1,3 and 2-positions of the glycerol moiety. Further oxidation of triacylglycerides results in a mixture of 1,2 and 1,3- bis hydroperoxides and tris hydroperoxides. These hydroperoxides on the fatty acids attached to the glycerol moiety are able to participate in autoxidation reactions in the same way as free fatty acid [Frankel, 2005].

Initiation

Since the direct reaction between oxygen in its ground state (triplet state) and lipids (ground state: singlet state) is spin forbidden, initiators are necessary to commence lipid oxidation. Initiation occurs when a hydrogen atom is extracted from an unsaturated fatty acid (LH), leading to the formation of a lipid alkyl radical ($L^{\bullet}$, Figure 1). This process can be triggered by a variety of different initiators such as transition metals, heat, already existing radicals (as indicated in Figure 1) and hydroperoxides. Since food always contain traces of metals, an important initiation mechanism in food emulsions is that trace metals catalyze the decomposition of hydroperoxides already present in the products [Frankel, 2005;Min et al., 1989], Scheme 1.

Trace metal as initiator

$$M^{(n+1)+} + LOOH \rightarrow M^{n+} + LOO^{\bullet} + H^{+} \qquad \textit{Reaction 1}$$

$$M^{n+} + LOOH \rightarrow M^{(n+1)+} + LO^{\bullet} + OH^{-} \qquad \textit{Reaction 2}$$

Scheme 1 Overview of the initiation reactions of lipid oxidation by trace metals. M: Metal ion; LOOH: Lipid hydroperoxides; $LOO^{\bullet}$: Lipid peroxyl radical; $LO^{\bullet}$: Lipid alkoxyl radical. Adapted from Frankel, 2005.

Propagation

Once alkyl radicals are formed, they react immediately with oxygen to form peroxyl radicals ($LOO^{\bullet}$). These radicals can then extract hydrogen atoms from other unsaturated lipids, thereby forming new alkyl radicals ($L^{\bullet}$) and propagating the chain reaction according to Figure 1 [Frankel, 2005;Chaiyasit et al., 2007].

The molecular structures of the lipids (mainly number and localization of double bonds) are important for the rate of lipid oxidation. The rate of autoxidation of PUFA increases approximately 2-fold for each bis-allylic methylene group present [Frankel, 2005;Kulås et al., 2003]. Moreover, the mixture of hydroperoxides becomes more complex with the number of bis-allylic sites [Frankel, 2005]. EPA and DHA have 4 and 5 bis-allylic methylene groups, respectively. Accordingly, EPA (20:5) and DHA (22:6) can oxidize to form mixtures of eight and ten positional hydroperoxide isomers, respectively. Oxidation of EPA can lead to the formation of 5-, 8-, 9-, 11-, 12-, 14-, 15- and 18-hydroperoxides, whereas oxidation of DHA gives 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17- and 20-hydroperoxides. These hydroperoxides can have either a *cis,trans* or a *trans,trans* configuration; thus, the lipid autoxidation of EPA and DHA is expected to yield 16 and 20 mono-hydroperoxides, respectively [Frankel, 2005;Kulås et al., 2003]. Lipid hydroperoxides are relatively unstable molecules and can decompose into a large variety of secondary oxidation products. This decomposition is described further in section 2.1.2.

Termination

The propagation of lipid oxidation is terminated when lipid radicals react with each other to form non-radical products. The termination reactions are listed in Scheme 2.

Termination

$L^{\bullet} + L^{\bullet} \rightarrow L\text{-}L$	Non radical products	*Reaction 3*
$LOO^{\bullet} + L^{\bullet} \rightarrow LOOL$		*Reaction 4*
$LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O_2$		*Reaction 5*
$LO^{\bullet} + LO^{\bullet} \rightarrow LOOL$		*Reaction 6*
$LO^{\bullet} + L^{\bullet} \rightarrow LOL$		*Reaction 7*

Scheme 2 Overview of reactions involved in the termination of autoxidation. $L^{\bullet}$: Lipid alkyl radical; $LOO^{\bullet}$: Lipid peroxyl radical; $LO^{\bullet}$: Lipid alkoxyl radical; LL, LOL and LOOL: Lipid dimers. Adapted from Frankel, 2005 and Yanishlieva and Marinova, 2001.

The propagation chain reactions can also be terminated by reactions between antioxidants and lipid radicals, whereby antioxidant radicals are formed. These radicals

are more stable and generally do not contribute further towards the propagation of autoxidation [Frankel, 2005;McClements and Decker, 2000]. Antioxidants and their mechanisms of action are described in Chapter 3.

2.1.1 FORMATION OF SECONDARY VOLATILE OXIDATION PRODUCTS

Unstable lipid hydroperoxides are transformed to alkoxyl radicals through homolytic cleavage. Alkoxyl radicals are intermediary radicals which lead to the formation of secondary oxidation products by β-scission. This results in the formation of both saturated and unsaturated aldehydes, alkyl- and alkenyl radicals. Thus, decomposition yields a complex mixture of secondary oxidation products (Figure 2) that is dependent on the structure of the original hydroperoxide molecules. Moreover, both alkyl- and alkenyl radicals can react further to form other compounds like alcohols, hydrocarbons, alkenes or 1-enols. The latter ones are normally transformed to saturated aldehydes. Furthermore, alkyl radicals can react with oxygen, which will result in the formation of shorter chain aldehydes [Frankel, 2005;Grosch, 1987;Frankel, 1982].

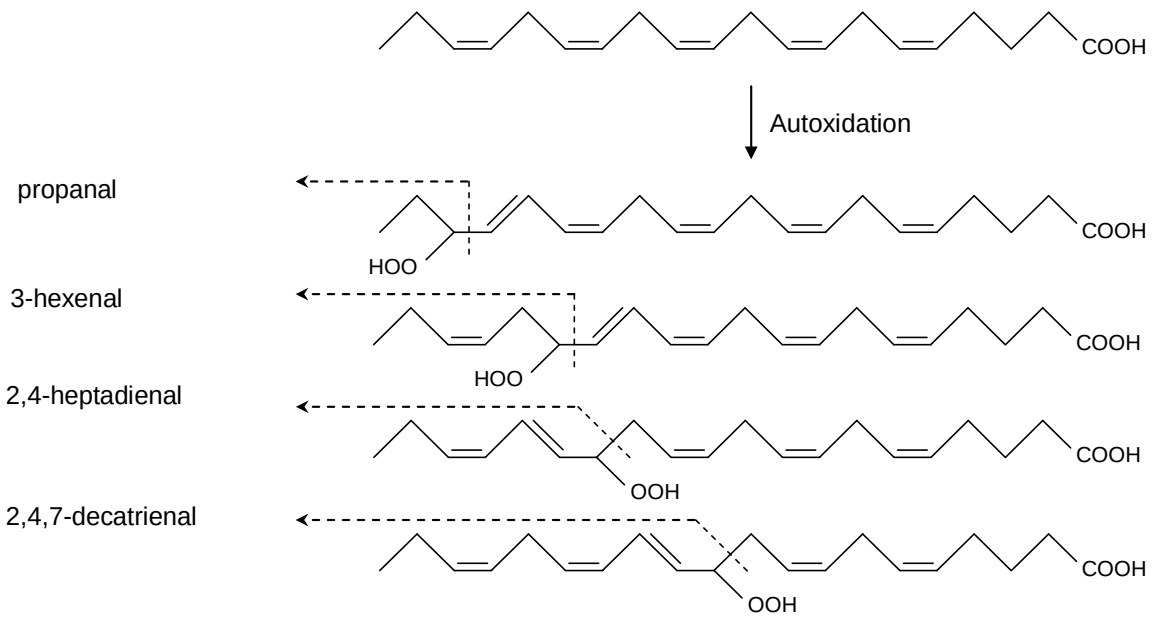

Figure 2 Autoxidation sites associated with decomposition of hydroperoxides from EPA to give different aldehydes. Adapted from Kulås et al., 2003.

2.1.2 SENSORY IMPACT OF OXIDATION PRODUCTS

Whereas lipid hydroperoxides are tasteless and odorless compounds, secondary volatile lipid oxidation products are responsible for changes in the flavor properties of food systems caused by oxidation. In Table 1 some of the volatiles originating from degradation of n-3 PUFA are shown and their associated odor listed.

Table 1 Volatiles originating from degradation of n-3 PUFA and their odors.

Types of oxidation product	Oxidation product	Odor description
Alkanals	Propanal	Sharp, irritating
Alkenals	*c*-3-Hexenal	Fresh green leaves
	c-4-Heptenal	Creamy, stale, burnt, fishy
Alkadienals	*t,t*-2,4-Heptadienal	Fatty, oily
	t,c-2,4-Heptadienal	Frying odour, fishy
	t,t-2,6-Nonadienal	Cucumber, tallowy
	t,c-2,6-Nonadienal	Fresh cucumbers, green, melon
Alkatrienals	*t,t,c*-2,4,7-Decatrienal	Burnt / fishy
	t,c,c-2,4,7-Decatrienal	Burnt / fishy
Vinyl ketones	1-Penten-3-one	Sharp, fishy
	1,*c*-5-Octadien-3-one	Metallic

The information is adapted from Frankel, 2005; Venkateshwarlu et al., 2004; Hartvigsen et al., 2000; Karahadian and Lindsay, 1989; Grosch, 1987.

The sensory impact of a given compound is governed by its chemical structure and odour thresholds, which differs according to the type of oxidation product under consideration. In order to have an impact on the flavor profile of the food product, volatiles need to be released from the food matrix. Thus, the overall flavor of the food products are governed by the way aroma compounds are distributed between the product and the gas phase [Savary et al., 2006]. Odor thresholds are higher in oil than in water due to the high affinity of the volatile compounds towards lipids [Druaux and Voilley, 1997]. In general, aldehydes and ketones have low threshold values and thus can have a high flavor impact [Frankel, 2005].

Several studies have been carried out in order to evaluate the flavor retention by different food ingredients. Results have shown that both polysaccharides and proteins are capable of increasing the retention of volatile compounds in food [Savary et al., 2006;Philippe et al., 2003;Rega et al., 2002;Fabre et al., 2002]. The retention of volatiles by polysaccharides has been suggested to be due to hindered molecule migration through its three-dimensional structure i.e. entrapping [Rega et al., 2002;Savary et al., 2006]. Retention of volatiles by proteins is suggested to be caused by direct molecular interactions [Fabre et al., 2002]. Moreover, different volatiles have been shown to interact differently with proteins and polysaccharides. Thus, the retention of volatiles in food products depends on both the composition of the product and on the flavor compounds present [Fabre et al., 2002;Jacobsen et al., 1999c;Savary et al., 2006].

Information about volatile profiles and their sensory impact in fish oil enriched emulsions and real food products, is very limited. However, the relationship between volatiles and sensory impact has been investigated in fish oil enriched mayonnaise and milk [Hartvigsen et al., 2000;Venkateshwarlu et al., 2004b;Venkateshwarlu et al., 2004a]. In fish oil enriched mayonnaise, 27 volatiles were suggested to contribute to the developed unpleasant fishy and rancid off-flavours. Two of these volatiles, *c*-4-heptenal and *t,c*-2,4-heptadeinal, were detected with distinct fishy odours. A combination of volatiles responsible for odours like cucumber and fishy have been suggested to be responsible for the unpleasant fishy off-flavour detected in fish oil enriched mayonnaise, whereas volatiles responsible for notes such as pungent, green, glue, burnt, fatty and deep-fried resulted in rancid off-flavour [Hartvigsen et al., 2000]. Similarly, several compounds contribute to the fishy and metallic off-flavour in fish oil enriched milk. The potent volatiles identified in this type of food product were 1-penten-3-one, *c*-4-heptenal, 1-octen-3-one, 1,5-octadien-3-one, *t,t*-2,4-heptadienal and *t,c*-2,6-nonadienal. Further experiments showed that the maximum intensity of fishy flavour was observed when the concentration of *c*-4-heptenal was at its maximum and *t,t*-2,4-heptadienal at its lowest concentration, or vice versa. Moreover, the metallic odour in milk is an interaction between *c*-4-heptenal and 1-penten-3-one in which *c*-4-heptenal enhances the effect of 1-penten-3-one [Venkateshwarlu et al., 2004b;Venkateshwarlu et al., 2004a].

CHAPTER 3 ANTIOXIDATIVE PROTECTION OF EMULSIONS

3.1 ANTIOXIDANTS: MECHANISM OF ACTION

Compounds that delay the onset, prevent or just slow down the rate of autoxidation are characterized as antioxidants. They are classified as primary or secondary antioxidants based on their mechanism of action. However, some antioxidants have more than one mechanism of action and are referred to as multiple-function antioxidants.

Retarding initiation and propagation

$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$ *Reaction 8*

$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}$ *Reaction 9*

$L^{\bullet} + AH \rightarrow LH + A^{\bullet}$ *Reaction 10*

Termination reactions

$LOO^{\bullet} + A^{\bullet} \rightarrow LOOA$ *Reaction 11*

$LO^{\bullet} + A^{\bullet} \rightarrow LOA$ *Reaction 12*

$L^{\bullet} + A^{\bullet} \rightarrow LA$ *Reaction 13*

$A^{\bullet} + A^{\bullet} \rightarrow AA$ *Reaction 14*

Scheme 3 Overview of antioxidant reactions with lipid radicals and other antioxidant radicals. AH: Antioxidant, LH: Lipid, LOOH: Lipid hydroperoxide, LOH: Lipid alcohol, $LOO^{\bullet}$: Lipid peroxyl radical, $LO^{\bullet}$: Lipid alkoxyl radical, $L^{\bullet}$: Lipid alkyl radical, $A^{\bullet}$: Antioxidant radical, LOOA, LOA and LA: Lipid conjugates with antioxidant and AA: Antioxidant dimer. Adapted from Chaiyasit et al., 2007.

Primary antioxidants, also referred to as chain-breaking antioxidants, are characterized by their ability to react directly with free radicals, such as lipid, alkoxyl and peroxyl radicals, and convert them to more stable, nonradical products. In the initiation and propagation steps antioxidants react with lipid, alkoxyl and peroxyl radicals as outlined in Scheme 3, reactions 8-10. In particular, the formation of stable lipid alcohols and the trapping of alkoxyl radicals with antioxidants are important reactions (reactions 9 and 12). These reactions inhibit further decomposition into aldehydes and other volatile oxidation products and the antioxidant radicals ($A^{\cdot}$) formed are less reactive than lipid, alkoxyl and peroxyl radicals. In addition, antioxidant radicals can terminate autoxidation by reacting

directly with the different lipid radicals (Scheme 3, reactions 11-14). In contrast, secondary antioxidants can inhibit lipid oxidation by several different mechanisms including chelation of transition metals, oxygen scavenging, synergism between antioxidants and singlet oxygen quenching. In all storage experiments within the present work, samples were stored in the dark. Since oxygen quenching antioxidants prevent photooxidation, this antioxidant mechanism will not be described further.

The natural presence of transition metals, such as copper and iron, in foods is an important factor in the promotion of lipid oxidation. Hence, metal chelating compounds are important antioxidants. Chelators are able to bind metals and thereby inactivate or reduce the activity of metal ions. Common metal chelating compounds are EDTA and some phenolics depending on their structure and number of hydroxyl groups on the benzene ring. Compounds that are able to change the redox properties of metals, e.g. ascorbic acid, may exert a prooxidative effect by reducing metal to its more reactive form. Also, different antioxidants present in a food system can reinforce each other by co-operative effects called synergism. One synergistic effect is the regeneration of one antioxidant by another e.g. regeneration of tocopherol by ascorbic acid. Regenerated antioxidants are capable of replenishing hydrogen to newly formed primary antioxidant radicals, and thereby renewing the ability of the primary antioxidants to react with lipid derived radicals. Oxygen scavengers are capable of trapping oxygen and thereby act as reductants [Frankel, 2005;McClements and Decker, 2000;Reische et al., 1998;Chaiyasit et al., 2007].

3.2 ANTIOXIDANT EFFICIENCY

The activity of antioxidant compounds in model or food systems depends not only on the structure and reactivity of the compound, but also on other factors such as their physical location, presence of other compounds e.g. proteins, emulsifiers, pro- and antioxidants and environmental conditions e.g. pH [McClements and Decker, 2000;Coupland and McClements, 1996;Rice-Evans et al., 1997;Sørensen et al., 2008]. In the present work, one aim was to lipophilize hydrophilic antioxidants in order to change their location (i.e. partitioning into different phases) and improve their antioxidant activity. Therefore, the

relationship between partitioning properties of antioxidants and their activity is summarized in the following section.

3.2.1 PARTITIONING

In emulsions, lipid oxidation is suggested to be initiated at the interface between the oil phase and the aqueous phase or air, and continued in the oil phase. In a multiphase system, such as an emulsion, antioxidants may partition into at least 3 different phases: the aqueous phase, the oil phase and the interface between oil and water. Figure 3 shows the primary location of the antioxidants according to their polarity in emulsions.

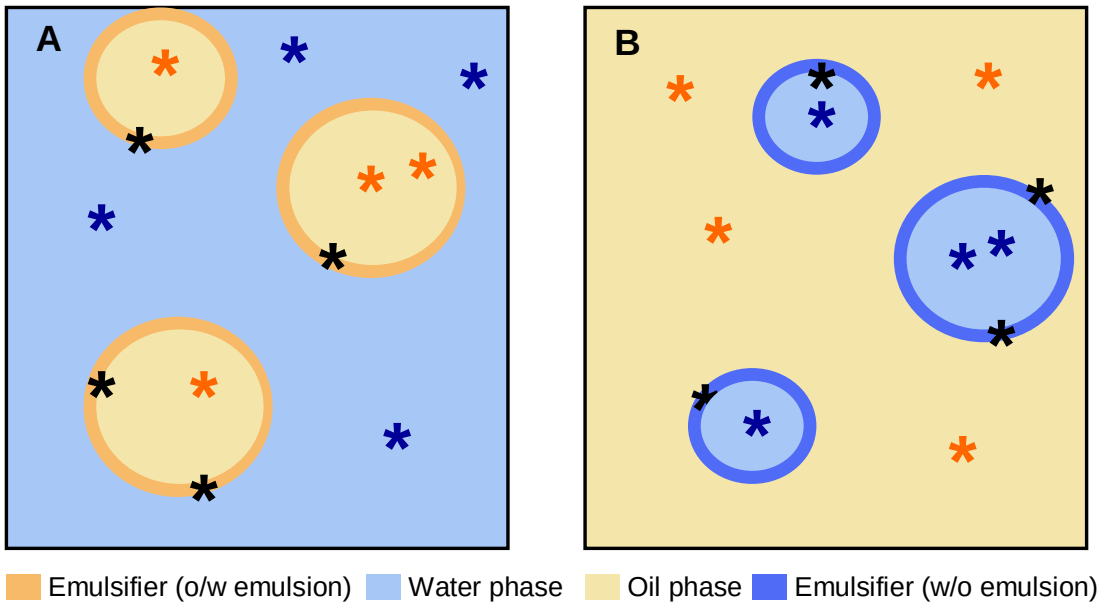

Figure 3. Simplified structure of A) o/w and B) w/o emulsions. The location of antioxidants in these emulsions is indicated according to their polarity. * Amphiphilic antioxidants, * Lipophilic antioxidants and * Hydrophilic antioxidants.

The partitioning of antioxidants into different phases in an emulsion is influenced by the hydrophilic vs. lipophilic character of the specific antioxidant, and by interactions with other components present in the emulsion e.g. emulsifier. In addition to the interfacial layer and the oil and water phases, a fourth phase may exist due to the formation of micelles by the emulsifier in the aqueous phase. This will occur if the emulsifier

concentration in the aqueous phase is above the critical micelle concentration (CMC) for the emulsifier [Schwarz et al., 1996;Huang et al., 1996c].

Antioxidant efficiency has been measured and compared in different systems e.g. bulk oil, o/w emulsions and w/o emulsions [Frankel et al., 1994;Frankel et al., 1996;Huang et al., 1996c;Huang et al., 1996a;Schwarz et al., 2000]. However, the efficiency of the antioxidants was dependent on the systems in which their effect was evaluated. Generally, the polarity of antioxidants in bulk oil and emulsions is considered to be decisive for their efficiency. This phenomenon is known as the polar paradox and states that hydrophilic antioxidants are more efficient in bulk oils than lipophilic antioxidants. In contrast, lipophilic antioxidants generally function better than hydrophilic antioxidants in emulsions [Porter, 1993]. This phenomenon is explained by differences in the affinity of the antioxidants towards the different phases. Hence, lipophilic antioxidants are more efficient in emulsions due to their ability to orient themselves closer to the oil-water interface, whereas hydrophilic antioxidants are diluted in the water phase [Frankel et al., 1994].

The polar paradox hypothesis has been supported by several studies [Frankel et al., 1994;Huang et al., 1996c;Huang et al., 1996b;Frankel et al., 1996]. According to the polar paradox hypothesis, esterification of hydrophilic antioxidants with fatty alcohols or acids to obtain more lipophilic antioxidants will improve their efficacy due to increased partitioning at the interface compared to the parent compound. However, recent studies have reported results that contradict the polar paradox hypothesis [Laguerre et al., 2009;Lue et al., 2010b;Sørensen et al., 2008;Yuji et al., 2007;Nenadis et al., 2003b]. Furthermore, Schwarz et al. (2000) studied the effect of different antioxidants in bulk oil with and without emulsifier, as well as w/o- and o/w emulsions. Based on findings they suggested that the observed differences in antioxidant activity for the same emulsion type may be influenced by specific interactions with the emulsifier dominating the oil-water interface. Sørensen et al. (2008) also found that the efficacy of antioxidants was influenced by the choice of emulsifier. These findings have led to the conclusion that the polar paradox hypothesis is too simple to explain antioxidant efficiency observed in emulsions [Laguerre et al., 2009;Sørensen et al., 2008].

Laguerre et al. (2009) observed that antioxidant capacity of chlorogenate increased as the esterified alkyl chain was lengthened with a maximum for the lauryl chain. Further increase of the alkyl chain length resulted in a drastic decrease in antioxidant capacity. Analysis of partitioning showed that lauryl chlorogenate was present at the lowest concentration in the aqueous phase compared to other lipophilized chlorogenic acid structures examined (alkyl chain from C1 to C20). Thus, results obtained with lipophilized chlorogenic acid with an alkyl chain from 1 to 12 carbon atoms confirmed the polar paradox hypothesis. However, the polar paradox was contradicted when the antioxidant hydrophobicity was increased by esterification with alcohols with a chain length of more than 12 carbon atoms long. This was suggested to be due to accumulation of the antioxidants in the aqueous phase possibly by a micellization process. These results allowed a new hypothesis to be suggested; a cut-off effect with special emphasis on the micellization process. The cut-off effect is related to the alkyl chain length esterified to the phenolic compound and is illustrated in Figure 4 [Laguerre et al., 2009]. When, the hydrophobicity of the lipophilized compound increases to above a certain level, the lipophilized compound was suggested to form micelles in the aqueous phase. This is supported by the fact that CMC decreases with increased alkyl chain length. Due to lower CMC of more hydrophobic antioxidants, these antioxidants may not be available at the interface [Yuji et al., 2007].

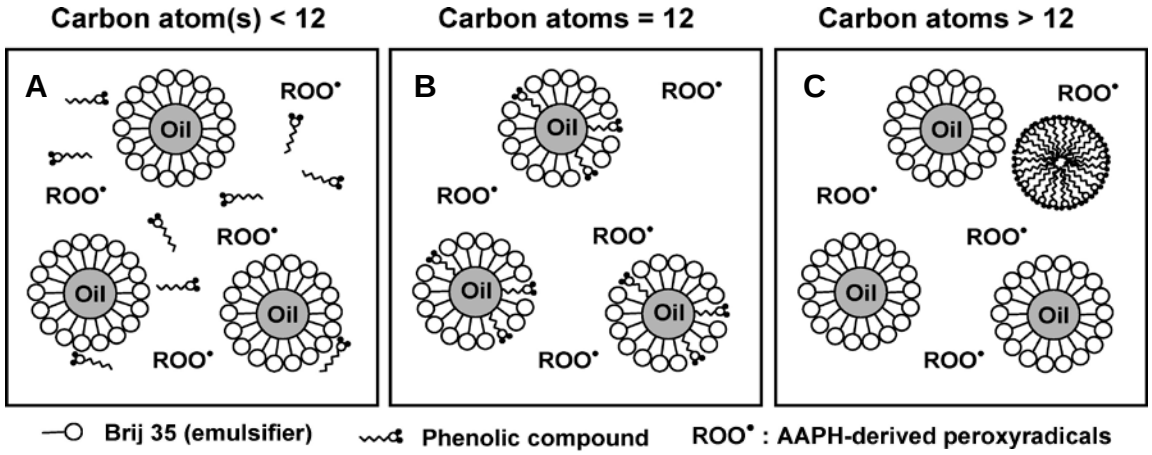

Figure 4 Distribution of lipophilized antioxidant in an emulsified system due to the cut-off effect hypothesized by Laguerre et al. (2009). A) Lipophilized chlorogenic acid, Alkyl chain < 12 carbon atoms, B) Lipophilized chlorogenic acid, Alkyl chain = 12 carbon atoms and C) Lipophilized chlorogenic acid, Alkyl chain > 12 carbon atoms. Adapted from Laguerre et al. (2009).

3.3 DESCRIPTION OF SELECTED ANTIOXIDANTS

The following sections describe antioxidant mechanisms and reported findings for the antioxidants employed in this PhD work. EDTA and BHT are well-known and very effective synthetic antioxidants; in the present work, they were used as control antioxidants with respect to metal chelation and chain breaking properties, respectively. The antioxidants employed are divided in 3 subgroups based on their origin: natural antioxidants, spice and spice extracts and modified antioxidants (lipophilized compounds).

3.3.1 NATURAL ANTIOXIDANTS

Chemical structures of the natural antioxidants employed, including ascorbic acid, conjugated linoleic acid (CLA), caffeic acid, dihydrocaffeic acid and rutin are shown in Figure 5.

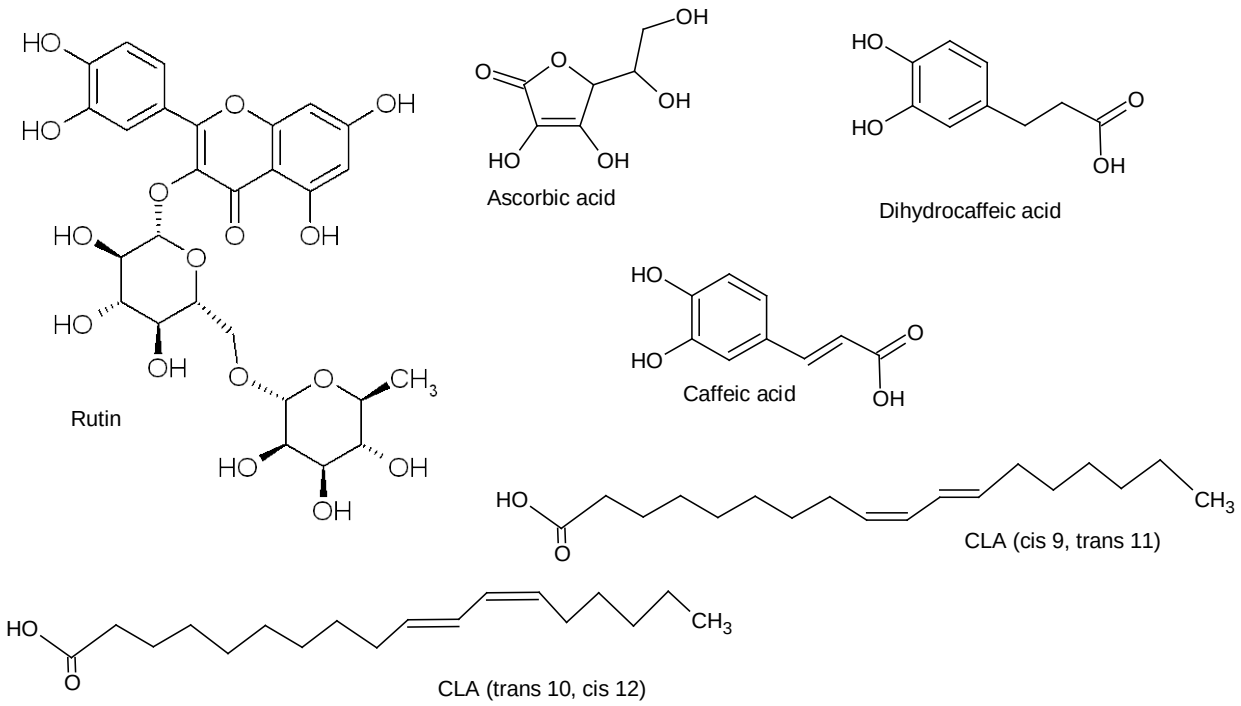

Figure 5. Chemical structures of natural antioxidants used in the present thesis. CLA: Conjugated linoleic acid. Only two CLA isomer is illustrated among several CLA isomers, however, the used CLA mixture consists primarily of these two CLA isomers.

Ascorbic acid

Ascorbic acid is a natural occurring hydrophilic compound and the source of ascorbic acid is fresh fruit and vegetables. Citrus fruits are an especially good source of ascorbic acid. Ascorbic acid is known to have multifunctional effects as an antioxidant due to its properties as a radical scavenger of hydrophilic radicals, oxygen scavenger, and its ability to donate an electron to reactive free radicals (reducing properties) [Niki, 1991]. Ascorbic acid can also act in synergy with tocopherol by regenerating tocopheroxyl radicals [Niki, 1991;Mäkinen et al., 2001]. However, the reducing properties of ascorbic acid can result in prooxidative effects if Fe^{3+} is reduced to Fe^{2+}, especially in the presence of already existing lipid hydroperoxides. For example, Jacobsen et al. (1999) have shown that the reducing properties of ascorbic acid resulted in prooxidative effects in fish oil enriched mayonnaise.

Conjugated linoleic acid

Conjugated linoleic acids (CLA) are a group of octadecadienoic acids containing conjugated double bonds, which are double bonds separated by one single bond. Molecular structures of two of the isomers are shown in Figure 5. Several CLA isomers exist, however, the illustrated ones are the primarily isomers in the CLA mixture used in the experimental work. CLA is naturally present in food from animal sources, such as dairy products and meat [MacDonald, 2000].

CLA exerted antioxidative effects in studies by Yu (2001) and Ip et al. (1991) as a radical scavenger and by suppressing formation of peroxides from unsaturated fatty acids in a model system. However, these findings conflict with results obtained by Chen et al. (1997), van den Berg et al. (1995) and Zhang and Chen (1997), who showed either no or prooxidative effects of CLA. No effect of CLA was observed in metal or radical initiated oxidation, whereas both a radical scavenging assay and oxygen consumption test indicated the prooxidative effect of CLA.

Caffeic acid and Dihydrocaffeic acid

Caffeic acid (3-(3,4-dihydroxyphenyl)-2-propenoic acid) is a natural antioxidant. King and Young (1999) have given an overview of the different sources of caffeic acid, showing

that it occurs in various agricultural products such as coffee beans, potatoes and vegetables. Caffeic acid belongs to the group of hydrocinnamic acids which are characterized by a 3-phenyl-2-propenoic acid structure with hydrogen, methoxyl or dihydroxyl groups in positions 3 and 5 and a hydroxyl group in position 4 [Belitz et al., 2004]. In addition, dihydrocaffeic acid (3-(3,4-dihydroxyphenyl)-2-propionic acid) is a degradation product of caffeic acid and is formed following reduction by intestinal bacteria [Peppercorn and Goldman, 1971]. The molecular structures of caffeic and dihydrocaffeic acids are shown in Figure 5. The 2,3- double bond in caffeic acid is the only structural difference between caffeic acid and dihydrocaffeic acid.

The hydroxyl groups of phenolic molecules are capable of scavenging free radicals and chelating metal ions. To chelate metal ions, two neighbouring OH groups (*o*-diphenol) are required in the structure [Andjelkovic et al., 2006;Chimi et al., 1991]. The ability to react with radicals correlates with the number of phenolic rings and catecholic structures (*o*-diphenol) in the molecule [Capitani et al., 2009]. The molecular structure of both caffeic acid and dihydrocaffeic acid includes a catechol moiety.

Caffeic acid-iron complex formation has been observed by UV spectrometry with the catechol moiety participating in this complex formation. Moreover, caffeic acid was able to form nanoparticles in the presence of iron [Sørensen et al., 2008]. The formation of a phenol-iron complex due to the catechol moiety has also been reported by Andjelković et al. (2006). However, in the presence of iron, the reduction of iron by the phenolic acid was more significant than chelation of iron by *o*-diphenols (phenols with catechol moiety) from olive oils [Keceli and Gordon, 2002]. In addition, some reports have shown that caffeic acid was capable of reducing Fe^{3+} to Fe^{2+}, thereby propagating lipid oxidation [Gülçin, 2006;Deiana et al., 1995]. In 10% o/w emulsions stabilized with citrem or tween, caffeic acid at a concentration of 100 µM acted as a prooxidant irrespective of emulsifier and iron addition. Moreover, caffeic acid was more prooxidative at pH 3 than pH 6 due to its reduction of iron at pH 3 [Sørensen et al., 2008].

Radical scavenging activity has been reported for both caffeic acid and dihydrocaffeic acid. Results obtained by Nenadis et al. (2003) showed that dihydrocaffeic acid exerted

a stronger radical scavenging effect than caffeic acid. Others have reported similar radical scavenging effects for caffeic acid and dihydrocaffeic acid in the concentration range from 3-350 µM, concluding that the double bond in the alkyl chain attached to the benzene ring had no influence on radical scavenging activity [Moon and Terao, 1998]. In addition, dihydrocaffeic acid lipophilized with mono- and diglycerol resulted in reduced radical scavenging activity compared to its phenol, which could indicate that the carboxylic acid group (–COOH) in dihydrocaffeic acid contributed to the donation of H-atoms [Sabally et al., 2007].

Caffeic and dihydrocaffeic acids have been evaluated in different model systems for their antioxidative activity. In the copper induced oxidation of low density lipoprotein (LDL), the induction period was longer following incubation of LDL with caffeic acid than with dihydrocaffeic acid. In contrast, dihydrocaffeic acid increased the oxidative stability of lard compared to caffeic acid. It was assumed that the double bond in the side chain attached to the benzene ring of caffeic acid affected the efficacy of caffeic acid as an antioxidant [Moon and Terao, 1998]. In 10% (o/w) emulsions caffeic acid at concentrations of 150, 300 and 1000 µM were more efficient in reducing lipid oxidation than dihydrocaffeic acid [Nenadis et al., 2003a].

Caffeic acid and dihydrocaffeic acid are hydrophilic compounds. Several studies have investigated the partitioning of caffeic acid and dihydrocaffeic acid in oil-water, water-octanol and emulsion systems [Nenadis et al., 2003b;Pekkarinen et al., 1999;Nenadis et al., 2003a]. In equal volumes of aqueous phase and octanol phase, the amount of caffeic and dihydrocaffeic acids in the aqueous phase was very high due to a low partitioning coefficient ($P_{octanol\ /\ aqueous}$): 0.18 (~ 10.5 % in octanol) and 0.16, respectively [Nenadis et al., 2003b;Nenadis et al., 2003a]. Pekkarinen et al. (1999) showed that only 1.2% caffeic acid was present in the lipid phase in an oil-water (1:9) system, whereas a high proportion of caffeic acid was found in the lipid phase (52.3%) with Tween 20 as an emulsifier. The results indicate that the emulsifier Tween 20 exhibited a high solubilisation capacity for caffeic acid and was thereby able to change the location of caffeic acid [Pekkarinen et al., 1999].

Rutin

Rutin (quercetin-3-rhamnosyl glucoside) belongs to the group referred to as flavonoids. Flavonoids are composed of two phenolic groups joined by a pyran (oxygen-containing) ring structure. A large number of flavonoid structures exist, differing in the number and position of hydroxyl (-OH) and methoxy ($-OCH_3$) groups on the skeleton. Some flavonoids such as rutin are also glycosylated [D'Archivio et al., 2007]. Rutin is a secondary plant metabolite found in a range of different foods e.g. apples, plums asparagus and citrus fruits [Lue et al., 2010a]. The structure of rutin is illustrated in Figure 5 [van Acker et al., 1996;Rice-Evans and Miller, 1996].

Since rutin has the same catechol moiety in its structure as caffeic and dihydrocaffeic acids, it may act by the same antioxidant mechanisms as these phenolics. This has been confirmed by the finding that rutin showed free radical scavenging activity [Afanas'en et al., 1989;Rice-Evans and Miller, 1996]. Additionally, van Acker et al. (1996) reported that a catechol moiety was required for good radical scavenging activity. At a concentration of 0.05 mg/mL (or 82 µM), rutin exhibited a strong DPPH radical scavenging activity of 90.4%. This was higher than for BHT (synthetic antioxidant, 227 µM) at 58.8% but slightly lower than ascorbic acid (284 µM) at 92.8% [Yang et al., 2008]. Several studies have shown the iron chelating properties of rutin [Sørensen et al., 2008;Afanas'en et al., 1989]; however, rutin was not as efficient as the synthetic metal chelating compound EDTA [van Acker et al., 1996]. The metal chelating effect of rutin was concentration dependent in the range of 100-900 µM, based on the iron initiated lipid oxidation of lecithin liposomes. Furthermore, the metal chelating activity of rutin increased with increasing concentration of rutin [Afanas'en et al., 1989].
In 10% o/w emulsions stabilized by citrem, rutin (100 µM) was prooxidative at pH 3 and had no effect at pH 6 based on the formation of secondary volatile oxidation products [Sørensen et al., 2008]. However, at a rutin concentration of 200 µM in a 5% o/w emulsion stabilized by citrem at pH 7, an antioxidative effect was observed [Lue et al., 2010b]. Due to the sugar moiety in the rutin molecule, rutin is not as hydrophilic as caffeic acid. In an oil-aqueous mixture (1:20) 72.5% of the rutin was found in the aqueous phase [Lue et al., 2010b].

3.3.2 DRY SPICES, EXTRACTS OF SPICES AND GREEN TEA

In the present thesis, thyme was employed as a dry spice, rosemary and oregano both as dry spices and as extracts and green tea as an extract. The main advantage of using extracts compared to dry spices is that the flavour can be removed from the extracts and a product containing a higher concentration of active compounds can be obtained.

Oregano, Thyme and Rosemary

Several experiments have indicated that different spices such as oregano, thyme and rosemary possess antioxidative activity [Tsimidou et al., 1995;Zheng and Wang, 2001;Bhale et al., 2007;Jimenez-Alvarez et al., 2008;Amarowicz et al., 2009]. Each herb contains different phenolic compounds and each compound possesses different antioxidant properties. A positive linear correlation (R = 0.986) between the phenolic content and the antioxidative capacity of the above mentioned spices has been observed [Zheng and Wang, 2001]. This indicates that the phenolic compounds contribute significantly to the antioxidant activity of herbs.

In mackerel oil, the addition of dry oregano (5,000 and 10,000 mg/kg) was shown to clearly inhibit the rapid lipid oxidation of the oil. Moreover, the effect of oregano was comparable to that of synthetic antioxidants BHA and TBHQ at a concentration of 200 mg/kg each. At a concentration of 5,000 mg/kg, rosemary exhibited an efficiency similar to oregano in retarding lipid oxidation, as indicated by PV measurements [Tsimidou et al., 1995]. The antioxidative effect of a methanolic extract of rosemary was greater than that of a corresponding oregano extract in retarding lipid oxidation of LC PUFA in menhaden oil [Bhale et al., 2007]. Furthermore, the efficacy of extracts of rosemary has been shown to be influenced by the type of system tested: bulk oil vs. o/w emulsion. In the bulk oil, the rosemary extracts inhibited formation of conjugated dienes and volatiles, whereas in the emulsions they were inactive or prooxidative [Frankel et al., 1996].

Thyme was shown to have antioxidant activity in the DPPH assay [Kulisic et al., 2005]. The antioxidant activity of carvacrol, thymol and *p*-cymene 2,3-diol, which are phenolic compounds from thyme leaves, was evaluated in bulk oil by monitoring the development of peroxides during storage. The most efficient phenolic was *p*-cymene 2,3-diol. Findings

were partly explained by the presence of two hydroxyl groups in contrast to other phenols having only one phenolic hydroxyl group within their molecular structures. However, the *p*-cumene 2,3-diol was evaluated in a higher concentration than the other compounds which may also have influenced the results [Bitar et al., 2008].

Both thyme and oregano extracts (600 and 1,200 mg/kg) result in reduced formation of hydroperoxides, hexanal and pentanal in bulk oil and emulsions compared to controls without extract added. Addition of thyme extracts result in better oxidative stability than the addition of oregano extract [Abdalla and Roozen, 1999]. Butter with thyme extract (5,000 and 10,000 mg/kg) also showed better oxidative stability than when oregano was added to butter. However, in another study, oregano extracts was exerted slightly higher radical scavenging activity in the DPPH assay and an improved capacity to act as a hydroxyl radical scavenger compared to extract from thyme [Amarowicz et al., 2009].

Green tea

Components of green tea extracts include epigallocatechin, catechin, caffeine, epicatechin, epigallocatechin gallate and epicatechin gallate [Frankel et al., 1997]. The antioxidative activity of green tea has been shown to be system dependent like other natural antioxidants. Thus, both prooxidative and antioxidative properties of green tea extracts have been reported [Shin et al., 2007;Azam et al., 2004;Frankel et al., 1997]. Results obtained by Shin et al. (2007) indicated that extracts of green teas exerted prooxidative activity at lower concentrations and antioxidative activity at higher concentrations. Antioxidant mechanisms reported for green tea extracts were metal chelation (iron), complex formation with copper, reducing potential (copper) and scavenging of peroxyl and hydroxyl radicals [Shin et al., 2007;Azam et al., 2004].

Green tea was an antioxidant in bulk oil and lecithin liposomes, whereas green tea was a prooxidant in o/w emulsions. The improved antioxidant activity observed in lecithin liposomes compared to emulsions was explained by the greater affinity of polar tea catechin gallates for the polar surface of the lecithin bilayer, thereby giving better protection against lipid oxidation [Frankel et al., 1997].

3.3.3 MODIFIED ANTIOXIDANTS

Generally, the solubility of polar antioxidants (e.g. ascorbic acid or phenolic acids) is good in an aqueous phase. Thus, the hydrophilic character of these antioxidants may reduce their effectiveness in stabilizing emulsions against oxidative deterioration. Therefore, lipophilization of these compounds with molecules such as fatty acids or alcohols can be used to alter their localisation in emulsions and may increase their antioxidative efficacy in these systems. The modified antioxidants employed are shown in Figure 6 and Figure 7.

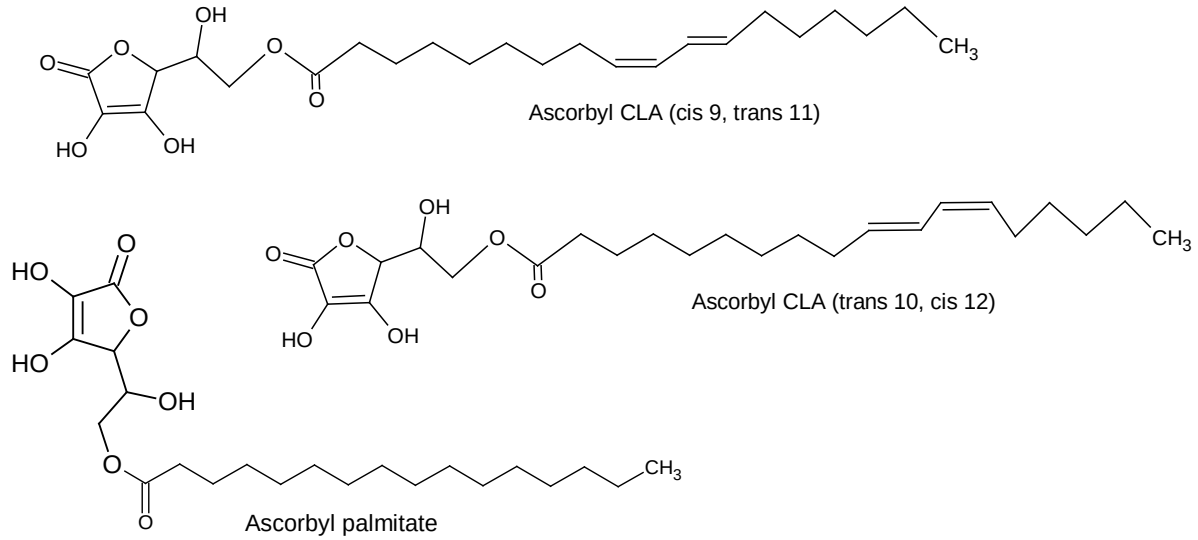

Figure 6 Chemical structures of ascorbyl palmitate and ascorbyl CLA, some of the modified antioxidants in the experimental work of the present PhD work. CLA: Conjugated linoleic acid.

Ascorbyl palmitate and Ascorbyl CLA

Ascorbyl palmitate (L-ascorbyl-6-palmitate) is a modified compound of ascorbic acid. Due to the molecular structure of ascorbyl palmitate (Figure 6), its antioxidative properties are most likely related to the ascorbyl group in this molecule. In contrast to ascorbic acid, ascorbic palmitate is an amphiphilic antioxidant. According to the polar paradox hypothesis, ascorbyl palmitate may thus be more efficient in an emulsion than ascorbic acid [Frankel et al., 1994;van Ruth et al., 1999].

In addition, other ascorbic acid esters have been evaluated for their antioxidative effects in different systems. Ascorbic acid esters exerted an increased protection against copper and radical initiated LDL oxidation compared to hydrophilic ascorbic acid at a concentration of 5 µM. The protection was greatest for the copper induced LDL oxidation assay and the efficacy of the esters were as follows: L-ascorbyl-6-laurate > L-ascorbyl-6-palmitate > L-ascorbyl-6-caprylate [Liu et al., 1998]. Moreover, L-ascorbyl-6-laurate, L-ascorbyl-6-myristate, L-ascorbyl-6-palmitate and L-ascorbyl-6-stearate (10,000 µM) showed significant antioxidative activity in a micellar substrate composed of linoleic acid. After 5 days, the degree of oxidation was 2-3 fold lower with the ester than without antioxidants [Stamatis et al., 1999].

Contrary to ascorbyl palmitate, the antioxidative effect of ascorbyl CLA (L-ascorbic-6-linoleate (conjugated linoleic acid)) is unknown. Molecular structure of ascorbyl CLA are shown in Figure 6. The lipophilization of ascorbic acid with CLA might result in a similar antioxidative activity compared with ascorbyl palmitate. However, its efficacy might depend on the partitioning of the antioxidant and interactions with other compounds in the particular system.

Lipophilized dihydrocaffeic acid and rutin

Generally, literature on antioxidant properties of lipophilized dihydrocaffeic acid and rutin is very limited. Octyl dihydrocaffeate and oleyl dihydrocaffeate are modified compounds of dihydrocaffeic acid esterified with either octanol or oleyl alcohol. The molecular structures of these compounds are illustrated in Figure 7. It may be suggested that these esters exhibit similar antioxidative properties as their parent compound, dihydrocaffeic acid, due to the presence of a catechol moiety.

A few studies have evaluated the radical scavenging activity of dihydrocaffeate esters by the DPPH assay. The esters evaluated were methyl-, ethyl- and propyl dihydrocaffeate at concentrations of 50-800 µM [Silva et al., 2000] and linoleyl dihydrocaffeate at a concentration of 20 µM [Sabally et al., 2005]. Radical scavenging efficacy of linoleyl dihydrocaffeate was comparable to that of α-tocopherol, exhibiting 12.9% and 16.1% respectively [Sabally et al., 2005]. Esterification of dihydrocaffeic acid with alkyl alcohol

resulted in a decrease in its radical scavenging activity. This was due to an occupied carboxyl group in dihydrocaffeic acid caused by esterification. The radical scavenging activities of dihydrocaffeate esters were independent of the alkyl chain length [Sabally et al., 2005;Silva et al., 2000].

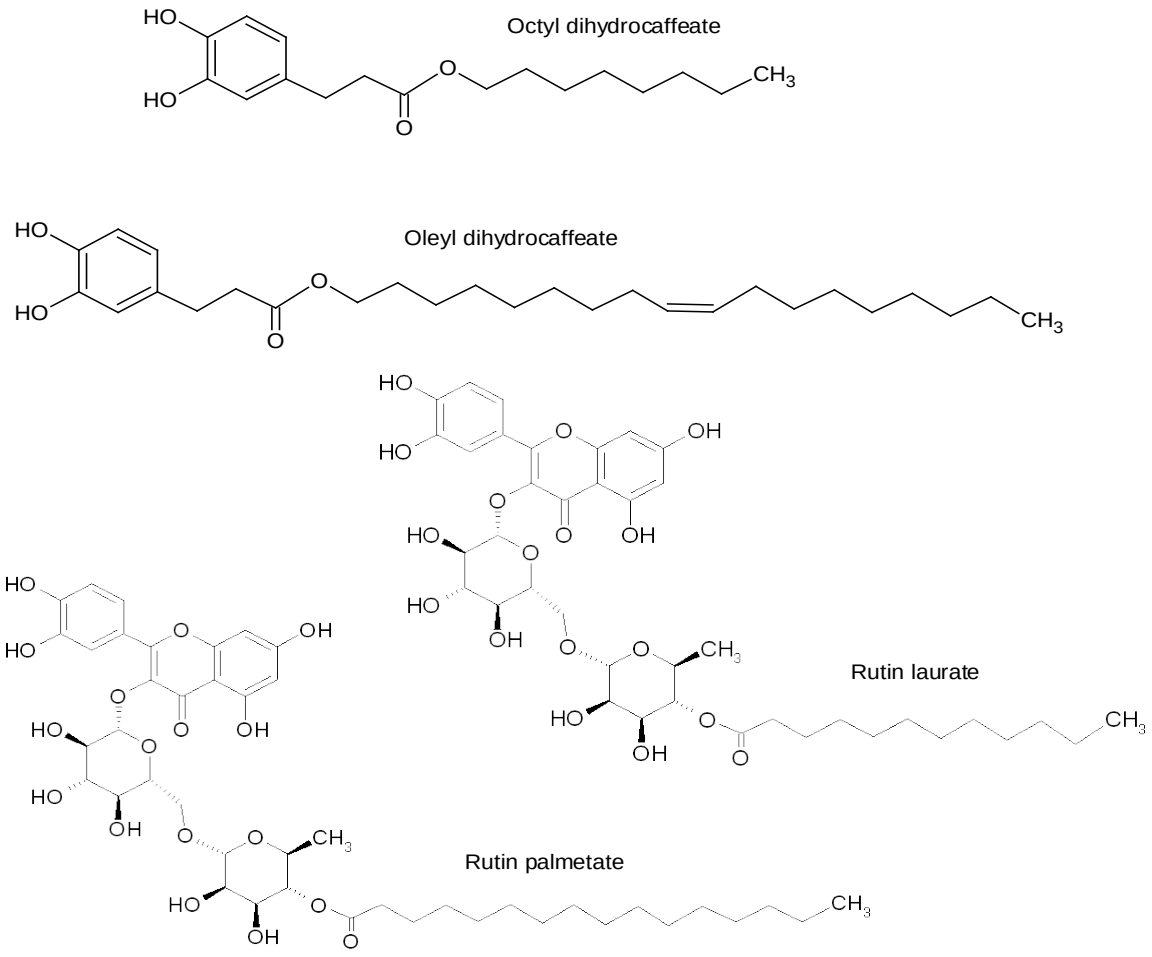

Figure 7 Illustration of the chemical structures of lipophilized dihydrocaffeic acid and rutin employed in the PhD work: octyl dihydrocaffeate, oleyl dihydrocaffeate, rutin laurate and rutin palmitate.

Rutin laurate and rutin palmitate are modified compounds of rutin, which have been esterified with either lauric or palmitic acids. The molecular structures of these compounds are illustrated in Figure 7.

In 5% w/o emulsions stabilized with citrem, both rutin laurate and rutin palmitate were found to effectively inhibit lipid oxidation. However, the esterification resulted in less effective antioxidants compared with rutin. A comparison of the effect of the rutin esters in an o/w emulsion and bulk oil revealed increased efficacy of the esters in emulsions compared with bulk oil. Thus, the findings supported the theory that lipophilic antioxidants have improved efficiency in emulsion systems [Lue et al., 2010b].

CHAPTER 4 FACTORS AFFECTING LIPID OXIDATION IN MODEL AND FOOD SYSTEMS

A variety of factors may affect lipid oxidation in model and food emulsions enriched with fish oil. These factors include: process and storage conditions, type and quality of ingredients, emulsifier type and concentration, concentration and quality of fish oil, presence of transition metal ions, structure of the emulsion and pH. In addition, these factors can also affect the efficacy of antioxidants. The complexity of a real food emulsion makes it difficult to predict the influence of different factors on lipid oxidation. This chapter briefly introduces the different model and food systems employed in this PhD work. Furthermore, the most important factors influencing lipid oxidation which were varied in the model or food emulsions experiments are described.

4.1 MAYONNAISE

The basic ingredients in mayonnaise are oil, water, vinegar, egg yolk, stabilizing agents and flavouring compounds. Traditional mayonnaise is an o/w emulsion consisting of egg yolk as emulsifier and has an oil content of 60-80%. In contrast, light mayonnaises may have a fat content as low as 40%. Thorough studies of fish oil enriched mayonnaise has shown that metal catalyzed lipid oxidation is an important factor influencing its oxidative stability. Of the metal chelating antioxidants evaluated (propyl gallate, lactoferin, phytic acid and EDTA), only EDTA inhibited the formation of free radicals [Jacobsen et al., 1999b;Jacobsen et al., 2001b;Jacobsen et al., 2001a;Nielsen et al., 2004].

In this PhD work, fish oil enriched mayonnaise was used in the mayonnaise based salads described in section 4.2. A light mayonnaise was used to evaluate factors that are known or expected to have an impact on lipid oxidation, including emulsifier type, storage temperature and fish oil concentration.

Type of emulsifier

Egg yolk is the most commonly used emulsifier in mayonnaise. Iron present in egg yolk proteins was shown to have a negative impact on the oxidative stability of fish oil enriched mayonnaise. It was suggested that the low pH in mayonnaise triggered the breaking of iron bridges between egg yolk proteins, which raised the iron concentration in the aqueous phase. The free iron released into the aqueous phase could subsequently catalyze the decomposition of existing lipid hydroperoxides located at the oil-water interface or in the aqueous phase. In addition, the presence of ascorbic acid was shown to increase the release of iron [Jacobsen et al., 1999a;Jacobsen et al., 2001b;Thomsen et al., 2000;Jacobsen et al., 2001b]. This work led to the conclusion that low pH releases iron from the egg yolk and thereby promotes lipid oxidation. At the same time, a hypothesis was forwarded that emulsifiers containing less iron than egg yolk may improve the oxidative stability of fish oil enriched mayonnaise.

Fish oil concentration

To the best of my knowledge, there are no published results on the effect of increased fish oil concentrations on lipid oxidation in mayonnaise or similar products. Based on the high susceptibility of PUFA to oxidation [Frankel, 2005], the oxidative stability is expected to decrease with increasing fish oil content.

Storage temperature

The typical storage temperature for mayonnaise is room temperature. The general theory about lipid oxidation states that lipid oxidation increases with increasing temperature [Frankel, 2005]. Therefore, decreased oxidation is expected with decreasing storage temperature. This has also been confirmed in other experiments with mayonnaise and milk enriched with fish oil [Let et al., 2005a;Hsieh and Regenstein, 1991;Jafar et al., 1994].

4.2 MAYONNAISE BASED SALADS

Mayonnaise based salads consist of mayonnaise and different ingredients such as vegetables and some kind of meat, fish or shellfish. In the present work, ingredients in

shrimp and tuna salads enriched with fish oil were evaluated. Mayonnaise based shrimp salads contain two ingredients besides the mayonnaise: shrimp and asparagus. In contrast, mayonnaise based tuna salads contain several ingredients besides from the mayonnaise, namely onion, maize, peas, red pepper and tuna. Current knowledge about the effect of these ingredients on oxidative stability is summarized below.

Influence of ingredients on the oxidative stability

Vegetables contain a variety of different phenolic compounds. Several phenolic compounds have been shown to exert different antioxidant activities (see chapter 3, section 3.3.1). Some studies have shown the antioxidative effects of asparagus, bell pepper and peas [Sun et al., 2007b;Rodriguez et al., 2005;Sun et al., 2007c;Ou et al., 2002;Lang and Ke, 2006]. However, antioxidative effects have only been evaluated from extracts of these ingredients in simple model system or *in vitro* assays. Thus, the antioxidative effects of these vegetables in a complex food matrix are still unknown.

4.3 MODEL FOOD EMULSIONS

Model food emulsions were employed as simple systems to evaluate the efficacy of different antioxidants. Two different emulsion types, namely o/w and w/o emulsions, were stabilized by citrem and PGPR (polyglycerol polyricinoleat), respectively. It is not possible to use the same emulsifier for o/w and w/o emulsions, thus these two were selected according to the similarities in their molecular structure, since both emulsifiers have a glycerol structure (Figure 8). Both emulsifiers are used as emulsifiers in commercial food products.

There is one difference between the two emulsifiers that might be important for the oxidative stability in emulsions. Citrem is an anionic emulsifier, whereas PGPR is a non-ionic emulsifier. Earlier experiments with citrem stabilized emulsion at pH 3 and pH 6 showed that citrem was negatively charged at pH 3 and became more negatively charged when pH was increased from pH 3 to pH 6 [Sørensen et al., 2008]. Therefore, it is assumed that citrem is also negatively charged when the pH increases to pH 7 as was the case in our model emulsion. The assumption of the negatively charged droplets with

citrem as emulsifier is supported by droplet charge measurements preformed by Lue et al. (2010) in similar emulsion systems as employed in this work. The influence of a charged emulsifier is briefly described below.

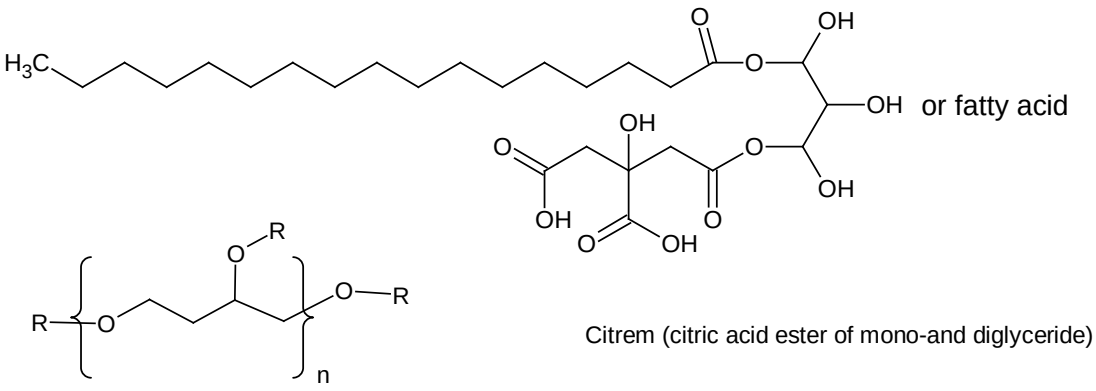

Figure 8 Structure of the two different emulsifiers used for stabilizing emulsions. Polyglycerol polyricinoleate (PGPR), R=H or a fatty alkyl group, n = degree of polymerization of glycerol (average > 1) used to stabilize w/o emulsions. Citrem (citric acid ester of mono- and diglyceride) used to stabilize o/w emulsions.

Impact of charged emulsifiers

The use of an ionic emulsifier (i.e. anionic or cationic) results in a charged interface between the oil and water phase. A charged surface can attract ions with opposite charge. Thus, a negatively charged surface may attract positively charged transition metal ions from the water phase. This means that the location of metals will be closer to the unsaturated fatty acids [McClements and Decker, 2000]. Therefore, the charge of the emulsifier may affect the oxidative stability. This has been confirmed in several experiments [Mei et al., 1998a;Mei et al., 1998b;Tong et al., 2000].

4.4 MILK

Whole milk (o/w emulsion) consists of approximately 87.2% water, 3.5% fat (95% triglycerides), 3.5% protein (80% casein and 20% whey protein) and 4.9% lactose.

Moreover, the milk also contains metal ions such as iron and copper. The pH of milk is higher than the pI of milk proteins; thus, the charge in of the fat droplets in milk is negative [Fox and McSweeney, 1998;Walstra and Jenness, 1984]. This may cause increased lipid oxidation due to attraction of metal ions. Furthermore, homogenization conditions and initial fish oil quality have been evaluated as important for the oxidative stability of fish oil enriched milk. Moreover, the addition of antioxidants such as ascorbyl palmitate and γ-tocopherol (rapeseed oil) to fish oil enriched milk has been shown to improve its oxidative stability [Let et al., 2004;Let et al., 2005a;Let et al., 2005b;Let et al., 2007b]. All milk samples used in this PhD work was produced similar.

CHAPTER 5 EXPERIMENTAL WORK

This chapter describes the approaches used to evaluate the influence of ingredients and efficacy of antioxidants on lipid oxidation. In the last part of this chapter, the methods employed are briefly summarized.

5.1 APPROACHES

5.1.1 INFLUENCE OF INGREDIENTS AND OIL CONTENT ON OXIDATIVE STABILITY

Due to the beneficial health effects of n-3 PUFAs from fish oil, there has been an increasing interest in fish oil enriched foods. A Danish mayonnaise salad company wished to evaluate possibilities for fish oil enriched mayonnaise based salads. They selected two types of salads for the evaluation: shrimp and tuna salads. These two types of salad were appropriate for enrichment due to the already existing content of marine sources, which seems more natural for the consumers.

The experimental design (Figure 9) was set up according to the hypothesis outlined in Chapter 1 and interest from the company. Mayonnaise used to produce the salads was evaluated based on oxidative stability in order to conclude on the overall effect of ingredients added to the fish oil enriched mayonnaise salads. All samples were stored in the dark at a temperature of 2°C for 8 weeks. The length of the storage period was set according to the shelf life of commercial mayonnaise based salads. Concentrations of lipid hydroperoxides and volatile oxidation products were measured during storage and a sub-set of salads were evaluated by a sensory panel. For more details refer to Paper I.

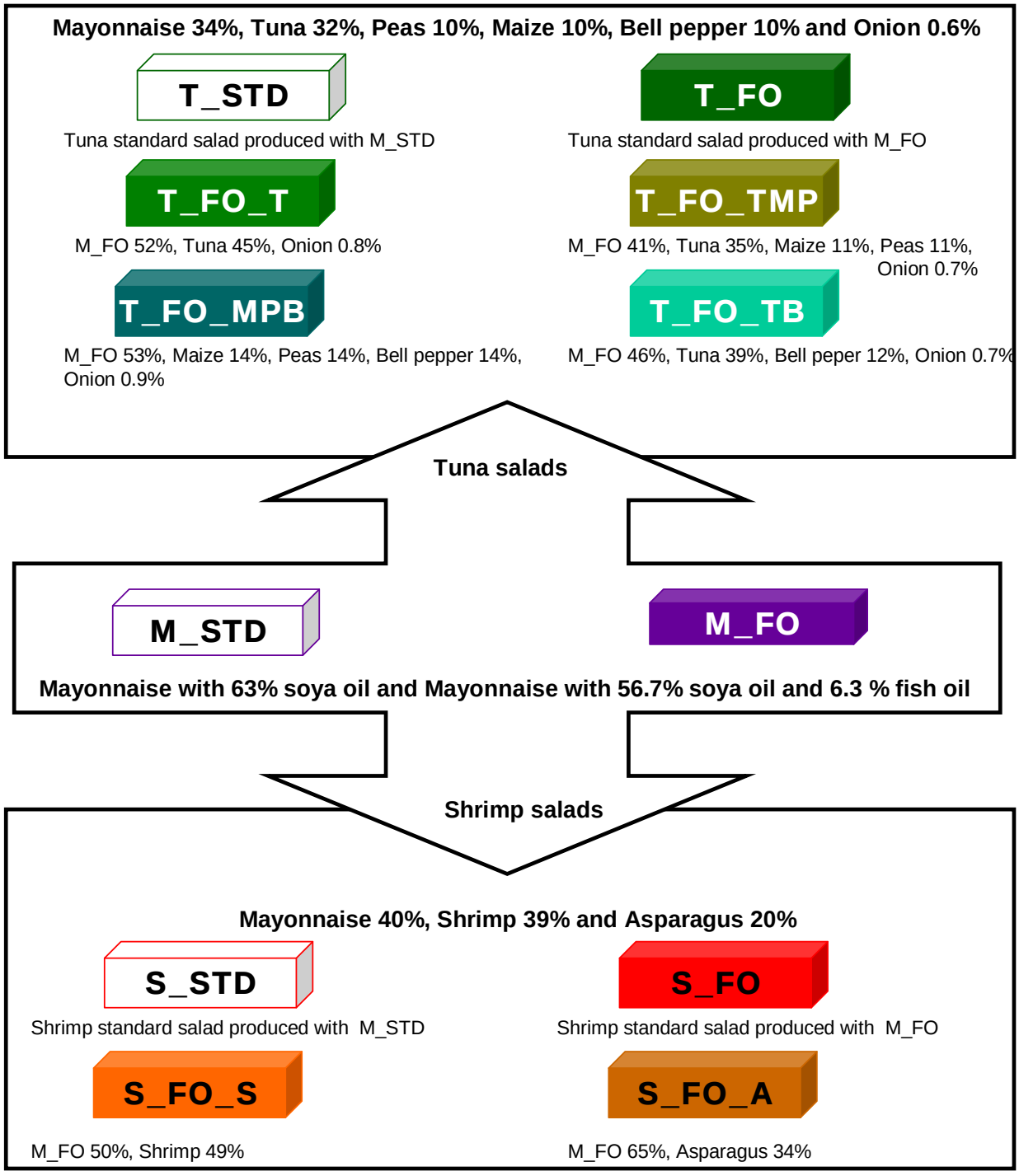

Figure 9 Experimental design (Paper I). Sample codes and ingredients [%] are listed. White boxes symbolize standard products produced without fish oil, whereas colored boxes symbolize fish oil enriched products. The total percentage of the ingredients in the salads is not 100%, but around 97-99%. The last 1-3% was made up of preservatives and acids used to adjust the sourness of the salad.

Based on the results from the fish oil enriched mayonnaise based salads, another objective was to improve the oxidative stability of fish oil enriched mayonnaise. A light mayonnaise with only 40% fat was selected for the present work because of the general consumer-driven demand for healthier products, such as low calorie products. The aim was to evaluate the influence of emulsifier (low and high iron content emulsifier), effect of fish oil concentration (4, 10 and 14% fish oil) and storage temperature (2 and 20°C) on lipid oxidation in fish oil enriched light mayonnaise.

The low and high iron containing emulsifiers in this study were milk protein based emulsifier and egg yolk, respectively. Fish oil concentrations were selected on the basis of earlier results and recommended daily intakes for EPA and DHA at 0.5 g/day proposed by various nutritional organizations. However, this experiment was designed before the newly proposed reference intake for n-3 at 0.25 g/day [Heller, 2009]. Consumption of 20 g of light mayonnaise containing a high concentration of fish oil should cover the daily needs of n-3 PUFA. Storage temperature was selected according to earlier results, with the high temperature used to simulate the storage temperature of commercial mayonnaise in the supermarket. Table 2 illustrates the experimental design.

Table 2 Experimental design for fish oil enriched light mayonnaise (Paper II).

Code	Emulsifier	Rapeseed oil [%]	Fish oil [%]
EY_STD	Egg yolk	40	0
EY_4%FO	Egg yolk	36	4
EY_10%FO	Egg yolk	30	10
EY_14%FO	Egg yolk	26	14
MP_STD	Milk protein†	40	0
MP_14%FO	Milk protein†	26	14

All samples were stored in dark at 2°C and 20°C. †Milk protein refers to a mixture of emulsifier and stabilizers (starch, skim milk powder, milk protein, sodium caseinate, guar gum, sodium alginate, xanthan gum) with milk protein as emulsifier.

Concentrations of lipid hydroperoxides and volatile oxidation products were measured during storage. Furthermore, the mayonnaises were evaluated by a sensory panel. For more details refer to Paper II.

5.1.2 EFFICACY OF ANTIOXIDANTS

The aim of the first three experiments was to investigate the limitations of the polar paradox hypothesis with respect to the effect of the lipophilicity of the antioxidants. Although some antioxidants are known to act in a concentration dependent manner, these experiments evaluated one concentration (100 µM). In order to be able to test the hypotheses, the experimental designs used were as described below.

The efficacy of antioxidants with different polarities was evaluated in two different emulsion types (oil-in-water and water-in-oil). The emulsifier, buffer and oil percentages in the two emulsion types were chosen according to previous experience. Compositions of the two types of emulsions are listed below.

W/O Emulsion: 98% oil, 1% buffer and 1% PGPR
Antioxidant: ascorbic acid, ascorbyl palmitate, ascorbyl CLA and CLA (only 68 µM due to impurities)
Reference (no antioxidant)

O/W Emulsion: 5% oil, 94% buffer and 1% citrem
Antioxidant: ascorbic acid, ascorbyl palmitate, ascorbyl CLA and CLA (only 68 µM due to impurities)
2 References A and B (no antioxidant)

Ascorbyl CLA was produced by enzymatic esterification and was performed at Aarhus University, Denmark. References A and B were similar and in the results, the average of these two was used as a reference.

The two emulsion types were stored in different containers; however, in both cases the temperature was 37°C and they were stored in darkness. Lipid hydroperoxides and

volatile oxidation products were measured during storage of the emulsions. In addition, the antioxidative properties of the employed antioxidants were evaluated in assays: radical scavenging, metal chelating and reducing effect, respectively. Moreover, the partitioning of the different antioxidants into the different phases of an o/w emulsion was studied. For more details, refer to Paper III.

In the second antioxidant experiment, an o/w emulsion similar to that described above was used along with four different antioxidants: caffeic acid, dihydrocaffeic acid, octyl dihydrocaffeate and oleyl dihydrocaffeate. Octyl and oleyl dihydrocaffeate were produced by enzymatic esterification performed at Aarhus University (Denmark). The synthesized oleyl dihydrocaffeate was only 60 % pure, and contained 40% free oleyl alcohol. Therefore, an oleyl alcohol containing the same amount of oleyl alcohol (29 mg/kg) as the emulsion with oleyl dihydrocaffeate was included to evaluate the effect of oleyl alcohol on lipid oxidation in o/w emulsions. Antioxidant properties, partitioning of the different antioxidants, lipid hydroperoxides and volatile oxidation products were measured. For more details, refer to Paper IV.

To test the last hypothesis based on the polar paradox, namely that lipophilization of phenolics can increase their antioxidant efficacy in a complex food matrix, fish oil enriched milk was used as a test system. Two different experiments were carried out, and some of the antioxidants were evaluated in both experiments. The experimental design was illustrated in Table 3. Milk samples were stored in the dark at 5°C for up to 12 days. Concentrations of lipid hydroperoxides and volatile oxidation products were measured at specific time points. For more details, refer to Paper V (Preliminary draft).

Table 3 Experimental design: Effect of phenolics and lipophilized phenolics in fish oil enriched milk (Paper V).

Antioxidants	Sample code	Concentration of antioxidant	
		[μM]	[mg/kg]
Experiment 1: Antioxidant + 1.5 mL acetone or 1.5 mL acetone with antioxidant			
Control	1Con	-	-
Rutin	1Rut	100	61.1
Rutin laurate (C12)	1Rut:C12	100	79.3
Rutin palmitate (C16)	1Rut:C16	100	84.9
Dihydrocaffeic acid	1DCA	100	18.2
Oleyl dihydrocaffeate (C18:1)	1DC:C18	100	72.1
Experiment 2: Antioxidant added without acetone			
Control	2Con	-	-
Dihydrocaffeic acid	2DCA	100	18.2
Octyl dihydrocaffeate (C8)	2DC:C8	100	36.8
Oleyl dihydrocaffeate (C18:1)	2DC:C18	100	72.1
Oleyl alcohol	2OLAL	-	28.8
Caffeic acid	2Caf	100	18.0

Besides antioxidant addition, another approach to improve oxidative stability of complex fish oil enriched food matrices was to use spices. As such, both dry spices and spice extracts were evaluated. The experimental design for these experiments in fish oil enriched tuna salads and milk are illustrated in Table 4. Concentrations of lipid hydroperoxides and volatiles were measured during storage.

In the first tuna salad experiment with dry spices added, soy oil was used to produce the salads, whereas rapeseed oil was used to produce the tuna salad with origanox. The change from soy oil to rapeseed oil was due to the supply of oil to the factory.

Table 4 Experimental design for the study on the effect of spices and spice extracts in fish oil enriched tuna salads and milk (Paper I, Appendices I and II).

Sample	Type of spice	Concentration [mg/kg]		
Tuna salads (Paper I)				
Control	-	-	-	-
Rosemary	DS	10,000	-	-
Thyme	DS	10,000	-	-
Oregano	DS	10,000	-	-
Tuna salads (Appendix I)				
Control	-	-	-	-
Origanox†	SE	75	750	1,500
EDTA†	-	75	-	-
Milk (Appendix II)				
Control	-	-	-	-
Green tea	SE	100	300	500
Rosemary 11	SE	100	300	500
Rosemary 201	SE	100	300	500

Abbreviations: DS dry spice and SE spice extract. Origanox is a spice extract based on oregano. †Concentrations in this experiment were expressed in mg/kg mayonnaise, thus the actual concentration in the tuna salad was lower than in the mayonnaise, approximately 1/3 (34% mayonnaise in tuna salad).

5.2 METHODOLOGIES

A major part of the experimental work has been in the form of storage experiments evaluating the influence of ingredients and efficacy of antioxidants in food and model systems. In addition, the properties and location of the antioxidants were characterized. For more detailed descriptions of the methods, refer to the specific papers.

5.2.1 OXIDATIVE STABILITY – STORAGE EXPERIMENTS

Evaluation of the oxidative stability of emulsion and food systems during storage was performed in order to detect changes in the oxidative stability over time. For experiments in real food systems, storage temperatures and time were selected to mimic normal storage conditions for the respective food products. The physical stability of the

emulsions was evaluated by the size distribution of the dispersed phase during storage. Furthermore, viscosity of the light mayonnaise was also measured to determine the physical stability.

Oxidative deterioration in emulsions and food products was evaluated through determination of peroxide values (PV) and levels of secondary volatile oxidation products. By measuring these two parameters, both the initial and later stages of lipid oxidation were evaluated. In addition sensory evaluation was carried out in some of the experiments.

Peroxide value (PV)

Primary oxidation products were determined by measuring lipid hydroperoxides. In the present PhD work, the colorimetric ferric thiocyanate method was employed on lipid extracts from the samples.

Secondary oxidation products

With the propagation of lipid oxidation, lipid hydroperoxides are decomposed to volatile secondary oxidation products. Measuring these volatiles during storage by a GC-MS instrument thus gives more detailed information about the propagation of lipid autoxidation. Volatiles were mainly determined using dynamic headspace sampling by a purge and trap method on Tenax tubes. In one case the volatiles were determined by static headspace sampling (Paper III). Concentrations of the individual volatile compounds were determined using calibration curves for each individual compound. In some experiments, differences in the release of volatiles from a real food matrix were also taken into account through the construction of calibration curves in the respective food product without fish oil. Calibration curves were constructed for light mayonnaises, milk, tuna salads with spices and the w/o emulsion. For the remaining experiments, the calibration curves were created by trapping the volatiles directly on the Tenax tubes i.e. for mayonnaise based salads and o/w emulsions.

Sensory analysis

A sensory evaluation can contribute significantly to the evaluation of the impact of the volatiles formed in the food product. Sensory analysis was therefore carried out in the experiments with real food products: mayonnaise salads, light mayonnaise and milk with spice extracts. Moreover, they were preformed either by a trained panel or by an expert panel. Project resources i.e. economy, time and amount of sample needed, did not allow for sensory evaluation by a trained panel in all experiments, therefore an expert panel was used. None of the experiments with lipophilized compounds in simple model emulsions were evaluated by sensory analysis; however, for one of the experiments with fish oil enriched milk containing lipophilized phenolics, the odor was evaluated by two persons.

5.2.2 PARTITIONING OF ANTIOXIDANTS

According to the polar paradox hypothesis, partitioning of the antioxidant into different phases of an emulsion has an impact on its efficacy. Therefore, both the partitioning and properties were included during evaluation of antioxidant efficacy. The partitioning was measured both in a buffer-oil mixture, buffer-emulsifier mixture and an o/w emulsion to obtain information on the impact of emulsifier in the emulsion.

5.2.3 ANTIOXIDANT PROPERTIES – IN VITRO ASSAYS

One aim was to evaluate the impact of lipophilization of the compounds. *In vitro* antioxidant assays is simple methods to determine the different antioxidative properties of an antioxidant. Thus, antioxidant assays were employed to give an indication of the effect of lipophilization on the radical scavenging and metal chelating properties as well as the reducing power of the antioxidants.

5.3 DATA ANALYSIS

5.3.1 STATISTICS

One- or two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test were employed to evaluate significant changes during storage

(progress of oxidation) and significant differences between samples at specific time points. The significance level was set at $p < 0.05$. When a significant difference was observed between two samples, they are denoted with different superscripts in the text, tables or graphs.

5.3.2 MULTIVARIATE DATA ANALYSES

In some cases, the results were subjected to principal component analysis (PCA) using Unscrambler version 9.0 (Camo, Oslo, Norway). The PCA model was built on the average of the measured data and full cross validation was used to validate the model. A PCA allows detection of similarities and dissimilarities between the different samples in a score plot, whereas correlations between the measured variables are visualized in a loadings plot. Comparing these plots in this case shows similarities and dissimilarities between samples and variables.

In one case, the chemical and sensory data were subjected to a partial least squares regression (PLSR) using Unscrambler. A PLSR model is an expansion of a PCA and two data matrices were modeled: X-variables (GC-MS data) and Y-variables (sensory), thus the PLSR holds the GC data up against the sensory data.

Pre-treatment of sensory results for multivariate analysis

Before using the sensory results, the performance of the panel and individual assessors was checked by the use of PanelCheck (MATFORSK (Norwegian Food Research Institute, Norway)) and Consonance analysis (Unscrambler version 9.0, Camo, Oslo, Norway) [Tomic et al., 2007;Dijksterhuis, 1995].

The preliminary treatment of the sensory results was necessary to remove the noise caused by scaling differences between the assessors. The noise was projected away by fitting a PLSR model to the sensory results for each sampling day (Unscrambler version 9.0, Camo, Oslo, Norway). The resulting residuals from these models were pooled into an overall average from each sample [Jacobsen et al., 1999b]. These averages were used for the PCA or PLSR on the sensory results.

CHAPTER 6 RESULTS AND DISCUSSION

In this chapter summaries of the experimental findings are presented and discussed in relation to relevant literature and the scientific hypotheses proposed in Chapter 1. For more detailed information about the results refer to the individual papers and appendices. The structure of the chapter is as follows:

Influence of ingredients and fish oil concentration:

- Influence of vegetables on lipid oxidation in fish oil enriched mayonnaise based salads (Paper I)
- Effect of emulsifier, fish oil concentration and storage temperature on lipid oxidation in light mayonnaise (Paper II)

Antioxidant protection:

- Antioxidant efficacy depending on emulsion type: w/o versus o/w emulsions (Paper III)
- *In vitro* antioxidative properties of lipophilized dihydrocaffeic acid and their partitioning in the phases of an o/w emulsion (Paper IV)
- Efficacy of lipophilized rutin and dihydrocaffeic acid in o/w emulsion and milk (Paper IV + V)
- Antioxidative effect of spices, spice extracts and green tea in tuna salads and milk (Paper 1, Appendix I and II)

6.1 INFLUENCE OF INGREDIENTS AND FISH OIL CONCENTRATION

6.1.1 INFLUENCE OF VEGETABLES IN FISH OIL ENRICHED MAYONNAISE BASED SALADS (PAPER I)

This research aimed at evaluating the influence of vegetables on lipid oxidation in two different kinds of fish oil enriched mayonnaise based salads; namely shrimp and tuna salad. The oxidative stability of these two salads was compared with fish oil enriched mayonnaise.

Comparison of fish oil enriched mayonnaise, shrimp and tuna salads

Figure 10 shows a scores plot and correlation loadings plot from a PCA model on the PVs and volatiles measured during storage of the different samples. Two samples were kept out of the PCA model, since they dominated the model and made interpretation of the rest of the samples impossible. These samples were also found to be significantly different from the others using univariate statistics.

From Figure 10 it is clear that the product type and the vegetables had more influence on lipid oxidation than substitution of some of soy oil with fish oil. Thus, the tuna salads were more oxidized than the mayonnaise and shrimp salads as seen from their closer location to the lipid oxidation variables (PV and volatiles). In contrast, mayonnaises were the least oxidized followed by the shrimp salads. The higher degree of oxidation in tuna salads compared to mayonnaise and shrimp salads may be due to the content of tuna, which contains heme iron [Monsen et al., 1978]. Iron can exist in different forms and can be a strong prooxidant [Monsen et al., 1978;Rhee et al., 2006]. However, further studies are required to investigate if iron play a significant role in the oxidation of tuna salad compared to pure mayonnaise.

Both salad types were more oxidized than mayonnaise alone. This could indicate that the vegetables did not have any antioxidative effect, but it could also indicate that the prooxidative effects of tuna or shrimp were strong and covered the potential antioxidative effects of vegetables.

Shrimp salads

Generally, the shrimp salads had a low concentration of lipid hydroperoxides, except for the shrimp salad without asparagus (S_FO_S, Figure 11A), possible indicating an antioxidative effect of asparagus. After 24 days of storage this shrimp salad (S_FO_S) had a significantly higher concentration of lipid peroxides compared to the other shrimp salads. Moreover, over time S_FO_S shrimp salad had the highest concentration of 1-penten-3-one, 2-pentenal (Figure 11B), 1-penten-3-ol, heptanal and 4-heptenal.

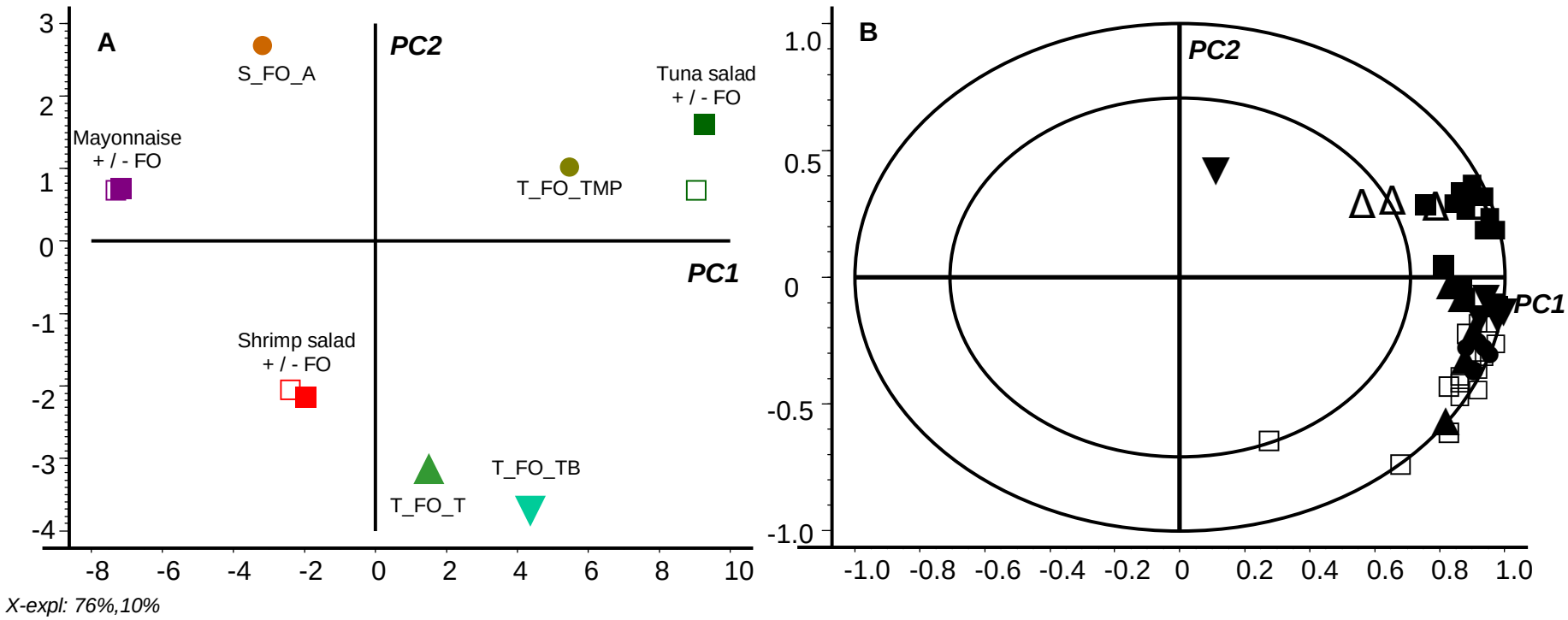

Figure 10 PCA model on PV and volatiles on mayonnaise, shrimp and tuna salads (mayonnaise with fish oil and shrimp and mayonnaise with fish oil, maize, peas and bell pepper were kept out of the model). A) Scores plot and B) Correlation loadings plot, the inner and outer circles indicate 50 and 100% explanation, respectively. The model was validated using full cross validation and the two first PC's explained 86% of the variation. Sample codes: □ Mayonnaise, ■ Mayonnaise with fish oil, □ Shrimp salad, ■ Shrimp salad with fish oil, ● Mayonnaise with fish oil and asparagus (S_FO_A), □ Tuna salad, ■ Tuna salad with fish oil, ▼Mayonnaise with fish oil, tuna and bell pepper (T_FO_TB), ▲ Mayonnaise with fish oil and tuna (T_FO_T) and ● Mayonnaise with fish oil, tuna, maize and peas (T_FO_TMP). Variable symbols: Δ PV, ■ Saturated aldehydes (pentanal, hexanal and heptanal), □ Unsaturated aldehydes (2-butenal, 2-pentenal and 4-heptenal), ▲ 1-penten-3-one, ▼ 2-pentyl furan and ● 1-penten-3-ol. FO: Fish oil.

However, the concentration of hexanal and pentanal in mayonnaise with fish oil and asparagus (S_FO_A) were generally higher than in the other types of shrimp salads, but already from the beginning of the storage.

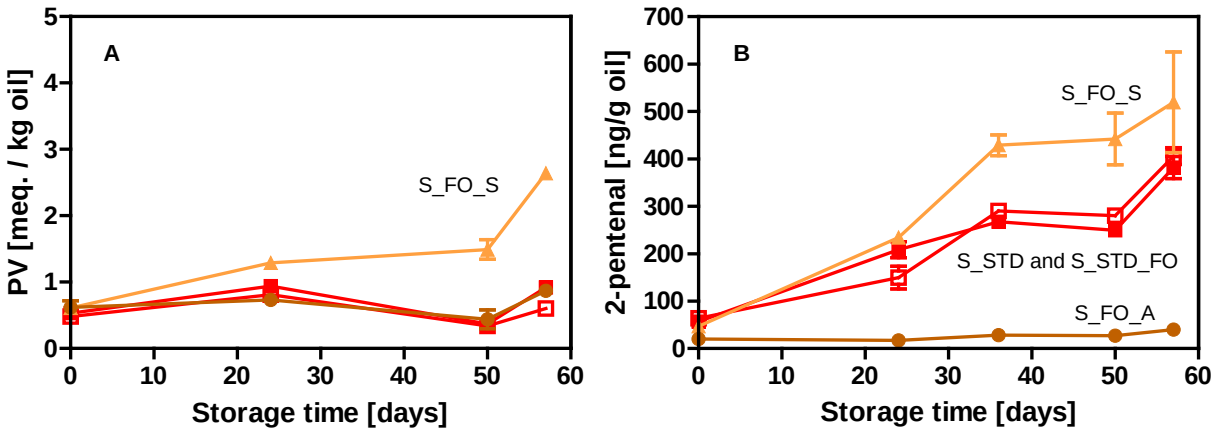

Figure 11 Lipid oxidation measured in fish oil enriched shrimp salads during storage: A) Concentration of lipid hydroperoxides, PV [meq. peroxides/kg oil] and B) Concentration of 2-pentenal [ng/g oil]. Sample codes: □ S_STD Shrimp salad; ■ S_STD_FO Shrimp salad with fish oil; ▲ S_FO_S Mayonnaise with fish oil and shrimp; ● S_FO_A Mayonnaise with fish oil and asparagus. Bars indicate SD of 2 and 3 measurements for PV and 2-pentenal, respectively.

Sensory evaluation was only performed on a sub-set of the samples: shrimp salad (S_STD), shrimp salad with fish oil (S_STD_FO) and mayonnaise with fish oil and shrimp (S_FO_S). Overall, the S_FO_S sample was evaluated as having a significantly more rancid and metallic taste and odor towards the middle or end of the storage compared with the evaluated intensities for S_STD and S_STD_FO. In contrast, a slight decrease in the intensity of shrimp (positive descriptor) and mayonnaise (significant at day 57) taste was observed for S_FO_S.

As indicated by a PLSR analysis (in Paper I), there was a very strong correlation between the measured volatiles and the sensory data. From the model it was concluded that sensory attributes like rancid, metallic and grass correlated positively with 1-penten-3-one, 1-penten-3-ol and 4-heptenal, whereas 2-pentylfuran showed negative correlations. Some of these compounds have also been reported in other studies to

contribute to the fishy and rancid flavour in fish oil enriched mayonnaise and milk [Hartvigsen et al., 2000;Venkateshwarlu et al., 2004b].

Interestingly, there was no significant difference between shrimp salad with (S_FO) and without fish oil (S_STD) for any of the volatiles. The data also suggested that shrimp, as an ingredient alone in the mayonnaise salad, had a prooxidative effect on lipid oxidation, whereas asparagus could prevent the prooxidative effect of shrimps in shrimp salads. The explanations for the prooxidative effect of shrimps are unclear, but might be attributed to the presence of metals such as iron and copper in the shrimp or in the shrimp brine. In addition, shrimps were in brine containing water, salt, citric acid, potassium sorbate and sodium benzoate and were drained before being mixing with mayonnaise. Therefore, the prooxidative effect of shrimps could also be due to these compounds diffusing in the shrimp salad. However, more studies are needed to confirm whether it is the iron and copper in shrimp, compounds from the brine or a combination that causes the prooxidative effect of shrimps. The antioxidative effects of asparagus have been reported in several studies [Sun et al., 2007b;Rodriguez et al., 2005;Lang and Ke, 2006]. However, from the label it was revealed that the asparagus used in the present study, was conditioned with citric acid. Whether, the antioxidative effect of asparagus observed was due to the citric acid, the phenolic compounds or other compounds present in the asparagus or a combination of these is unknown and deserves further investigations.

Tuna salads

For all tuna salads similar concentrations of primary oxidation products were observed during storage, but concentrations varied between tuna salads. The PV measured in tuna salads was generally higher than PV measured in mayonnaise alone, except for two of them: mayonnaise with fish oil and tuna (T_FO_T) and mayonnaise with fish oil, tuna and bell pepper (T_FO_TB). No increase in PV during storage means, either no development in lipid oxidation or that decomposition of peroxides to secondary oxidation products was as fast as the formation rates of the lipid hydroperoxides. However, from the concentration of volatiles it is difficult to conclude if any of the ingredients influenced oxidation of the lipids. This is due to the fact that the different ingredients resulted in

different amounts of volatiles already from the beginning of the storage period and that no further development during storage was observed. Only 3 of the 9 volatiles measured effectively increased in concentration during storage. However, concentration of these volatiles did not differ significantly irrespective of the salad type. Concentrations of the different volatiles depended on the ingredients in tuna salads as illustrated in the PCA model (Figure 10) with the different tuna salads being located close to different volatiles. In contrast to shrimp salads, it was more difficult to detect any sensory differences between tuna salads. Significant differences in rancid taste was first observed at the end of the storage period, and the mayonnaise with fish oil and tuna was evaluated to be more rancid than the two standard tuna salads without and with fish oil (T_STD and T_FO, respectively). As observed for the shrimp salads, the mayonnaise odour and taste decreased significantly at day 57 for T_FO_T when rancid taste increased.

One tuna salad, mayonnaise with fish oil, maizes, peas and bell peppers (T_FO_MPB) i.e. without tuna, was found to be significantly different from the other tuna salads. This sample had the highest concentration of heptanal, pentanal and hexanal and lowest concentration of 1-penten-3-ol compared to the other salads. None of the volatiles found in high concentration in this salad correlated with rancidity in the PLSR model from the shrimp salad data. It is therefore doubtful that this tuna salad was more oxidized than the tuna standard salads prepared with or without fish oil (T_FO or T_STD).

A higher concentration of volatiles was found in the standard tuna salads containing all the ingredients compared to the tuna salad without any vegetables. This finding suggests that addition of vegetables to the tuna salad promoted oxidation. However, this is contradicting the findings from the sensory analysis, which suggested that the tuna salad without vegetables was more rancid. Based on the results from the sensory evaluation it could then be speculated that the vegetables may have masked the rancid taste. According to the PCA model (Figure 10), tuna salad without vegetables (T_FO_T) was not located near the standard tuna salads and this was most likely due to a higher concentration of volatiles in the standard tuna salads, which may originate from the vegetables them-selves rather than from oxidation. 4-Heptenal, one of the two volatiles that increased in concentrations in tuna salads during storage, was found to correlate to

rancid odor and flavor in shrimp salad. The concentration of this volatile compound in the different tuna salads was not significantly different except for the concentration in T_FO_MPB, which was significantly lower than in the other salads. Thus, the biggest differences in the concentration of volatiles in tuna salad may be due to the vegetables present in the tuna salad and not due to oxidation of the samples.

Tuna salads prepared without one or more vegetables seemed to reach concentrations of volatiles between those observed for the standard tuna salads (T_STD and T_STD_FO) and the tuna salad prepared without vegetables (T_FO_T). For the same reasons as mentioned previously, it is difficult to conclude whether these finding are due to volatiles naturally present in vegetables or to differences in the degree of lipid oxidation in the different products. Nevertheless, several other studies have found that bell pepper [Sun et al., 2007c;Materska and Perucka, 2005;Ou et al., 2002;Lang and Ke, 2006] and peas [Lang and Ke, 2006] have good antioxidative activity. A similar effect was not obvious in our study.

6.1.2 EFFECT OF EMULSIFIER, FISH OIL AMOUNTS AND STORAGE TEMPERATURE (PAPER II)

In previous work with fish oil enriched mayonnaise, iron from egg yolk combined with low pH were found to be the predominant factors responsible for oxidation and it was suggested that oxidation may be reduced by using an alternative emulsifier to replace egg yolk [Jacobsen et al., 2001b]. Therefore, the aim of the present work was to compare the effect of egg yolk as an emulsifier with a milk protein based emulsifier on the oxidative stability of fish oil enriched light mayonnaise (40% fat) containing 14% fish oil. Additionally, the influence of fish oil concentrations and storage temperatures on lipid oxidation was evaluated.

Emulsifier

Iron concentration was 7.2 fold higher in the egg yolk compared to milk protein based emulsifier. This resulted in a 4 fold higher iron concentration in the mayonnaise produced with egg yolk compared to the mayonnaise produced with milk protein based emulsifier. The concentration of primary oxidation products increased during storage and was highest for the mayonnaise prepared with milk protein and fish oil (EY_14%FO),

data shown in Paper II. Similarly concentrations of 1-penten-3-one (Figure 12A), 2(t)-butenal, 1-penten-3-ol and 2,4(t,t)-heptadienal were also significantly higher in MP_14%FO mayonnaise. In contrast, concentrations of pentenal (Figure 12B), nonanal, heptanal, hexenal and pentyl furan were significantly higher in EY_14%FO mayonnaise. For many of the volatiles measured, their concentrations was significantly higher in the MP_14%FO mayonnaise already after 28 days of storage compared to the other mayonnaise samples. Exceptions were observed for pentyl furan and the saturated volatiles (pentanal, hexanal, heptanal and nonanal).

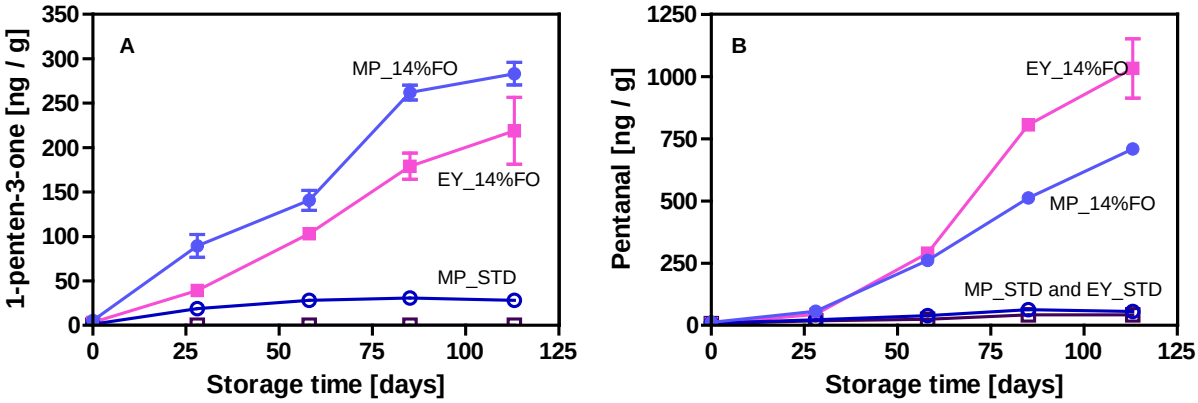

Figure 12 Concentration of two secondary volatile oxidation products in light mayonnaise [ng/g] stored at 20°C A) Concentration of 1-penten-3-one and B) Concentration of pentenal. Sample codes: □ EY_STD Mayonnaise stabilized with egg yolk; ■ EY_14%FO Mayonnaise with 14% fish oil stabilized with egg yolk; ○ MP_STD Mayonnaise stabilized with milk protein; ● MP_14%FO Mayonnaise with 14% fish oil stabilized with milk protein. Bars indicate SD of 3 measurements.

The reasons for the increased formation of pentyl furan and saturated volatiles in mayonnaise with egg yolk could be that these volatiles were released more easily from the matrix in the egg yolk stabilized mayonnaise, when lipid oxidation proceeds.

Irrespective of the emulsifier type, the two mayonnaises without fish oil (EY_STD and MP_STD) were evaluated as being sweet with flavor of egg yolk even though MP_STD was produced without egg yolk. These attributes were negatively correlated with rancidity, indicating less lipid oxidation in the mayonnaises without fish oil. Furthermore, the sensory evaluation indicated that MP_14%FO was more rancid than EY_14%FO. Surprisingly, the present study thus showed that mayonnaises with milk protein oxidized

faster than mayonnaise prepared with egg yolk as emulsifier, even though the iron content was much higher in egg yolk stabilized mayonnaise.

Food emulsions such as mayonnaises are complex matrixes where several factors might influence oxidation such as the quality and the anti- and prooxidative properties of the ingredients, the presence of trace metals and the viscosity of the emulsion [McClements and Decker, 2000;Coupland and McClements, 1996]. Most likely the increased oxidation observed for MP_14%FO compared with EY_14%FO was due to the quality of the emulsifier applied and interactions between ingredients more than droplet charge or viscosity changes. The only difference between the two fish oil enriched mayonnaises in the present study was the emulsifiers. To investigate whether the quality of the emulsifiers could have influenced the oxidative stability of the mayonnaises, their peroxide value was measured. The milk protein emulsifier had a much higher peroxide value than the egg yolk: 9.8 vs. 0.01 meq. peroxides/kg oil. Taking the different oil contents in the emulsifiers into consideration the concentrations in meq. peroxides/kg emulsifier 0.156 and 0.003 for milk protein mixture and egg yolk respectively, which means that the milk protein mixture was not of high quality from the start. This might explain the increased lipid oxidation observed in fish oil enriched mayonnaise with milk protein compared with the mayonnaise emulsified with egg yolk. Moreover, the importance of initial oil quality was reported by Let et al. (2005) who showed that a poor oil quality significantly increased lipid oxidation [Let et al., 2005a]. Therefore, it seems reasonable to assume that the quality of the emulsifier can also have a great impact on the oxidative stability of the final product.

Moreover, both types of mayonnaise contained xanthan gum, which has antioxidative properties due to its metal chelating properties [Paraskevopoulou et al., 2007;Sun et al., 2007a;Shimada et al., 1996]. However, studies have also indicated that it can have prooxidative properties by interacting with unabsorbed whey protein isolate (WPI) in the continuous phase in WPI stabilized o/w emulsions. Thereby, xanthan gum prevents WPI from acting as an antioxidant and it might also lose its metal binding properties it-self [Sun et al., 2007a]. Taken together, the suggested negative effect of the reduced quality of the milk protein based emulsifier and the prooxidative effect of xanthan gum may both

be responsible for the findings that milk protein stabilized mayonnaise was more oxidized than egg yolk stabilized mayonnaise.

Fish oil concentrations and storage temperature

Generally, the concentration of lipid oxidation products increased with increasing concentration of fish oil (4, 10 and 14% fish oil), see Figure 13 and Paper II. However, the concentration for 1-penten-3-one (Figure 13A) and 1-penten-3-ol was measured in equal amounts in mayonnaise with 10 and with 14% fish oil.

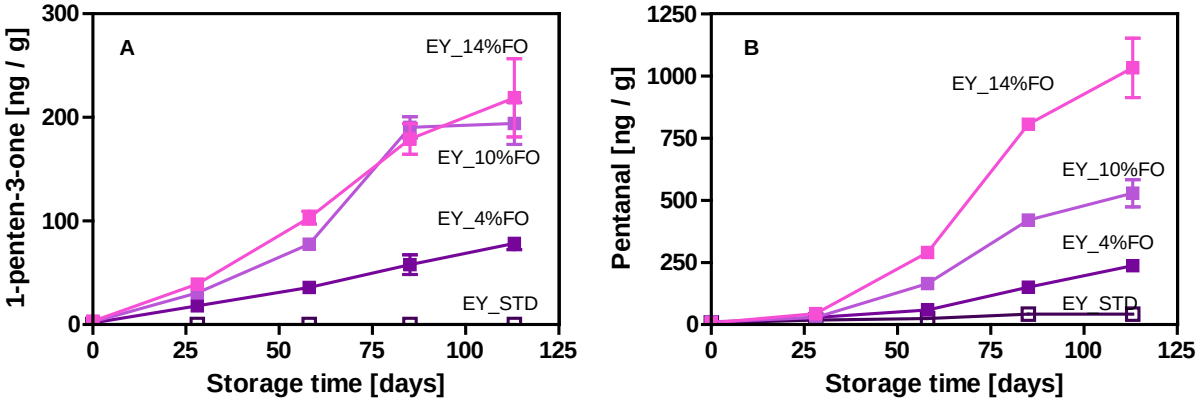

Figure 13 Concentration of two secondary volatile oxidation products [ng/g] in light mayonnaise stabilized with egg yolk stored at 20°C: A) Concentration of 1-penten-3-one and B) Concentration of pentenal. Sample codes: □ EY_STD Mayonnaise standard; ■ EY_4%FO, ■ EY_10%FO and ■ EY_14%FO Mayonnaise with 4, 10 and 14% fish oil, respectively. Bars indicate SD of 3 measurements.

The sensory evaluation overall confirmed the results from the chemically measured oxidation products, that increasing fish oil concentration increased lipid oxidation. This was indicated by the higher intensity in rancidity for mayonnaises with the higher fish oil concentration. However, the sensory results indicated that 10 and 14% fish oil resulted in the same level of rancidity. In contrast, the mayonnaise with 4% fish oil was evaluated to be similar to the standard mayonnaise without fish oil added. Hence the hypothesis that increased fish oil concentration results in increased lipid oxidation was confirmed.

Taken together, the data in the present study showed that substituting 4% rapeseed oil with fish oil to mayonnaise with egg yolk resulted in increased volatiles concentrations,

but did not result in increased sensory scores for rancidity compared to mayonnaise without fish oil when stored at 20°C for 113 days (approximately 4 months). Hence, it seems possible to substitute 4% of the rape seed oil with fish oil. Using such formulation, one portion of mayonnaise (20 g) would provide 0.16 g EPA + DHA.

Reducing the storage temperature from 20°C (normal storage temperature for mayonnaise) to 2°C markedly improved the oxidative stability of the mayonnaises (Paper II). This has also been shown in other experiments with mayonnaise and milk enriched with fish oil [Let et al., 2005a;Hsieh and Regenstein, 1991;Jafar et al., 1994]. Results from both lipid hydroperoxides and volatiles measurements showed significant oxidation at 20°C after 4 months in all mayonnaises. Hence, it is not recommended to store mayonnaise at 20°C for 4 months, even when fish oil is not added. This is common practice in many supermarkets today. However, mayonnaise enriched with 4% fish oil and stored at 2°C was not more oxidized than mayonnaises without fish oil as concluded from PV and volatiles. Therefore, it seems possible to enrich light mayonnaises with 4 % fish oil without adding antioxidant if stored at low temperature (2°C), but this has to be confirmed further by sensory analysis.

Despite the lower total oil content in light mayonnaise, it oxidized faster than the 63% mayonnaise (Paper I) and at the same rate as full fat mayonnaise (80%) [Jacobsen et al., 1999b]. Thus, it seems that other factors than the oil content impact the oxidation rate in such light products and this has to be investigated further.

6.1.3 DISCUSSION OF FINDINGS IN RELATION TO HYPOTHESES

For the fish oil enriched mayonnaise based salads, the hypothesis was that vegetables present in the salads could improve the oxidative stability of mayonnaise. The findings did not fully confirm this hypothesis, although asparagus exerted an antioxidative effect. However, the antioxidative effect of asparagus was counterbalanced by the prooxidative effect of shrimps. The overall effect of ingredients in mayonnaise salads were prooxidative compared to mayonnaises alone prepared without or with fish oil. Although, the presence of vegetables did not improve the oxidative stability of fish oil enriched mayonnaises, the substitution of 10% soy oil with fish oil did not affect the oxidation

markedly. Thus, according to the chemical and sensory results obtained it seems possible to enrich shrimp and tuna salads with fish oil – if the required shelf-life is reduced from 8 to 6-7 weeks – without affecting the quality and taste compared to the standard salads without fish oil. The shelf-life of mayonnaise salads has recently been reduced from 8 to 6 weeks by the manufacturer. This additionally increases the possibilities for fish oil enriched shrimp and tuna salads.

Tuna salads had the highest concentration of volatiles, and many of them were present in high concentration from the start with no further increase during storage. The natural flavor profile of the vegetables was suggested to explain these findings and thus suggested that no significant lipid oxidation was taking place in this type of salad. For determination of individual volatile, the calibration curve was performed directly on the Tenax tubes, because all samples in the experiment were prepared with different ingredients. Thus, the release of volatiles from the food matrix and the contribution of the food matrix on the volatile release were not taken into consideration. Constructing a calibration curve for each tuna salad might have given deeper understanding of the effect of ingredients on oxidation during storage and could have indicated, if the high concentration of volatile in this experiment was due to the flavor profile of the ingredients rather than lipid oxidation.

One ingredient in fish oil enriched mayonnaise, the egg yolk, has been shown to have a great influence on oxidative stability. This led to our hypothesis, that milk protein could improve the oxidative stability of light mayonnaise compared to mayonnaise stabilized with egg yolk. This hypothesis was based on the lower iron content in milk protein based emulsifier compared to egg yolk. Results obtained in this study did not confirm our hypothesis, and the main reason was suggested to be the low quality of the added milk protein based emulsifier. Although, it was not possible to confirm our hypothesis, it is still possible that this hypothesis can be confirmed with an emulsifier of the same quality as the egg yolk.

6.2 ANTIOXIDANT PROTECTION

6.2.1 ANTIOXIDANT EFFICACY DEPENDING ON EMULSION TYPE (PAPER III)

This part of our research aimed at investigating the antioxidative efficacy of different ascorbic acid derivatized antioxidants with different polarities in w/o and o/w emulsions. Moreover, the effect of antioxidants measured in emulsions was related to their partitioning into different phases in an o/w emulsion and their antioxidant properties in *in vitro* antioxidant assays.

W/O Emulsions

A clear lag phase was observed for lipid hydroperoxide and propanal concentrations in all samples (Figure 14).

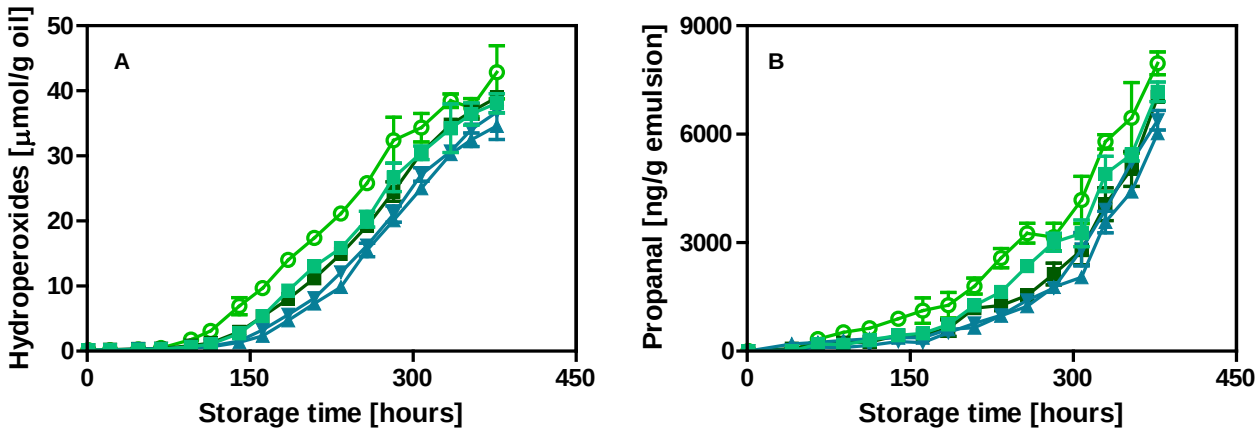

Figure 14 Concentrations of A) lipid hydroperoxides [µmol/g oil] and B) Propanal [ng/g] in w/o emulsions. Sample codes: ○ Reference (no antioxidant); ■ Ascorbic acid; ■ CLA; ▲ Ascorbyl palmitate and ▼ Ascorbyl CLA. Bars indicate SD of 3 measurements.

The lag phase was shortest for the reference emulsion (no antioxidant added) followed by CLA and ascorbic acid and the longest lag phase was observed for emulsions with ascorbyl CLA and ascorbyl palmitate added. No significant differences between the antioxidative effect of ascorbyl palmitate and ascorbyl CLA was observed. During the entire storage period there was a lower concentration of lipid hydroperoxides in the emulsions with antioxidants compared to the reference emulsion. However, at the end of

the storage period the efficacy of the tested compounds in retarding lipid oxidation was reduced indicating that they may be depleted (Table 3 in Paper III). Based on the concentration of hexanal, no significant differences between the efficacies of the different compounds were observed. However, CLA had the tendency to give higher concentration of hexanal compared to the other antioxidants. However the hexanal concentration in the CLA emulsion was not significantly different from the reference sample.

O/W Emulsions

In contrast to our findings in w/o emulsions, no lag phase was observed for lipid hydroperoxide formation in the o/w emulsions (Paper III). However, there was a reduced concentration of lipid hydroperoxides from day 0 to 4 in emulsions with ascorbyl CLA, ascorbic acid and ascorbyl palmitate added compared to the reference emulsion (no antioxidant added). The development of the different volatiles was significantly affected by the antioxidant addition compared with the reference emulsion. Similar to the findings for lipid hydroperoxide, none of the volatiles showed a lag phase in any of the emulsions. In the beginning of the storage period ascorbic acid and ascorbyl palmitate slow down the formation of 1-penten-3-one (Figure 15A), hexanal, 1-penten-3-ol and 4-heptenal but had no effect on the development of 2-hexenal and 2-octenal.

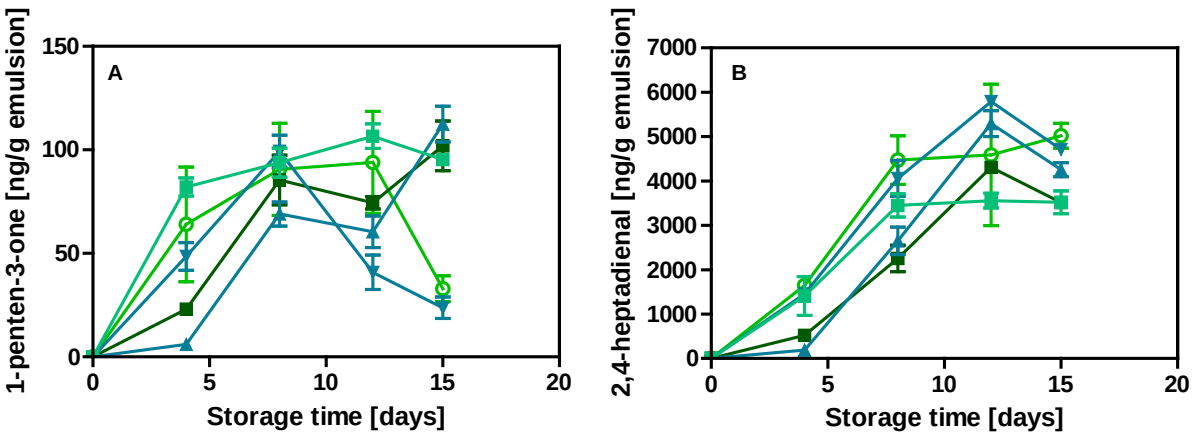

Figure 15 Concentrations of A) 1-penten-3one and B) 2,4-heptadienal [ng/g] in o/w emulsions. Sample codes: ○ Reference (no antioxidant); ■ Ascorbic acid; ■ CLA; ▲ Ascorbyl palmitate and ▼ Ascorbyl CLA. Bars indicate SD of 3 measurements.

In contrast, ascorbyl CLA and CLA had no or prooxidative effect on all volatiles except for 2,4-heptadienal (Figure 15B). At the end of the storage period all the antioxidants tested resulted in a significantly increase in the formation of volatiles compared to the reference, except for the formation of 2,4-heptadienal. Ascorbyl palmitate was the most efficient at reducing the development of volatiles in the beginning of the storage period; however, at the end of the storage it was the most prooxidative of all antioxidant tested.

Antioxidant efficacy in multiphase systems: w/o versus o/w emulsions

Both the w/o and o/w systems are multiphase systems and according to the polar paradox hypothesis, amphiphilic antioxidants are more efficient compared to hydrophilic and lipophilic antioxidants in protecting against lipid oxidation in emulsions [Porter, 1993;Frankel et al., 1994]. In the w/o emulsions ascorbyl palmitate and ascorbyl CLA were more efficient than ascorbic acid and CLA, which is in accordance with the polar paradox for emulsions. In contrast, the lack of effect of ascorbyl CLA in the o/w emulsion was not in accordance with the polar paradox. Ascorbyl CLA was not a completely purified product after esterification and contained free fatty acids, which can have a prooxidative effect in o/w emulsions together with iron [Waraho et al., 2009]. Thus, the antioxidative activity of ascorbyl CLA may be neutralized due to its content of free fatty acids and this could explain its poor antioxidant effect in o/w emulsion. However, this might be more important in the w/o emulsion, where the droplets were not negatively charged, since the fatty acids are expected to be located closer to the interface. However, ascorbyl CLA had a significant antioxidative effect in the w/o emulsion.

In the o/w emulsion the effect of ascorbyl CLA differed from the effect of ascorbyl palmitate. This could indicate that the chain length and saturation of the fatty acids esterified to ascorbic acid influenced its efficacy as antioxidant. Inhibition or delay of copper initiated LDL oxidation has been evaluated using ascorbic acid esters, and the chain length of the fatty acid was shown to affect the development of oxidation. The efficacy of the different ascorbic acid esters was reported as follows: ascorbyl laurate > ascorbyl palmitate > ascorbyl caprylate [Liu et al., 1998]. These findings thus agreed with our findings. Taken together, these findings indicated that the polar paradox is too simple to explain the effect of antioxidants in multiphase system as emulsions. This is in

agreement with earlier studies on o/w emulsions, which also showed that antioxidative effects were not in accordance with the polar paradox due to interactions between iron, emulsifiers and antioxidants [Sørensen et al., 2008;Lue et al., 2010b].

One of our hypothesis was that hydrophilic antioxidants could offer a better protection in a w/o emulsion compared to the reverse o/w emulsions. A prerequisite for this hypothesis was that the concentration of the hydrophilic antioxidant would be higher in the aqueous phase of a w/o emulsion than in an o/w emulsion. It was observed that ascorbic acid inhibited oxidation in the w/o emulsion better than in the o/w emulsion. In addition, the relative proportion of the disperse phase in our w/o emulsion (1%) was smaller than in our o/w emulsion (5%), which leads to even higher concentration of ascorbic acid in the aqueous phase of the w/o emulsions (10,000 μM) compared to the aqueous phase in the o/w emulsions (106 μM). Therefore, it was suggested that ascorbic acid was located closer to the interface in the present w/o emulsion due to its higher concentration in the aqueous phase compared to o/w emulsion studied here and therefore ascorbic acid exhibits better antioxidative efficacy. However, interactions with other compounds such as emulsifiers should also be considered [Sørensen et al., 2008].

Partitioning of the antioxidants related to their efficacy

As expected, ascorbic acid was found in highest concentration in the aqueous phases of the oil-buffer and the emulsion systems. At pH 7 both citrem and ascorbic acid are expected to be negatively charged, hence repulsive forces existed between the interface and ascorbic acid [Frankel and Meyer, 2000]. Thus, it is likely that ascorbic acid was not located close to the interface due to repulsive forces. In contrast, the emulsifier in the w/o emulsion, PGPR, is a non-ionic emulsifier, which enables ascorbic acid to be closer to the interface in this emulsion system. Therefore, the differences in charge of the emulsifier used in o/w and w/o emulsions might also explain the higher efficiency of ascorbic acid in the w/o emulsions compared to the o/w emulsions. However, partitioning studies with PGPR and ascorbic acid are required before further conclusions can be drawn.

The partitioning data for the o/w system showed that in the buffer-oil system a little less than half of the amount of the antioxidant added (ascorbyl palmitate and ascorbyl CLA) was detected in the buffer phase. However, when citrem was present ascorbyl palmitate and ascorbyl CLA were not detected in the aqueous phase. This indicated that ascorbyl CLA and ascorbyl palmitate may have interacted with citrem located at the interface or participated in micelles formed with citrem in the aqueous phase, but also that some ascorbyl CLA and ascorbyl palmitate may have partitioned into the oil phase. The CMC of citrem (≈ 15 mg / L [Semenova et al., 2007]) is higher than the concentration of citrem (10 mg / L) in our experiment, hence it might generally be assumed that citrem is mainly located at the interface. Even though the structure of ascorbyl palmitate and ascorbyl CLA only differs in the fatty acyl group by the chain length and degree of saturation, it can be speculated that structural differences resulted in different interactions between citrem and ascorbyl CLA than between citrem and ascorbyl palmitate. This may account for the reduced ability of ascorbyl CLA to act as an antioxidant and thereby reduce lipid oxidation. Data from the oil-buffer system indicated a higher concentration of ascorbyl palmitate in the oil phase compared to ascorbyl CLA. This can perhaps explain why ascorbyl palmitate was more efficient than ascorbyl CLA in the beginning of the storage period.

Ascorbyl CLA was located closer to the lipid than ascorbic acid, and therefore it can be expected to be more efficient than ascorbic acid. Nevertheless, ascorbic acid was a more efficient as antioxidant compared to ascorbyl CLA in o/w emulsion in the beginning of the storage period. Ascorbic acid was suggested to be repelled from the interface by citrem due to the negative charges of both ascorbic acid and citrem at pH 7. The repulsive forces may not be the only important factors, since ascorbic acid acted as a better antioxidant than ascorbyl CLA in o/w emulsions. Further studies on interaction of these antioxidants with citrem are required to improve our understanding of the antioxidative activity of ascorbyl CLA and ascorbyl palmitate. This might also explain the better protection obtained with ascorbic acid than with ascorbyl CLA in o/w emulsions.

6.2.2 THE EFFECT OF LIPOPHILIZATION OF DIHYDROCAFFEIC ACID ON ITS PARTITIONING AND ANTIOXIDATIVE PROPERTIES (PAPER IV + V)

The experiment aimed at evaluating the antioxidative effect of lipophilized dihydrocaffeic acid in simple emulsions and related the effect with their partitioning in the different phases of an o/w emulsion and with their antioxidative properties (*in vitro* assays). A second aim was to evaluate the antioxidative effect of lipophilized dihydrocaffeic acid and rutin in a complex food system (fish oil enriched milk).

Partitioning and Antioxidant properties (In vitro)

As expected, lipophilization of dihydrocaffeic acid with either octyl or oleyl alcohol changed their location, since neither of these compounds were detected in the aqueous phase independent of the system analyzed (oil-buffer, emulsifier-buffer and emulsion), whereas dihydrocaffeic acid was found in the aqueous phase in all three systems. This is probably a result of interactions between octyl dihydrocaffeate or oleyl dihydrocaffeate and citrem and / or location of these lipophilized phenolics in the oil phase.

Radical scavenging, iron chelating and reducing power activities increased with increasing antioxidant concentration from 25-200 µM (Figure 16). The lipophilization had a negative effect on the antioxidative activity in all three assays. This indicates that the esterified acid group may contribute to the ability of the compounds to donate H-atoms or electrons and to chelate iron. These findings are in accordance with earlier results obtained by Sabally et al. (2007) and Lue et al. (2009). Sabally et al. (2007) reported that the reduced radical scavenging activity observed for lipophilized dihydrocaffeic acid indicated that the acid group in dihydrocaffeic acid contributed to the donation of H-atoms. Despite the fact that the catechol moiety has been suggested to be the site of metal chelation [Andjelkovic et al., 2006;Chimi et al., 1991], lipophilization of dihydrocaffeic acid resulted in markedly reduced chelation activity, which is in accordance with results obtained by Lue et al. (2009) with lipophilized rutin. Thus, the findings may indicate that the binding site of the alkyl chain may play a role for the ability of the compound to chelate metal.

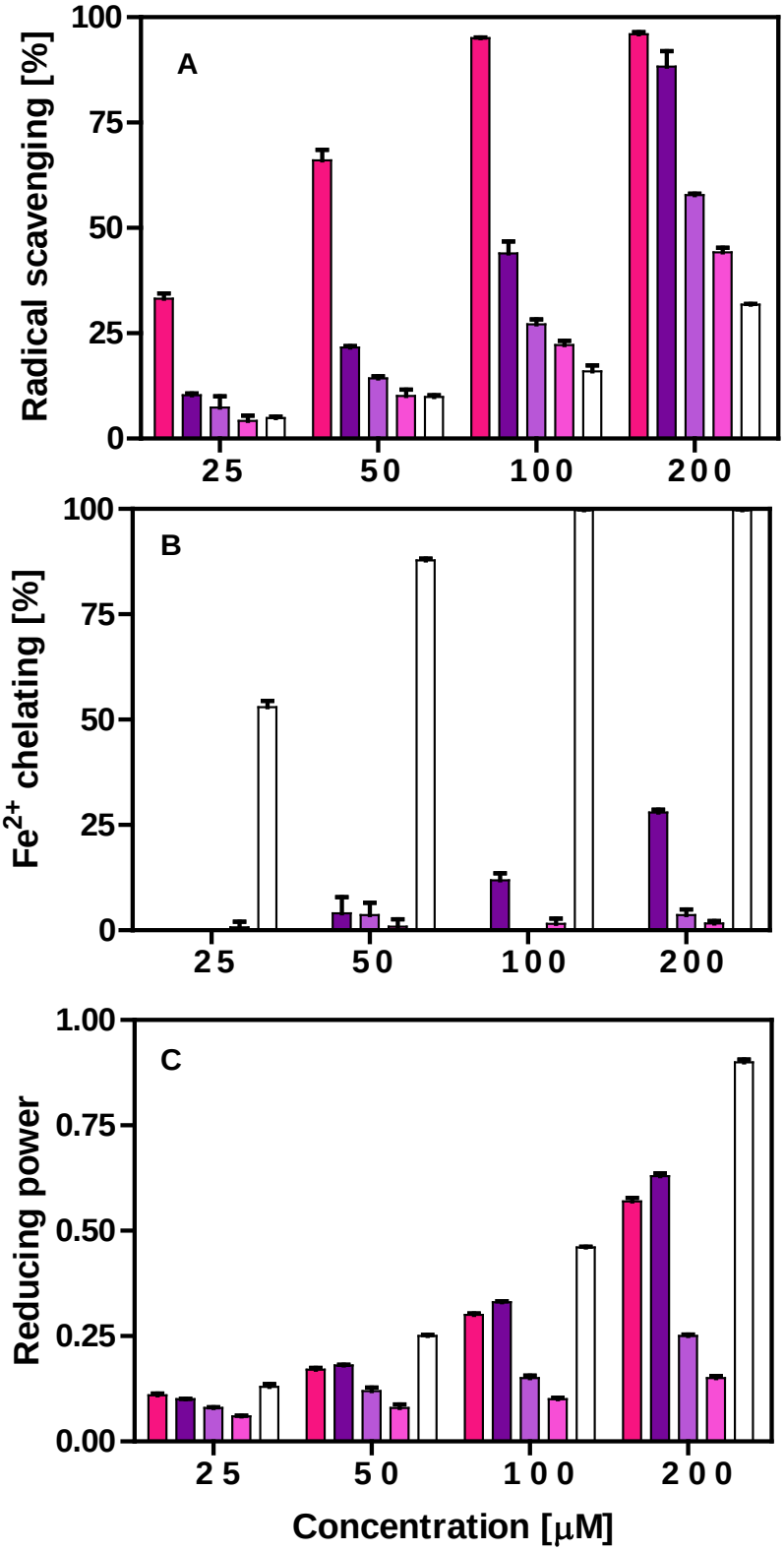

Figure 16 Antioxidant activities measured in different *in vitro* assays: A) Radical scavenging [%], B) Fe^{2+} chelating [%] and C) Reducing power. Sample codes: ■ Caffeic acid; ■ Dihydrocaffeic acid; ■ Octyl dihydrocaffeate, ■ Oleyl dihydrocaffeate and □ Positive control: A) BHT, B) EDTA and C) Ascorbic acid, respectively. Bars indicate SD of 3 measurements.

Moreover, in all three antioxidant assays, the activity was higher for octyl dihydrocaffeate than oleyl dihydrocaffeate. Lue et al. (2009) has reported the same tendency for the reducing power assay for rutin esterified with two acyl chains of different length: 12-carbon and 16-carbon. They suggested that the formation of micellar structures in the aqueous environment of the assay could explain the different effects of the two rutin esters. Thus, rutin palmitate was anticipated to form micelles at lower concentrations due to the longer acyl chain compared to rutin laurate, resulting in less free ester and consequently lower reducing power [Lue et al., 2009]. Furthermore, Yuji et al. (2007) has reported that CMC decreases with increasing alkyl chain length. Thus, the reduced antioxidant activity observed in this study for oleyl dihydrocaffeate with a long acyl chain compared to octyl dihydrocaffeate with a short acyl chain may be due to different degree of micelle formation.

Antioxidant efficacy of lipophilized dihydrocaffeic acid in o/w emulsion

Caffeic acid had the highest antioxidative activity followed by dihydrocaffeic acid, when measured with the DPPH and the reducing power *in vitro* assays. These two compounds also gave the lowest concentration of lipid hydroperoxides in o/w emulsions, which could indicate a slower formation of lipid hydroperoxides in emulsions with either caffeic acid or dihydrocaffeic acid. However, in this case it might more likely be due to a fast decomposition of lipid hydroperoxides, as deduced from the high concentration of volatiles generally observed in emulsions with these two compounds despite their high antioxidant activity measured in the different *in vitro* assays (Figure 17). Additionally, only these two compounds and not the lipophilized compounds were detected in the aqueous phase in the emulsion system. In contrast to non lipophilized compounds, the two lipophilized dihydrocaffeate had a poor antioxidative activity when measured with *in vitro* assays. However, o/w emulsions with these lipophilized compounds resulted in higher concentration of PV in the end of storage when compared to the more hydrophilic compounds, but a decreased concentration of volatiles. This seemed to indicate that decomposition of lipid hydroperoxides was slower than the formation of lipid hydroperoxides in these emulsions.

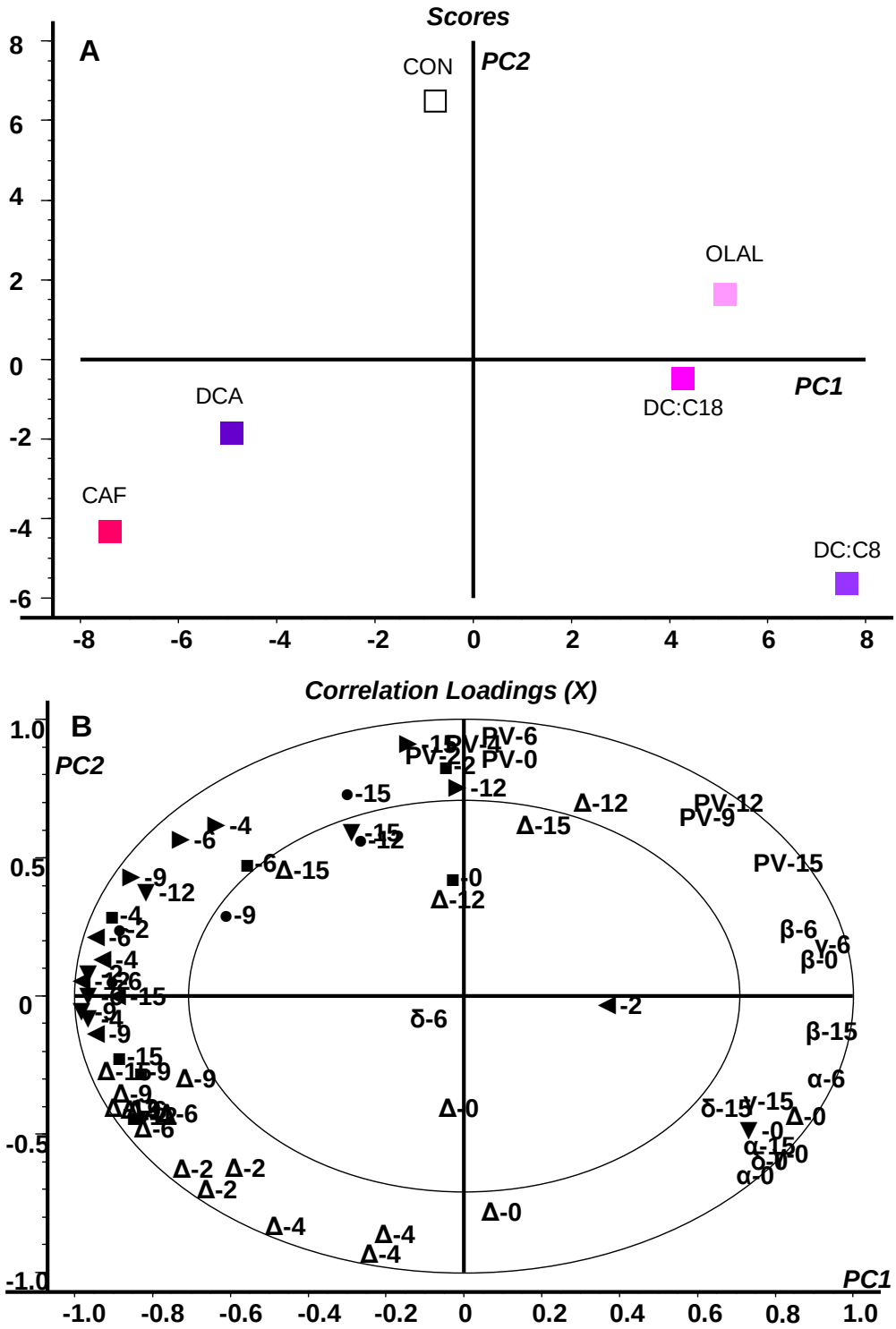

Figure 17 Sores (A) and Correlation loadings (B) graphs obtained by principal component analysis (PC 1 vs. PC 2) on PV, volatile and tocopherol results (full cross validation, X-explained variance: 50%, 27%). □ CON: Control (no antioxidant); ■ DCA: Dihydrocaffeic acid; ■ DC:C8: Octyl dihydrocaffeate; ■ DC:C18: Oleyl dihydrocaffeate; ■ OLAL: Oleyl alcohol and ■ CAF: Caffeic acid. Correlation loadings, the measured variables; PV: Lipid hydroperoxides; α-, β-, γ- and δ-: The 4 different tocopherol homologues; Δ: Saturated aldehydes (pentanal, hexanal and nonanal);●: 2 -pentenal; ■: 1-penten-3-ol; ▶: 4-heptenal; ◀: 1-octen-3-ol; ▼: 2,4-heptadienal. Numbers indicate storage time [days].

Our results partly support the polar paradox hypothesis, since emulsion with lipophilized dihydrocaffeic acid had a better oxidative stability when compared with emulsion prepared with the more hydrophilic parent compound, dihydrocaffeic acid. Comparing the two lipophilized compounds it was observed that octyl dihydrocaffeate worked better as an antioxidant than oleyl dihydrocaffeate even though neither of the compounds was detected in the aqueous phase in either of the systems used for measuring partitioning. Since oleyl alcohol was observed to be inactive or had an antioxidative effect in o/w emulsions, the slightly reduced effect observed for oleyl dihydrocaffeate in o/w emulsion compared with octyl dihydrocaffeate was most likely not due to impurities.

Lately the effect of lipophilized chlorogenic acids has been evaluated as antioxidant in o/w emulsion, where the effect of alkyl chain lengths from 1 to 20 carbon atoms were evaluated [Laguerre et al., 2009]. The highest oxidative stability was obtained with a 12 carbon atoms alkyl chain for lipophilized cholorogenic acid.

A more lipophilic chain with more than 12 carbon atoms esterified to chlorogenic acid decreased the oxidative stability of the emulsions. This was suggested to be due to micellization by the lipophilized compounds in the aqueous phase since the CMC is reduced when the compounds are more lipophilic. This phenomenon was termed the cut-off effect. Due to micellization these compounds may not be available at the oil-water interface [Laguerre et al., 2009]. The hypothesis was supported by the finding that higher concentrations of these compounds was observed in the aqueous phase of the emulsion [Laguerre et al., 2009;Yuji et al., 2007]. Another experiment with rutin and lipophilized rutin (rutin laurate (C12) and rutin palmitate (C16)) as antioxidants in o/w emulsion performed by Lue et al. (2010) did, however, not support the cut-off effect, since the esters were consistently less effective when compared with rutin.

In the present study, an alkyl chain length of 8 carbon atoms was esterified to dihydrocaffeic acid and this resulted in a better antioxidative protection than when esterified with a chain length of 18 carbon atoms. Hence, although, the present study only evaluated two alkyl chain lengths, it supported the new cut-off effect hypothesis previously suggested by Laguerre et al. (2009). The obtained partitioning results

indicated that none of the lipophilized dihydrocaffeic acid was present in the aqueous phase independent of alkyl chain lengths. These findings thus seemed to contradict the hypothesis that oleyl dihydrocaffeate formed micelles in the aqueous phase. Scince the lipid oxidation results supported the cut-off hypothesis, it may be suggested that possible formed oleyl dihydrocaffeate micelles were retained in the filter of the centrifuge tubes together with the oil and this could explain the findings for the partitioning experiment.

Differences in cut-off effects between different lipophilized compounds could be explained if rutin is a more hydrophobic compound than chlorogenic and dihydrocaffeic acids. The findings with lipophilized dihydrocaffeic acid and chlorogenic acid could then suggest that the cut-off effect for an ester produced from rutin occurs with a shorter alkyl chain length than 12 carbon atoms. Thus, it is possible that rutin laurate also participated in micellization in the aqueous phase and that this could explain why lipophilization of rutin did not increase its efficacy as an antioxidant in fish oil enriched o/w emulsions.

Antioxidant efficacy of lipophilized dihydrocaffeic acid and rutin in milk

The results from PV and volatile analyses indicated that both lipophilized dihydrocaffeic acid and rutin had a better antioxidative effect in fish oil enriched milk compared with their more hydrophilic parent compound: dihydrocaffeic acid and rutin, respectively (Figure 18).

Both octyl dihydrocaffeate and oleyl dihydrocaffeate exerted stronger antioxidative effects than dihydrocaffeic acid in fish oil enriched milk. The efficacy of octyl dihydrocaffeate was slightly better than oleyl dihydrocaffeate. These findings are similar to the findings obtained for these compounds in a simple o/w emulsion. Thus, the cut-off effect with the optimal alkyl chain length below C18 long seemed to be confirmed for dihydrocaffeate in fish oil enriched milk. However, oleyl dihydrocaffeate contained impurities such as oleyl alcohol. Since oleyl alcohol was inactive or acted as a prooxidant in fish oil enriched milk, it might have reduced the antioxidative effect of oleyl dihydrocaffeate. Thus, the antioxidative effect may have been better for oleyl dihydrocaffeate if a more purified compound was used, but this needs to be further studied.

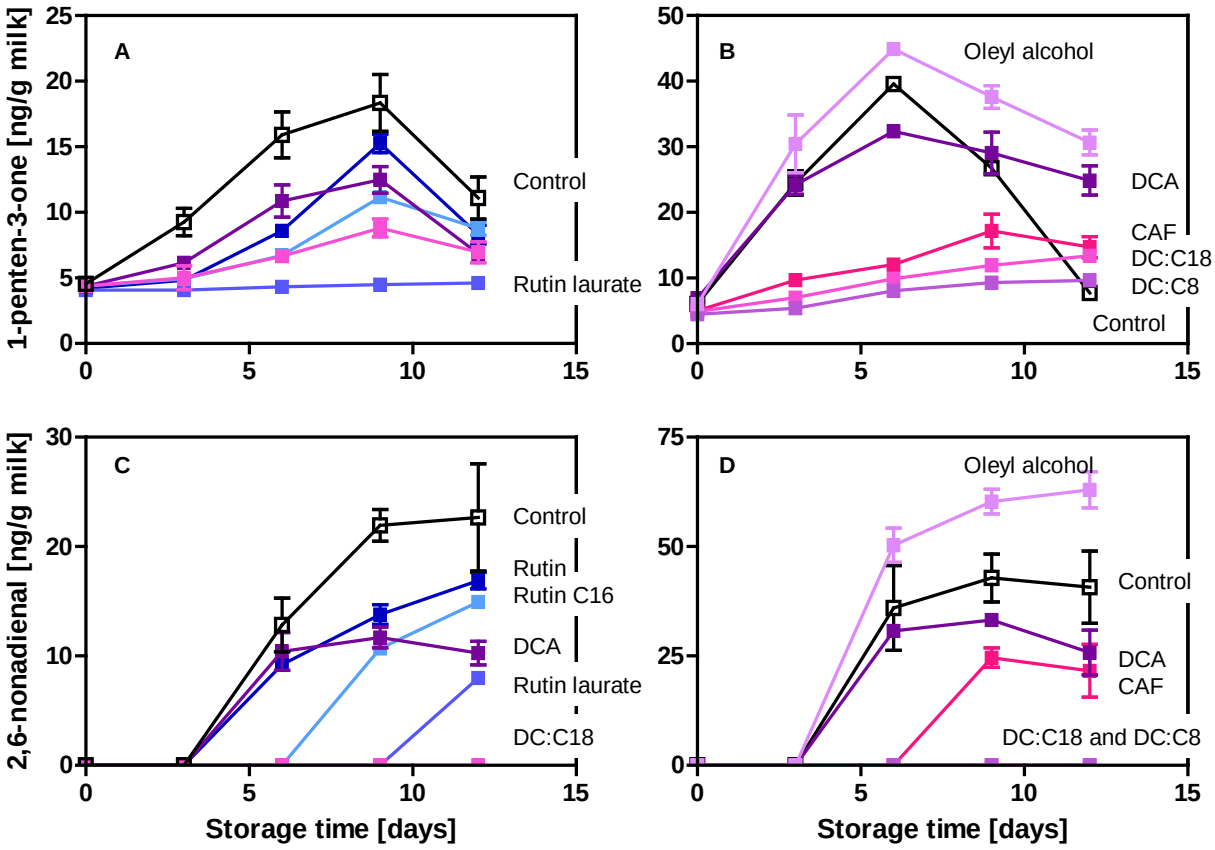

Figure 18 Concentrations [ng/g milk] of A) 1-penten-3-one (acetone added), B) 1-penten-3-one (no acetone), C) 2,6-nonadienal (acetone added) and D) 2,6-nonadienal (no acetone). Sample codes: □ Control (no antioxidant); ■ Rutin; ■ Rutin laurate (C12); ■ Rutin palmitate (C16); ■ DCA Dihydrocaffeic acid; ■ DC:C8 Octyl dihydrocaffeate; ■ DC:C18 Oleyl dihydrocaffeate; ■ CAF Caffeic acid and ■ OLAL Oleyl alcohol. Bars indicate SD of 3 measurements.

Interestingly, findings regarding the efficacy of rutin esters as antioxidants were different in fish oil enriched milk compared with the simple o/w emulsion. In fish oil enriched milk, both rutin esters had an antioxidative effect, but rutin laurate was a more efficient antioxidant than rutin palmitate. In contrast, these rutin esters were less effective antioxidants when compared with rutin in o/w emulsion [Lue et al., 2010b]. Moreover, rutin laurate also exerted stronger antioxidative activity than rutin and rutin palmitate in a LDL assay which is a more complex system than an o/w emulsion [Lue et al., 2009]. Thus, these findings indicate that the cut-off effect might be influenced by the system i.e. simple o/w emulsion or more complex emulsion systems such as LDL and milk and that complexity of the system affect the cut-off effect.

6.2.3 EFFECT OF SPICES, SPICE AND GREEN TEA EXTRACTS ON THE OXIDATIVE STABILITY OF FISH OIL ENRICHED FOODS

Investigations on spices, spice and green tea extracts in fish oil enriched tuna salads and milk aimed at revealing if natural antioxidants could increase the oxidative stability of complex fish oil enriched food emulsions.

Fish oil enriched tuna salads (Paper I and Appendix I)

Several experiments have shown that spices or extracts based on spices possess antioxidative activity [Bhale et al., 2007;Jimenez-Alvarez et al., 2008;Kulisic et al., 2005;Tsimidou et al., 1995;Zheng and Wang, 2001]. However, no studies have been performed in complex food matrices such as tuna salads.

Our studies showed that addition of 1% of oregano, thyme or rosemary to fish oil enriched tuna salads slowed down the formation of volatiles during storage (Figure 19), indicating that addition of spices could increase the oxidative stability compared to the standard tuna salad with fish oil. On the basis of the volatiles data it could be concluded that oregano had the strongest antioxidative effect followed by rosemary and thyme. Thus, the hypothesis that dry spices improve the oxidative stability of fish oil enriched tuna salad was confirmed.

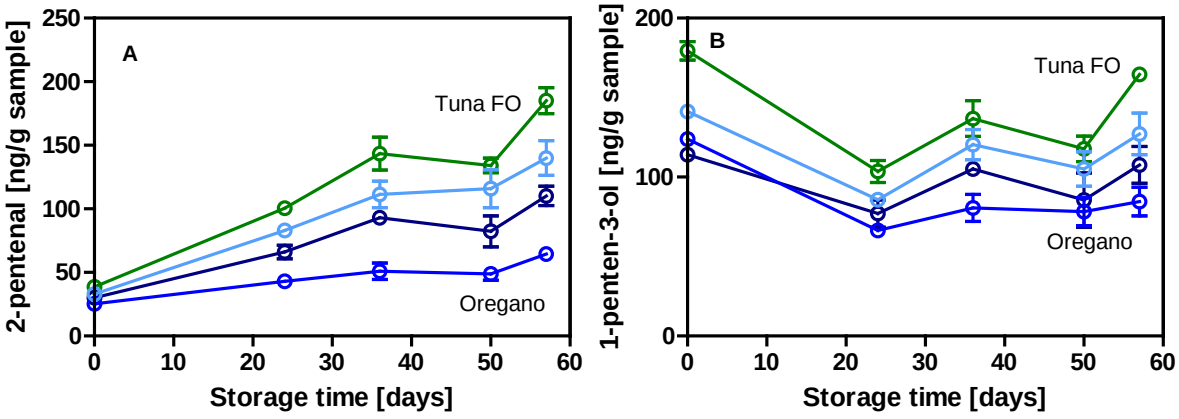

Figure 19 Lipid oxidation measured in fish oil enriched tuna salads during storage: concentration [ng/g sample] of 2-pentenal A) and 1-penten-3-ol B). Sample codes: ○ Tuna FO Tuna standard salad with fish oil; ○ Tuna FO + 1% rosemary; ○ Tuna FO + 1% oregano and ○ Tuna FO + 1% thyme. Bars indicate SD of 3 measurements.

However, the dry spices lead to undesirable flavors from the spices in the traditional tuna salads. Therefore the antioxidative effect of a flavor-less oregano extract, origanox, was evaluated. Three different concentrations were evaluated and compared with a standard tuna salad with fish oil. In addition, the effect of EDTA (75 mg/kg mayonnaise) was compared with the effect of origanox. Results from *in vitro* antioxidant assays showed that origanox possessed metal chelating, radical scavenging and reducing activity. Moreover, a sensory expert panel could not detect significant lipid oxidation odor or flavor and oregano flavor when origanox was used in tuna salads. However, results of volatile oxidation products measured during storage revealed that addition of EDTA or origanox to tuna salads did not affect the formation of secondary lipid oxidation products when compared with standard tuna salad. In the tuna salad used for this experiment, rapeseed oil was used and in the tuna salads prepared with dry spices, soy oil was used. Rapeseed oil is less unsaturated than soy oil and this may explain why oxidation was less pronounced in the experiment with origanox and therefore why it was not possible to detect any clear effect of origanox. Even though the producer of origanox has shown an antioxidative effect on origanox and other studies reported with oregano extracts has shown antioxidative effects, this could thus not be confirmed in the present study.

Fish oil enriched milk (Appendix II)

The antioxidative effect of two different rosemary extracts and one green tea extract was evaluated in fish oil enriched milk in three different concentrations (100, 300 and 500 mg/kg product). Irrespective of the concentration used, all three extracts increased the oxidative stability of fish oil enriched milk. Differences in concentration of oxidation products immediately after production indicated that the extracts acted as antioxidants under the production of the fish oil enriched milk. Of the evaluated extracts, the most efficient was green tea at a concentration of 500 mg/kg, and the effect of this concentration of extracts is illustrated in Figure 20.

The sensory evaluation performed by an expert panel showed that all the milk samples had a fishy taste, but milk samples containing natural extracts also tasted of either green tea or rosemary. However, all milk samples with natural extracts were evaluated to have a lower fishy taste. Thus, the results confirmed the hypothesis that the extracts could

improve the oxidative stability of fish oil enriched milk. However, these natural extracts are not the most appropriate as antioxidants in fish oil enriched milk since they gave an after-taste of rosemary or green tea.

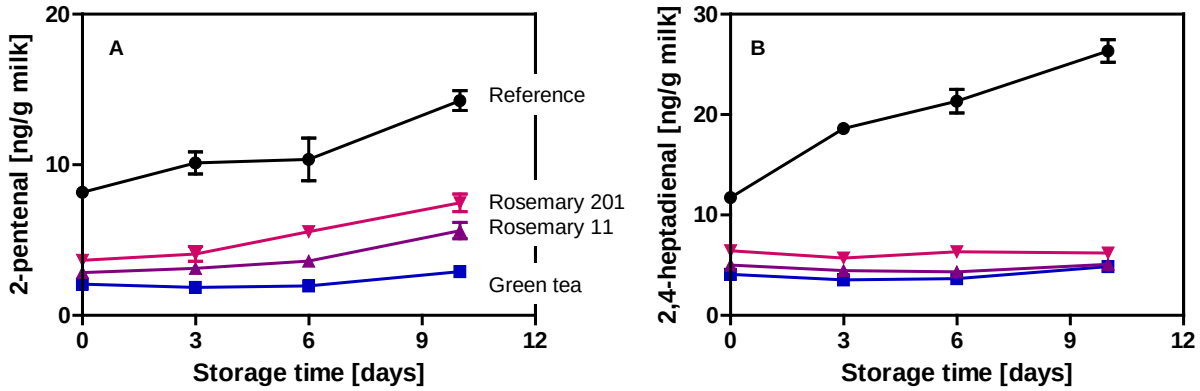

Figure 20 Lipid oxidation measured in fish oil enriched milk during storage: concentration [ng/g milk] of 2-pentenal A) and 2,4-heptadienal B). Sample codes: ● Reference (no extract added); ■ Milk with green tea (500 mg/kg); ▲ Milk with rosemary 11 (500 mg/kg) and ▼ Milk with rosemary 201 (500 mg/kg). Bars indicate SD of 3 measurements.

6.2.4 DISCUSSION OF FINDINGS IN RELATION TO HYPTHESES

As hypothesized ascorbic acid (hydrophilic antioxidant) was more efficient in preventing lipid oxidation in w/o than o/w emulsions. This was explained by the higher concentration of ascorbic acid in the aqueous phase in w/o emulsion and hence closer locations to the oil-water interface. Additionally, the findings indicate that the polar paradox is not always valid even in simple emulsions, since ascorbyl palmitate and ascorbic acid were the most efficient antioxidant in the beginning of the storage period and because ascorbyl CLA was practically inactive in the o/w emulsions. This will be discussed further below.

It was confirmed that lipophilization of dihydrocaffeic acid changed the partitioning of the antioxidants into different phases of an o/w emulsion compared with its parent compound. None of the lipophilized compounds were detected in the aqueous phase of an o/w emulsion. This was suggested to be due to the partitioning of the lipophilized compound into the oil-water interface or oil phase. Another possibility could be micelle formation in the aqueous phase, if micelles formed in the aqueous phase were retained

in the filter of the centrifuge tube together with the oil phase. However, further partitioning studies has to be performed in order to confirm if the micelle were formed in the aqueous phase.

Lipid oxidation in o/w emulsion was affected by the type of antioxidants employed. The hypothesis that lipophilization of phenolic compounds will increase the oxidative stability of o/w emulsion compared with their parent compound was confirmed. Octyl dihydrocaffeate and oleyl dihydrocaffeate were stronger antioxidants than dihydrocaffeic acid, which acted as a prooxidant. The stronger antioxidative effect of octyl dihydrocaffeate compared to oleyl dihydrocaffeate supported the cut-off effect suggested by Laguerre et al. (2009) in relation to the length of the acyl chain esterified to the phenolic compound. In fish oil enriched milk octyl dihydrocaffeate was also a more efficient antioxidant compared to oleyl dihydrocaffeate, however, differences in their antioxidant efficacy was not as big as observed in the o/w emulsions. Oleyl alcohol had no effect or behaved like an antioxidant in o/w emulsions, whereas in fish oil enriched milk oleyl alcohol acted as a prooxidant. Thus, the optimal acyl chain length for lipophilized dihydrocaffeic acid may be different for fish oil enriched milk than for o/w emulsion, which was below C18 long for the o/w emulsion. Despite the lack of antioxidative effect of the rutin esters in o/w emulsions [Lue et al., 2010b], they were better antioxidants than rutin in fish oil enriched milk. Due to the antioxidative effect of lipophilized phenolics in o/w emulsions and in fish oil enriched milk our findings supported the suggested cut-off effect. Interestingly, the cut-off effect seems not only to be specific to individual phenolic compound that is lipophilized, but seemed also to depend on the emulsion system used e.g. simple emulsions compared to complex food emulsions.

The finding that the cut-off effect also depended on the system, in which the lipophilized compounds was evaluated, may also help at explaining the lack of antioxidative effect of ascorbyl CLA in o/w emulsion compared to w/o emulsion. Moreover, the earlier findings with ascorbyl esters by Liu et. Al. (1998) indicated that the optimal chain length at least in LDL oxidation was C12, which was more efficient than acorbyl ester with C16 or C8. Thus, it may be plausible that ascorbyl esters used in our experiment had too long acyl

chains and therefore the effect was decreasing with increasing chain length due to micelle formation in the aqueous phase instead of being located at the oil-water interface or in the oil phase. However, this has to be supported by further research with oxidation studies for optimal chain length and additional partitioning studies.

The last hypotheses deal with the antioxidative effect of spices, spice and green tea extracts in improving the oxidative stability of complex food systems such as fish oil enriched tuna salads and milk. Oregano, thyme and rosemary increased the oxidative stability of fish oil enriched tuna salads. Moreover, rosemary and green tea extracts also improved the oxidative stability of fish oil enriched milk. Thus, these findings then confirmed the hypotheses. However, dry spices and extracts led to undesirable spice flavors in the traditional tuna salads and milk. Therefore, a tasteless oregano extract, origanox, was additionally evaluated in tuna salads. Although oregano had the strongest antioxidative activity, origanox did not improve the oxidative stability of fish oil enriched tuna salads. This may be explained by the change in oil used for the mayonnaise, from soy oil to less unsaturated oil, rapeseed oil. The changes in type of oil might have resulted in less lipid oxidation, and thereby resulted in such an unclear effect of origanox. However, the effect of origanox should be evaluated further for its antioxidative effect in tuna salads with soy and fish oil in order to clarify our findings.

CHAPTER 7 CONCLUSIONS AND PERSPECTIVES

The main objective of this PhD work was to expand current knowledge on oxidative stabilization of fish oil enriched food systems. Many new findings were reported through this research and may influence future research in this area.

Influence of ingredients

The hypothesis that individual ingredients in fish oil enriched mayonnaise salads can improve the oxidative stability was only partly confirmed.

Pure mayonnaise with and without fish oil was less oxidized than the mayonnaise salads. Although the asparagus had an antioxidative effect in fish oil enriched shrimp salads, its effect was neutralized by the prooxidative effect of shrimps. In fish oil enriched tuna salads it was unclear if the vegetables resulted in more lipid oxidation due to the high concentration of volatiles already from the beginning. It was suggested that the higher level of volatiles more likely was due to the natural flavor profile of the vegetables. For further conclusion on the influence of vegetables in tuna salads more research is needed. As described earlier, the evaluation of the influence of ingredients in tuna salads could be improved by creating a calibration curve for the volatiles directly in the specific tuna salad, since the natural flavor profile of the vegetables would then be subtracted.

Interestingly, these experiments conclude that it is possible to substitute 10% soy oil with fish oil in tuna and shrimp salads stored at 2°C with a shelf-life of 6 weeks, which are the current shelf-life used by several manufactures. However, additional experiments are needed to evaluate the effect of different factors such as storage temperature in the super market and exposure to light, which might also influence the oxidative stability.

One ingredient in the mayonnaise, the egg yolk, was substituted with a milk protein based emulsifier with lower iron content. It was hypothesized that this substitution could

improve the oxidative stability of light mayonnaise. However, the hypothesis was not confirmed with this milk protein based emulsifier. The main reason is most likely the low quality of the milk protein based emulsifier, which had 50 times higher lipid hydroperoxide content than the egg yolk. Thus, it is concluded that not only the iron content, but also the initial oxidative quality of the ingredients is crucial for the final oxidative stability of fish oil enriched mayonnaise. The milk protein based emulsifier is used in some mayonnaises instead of egg yolk. However, the quality of the milk protein should be measured and taken into consideration if a good oxidative stability of mayonnaise, even without fish oil, should be maintained throughout the storage period. To determine whether the oxidative stability can be improved by substitution of the egg yolk with a milk protein mixture of a better quality than the milk protein based emulsifier used here needs further investigated.

Both the storage temperature (2 and 20°C) and fish oil concentration (4, 10 and 14%) influenced the oxidative stability of the fish oil enriched light mayonnaises. As hypothesized, increased storage temperature and increased fish oil concentration both led to oxidatively less stable mayonnaise. Due to the marked lipid oxidation measured in light mayonnaises stored at 20°C, this storage temperature is not recommended, although this is common practice in supermarkets today.

Interestingly, light mayonnaises enriched with 4% fish oil stored at 2°C was not more oxidized than mayonnaises without fish oil as concluded from both the PV and levels of volatiles. Thus, it was possible to enriched light mayonnaise with 4% fish oil without adding antioxidants if the mayonnaises were stored at low temperature. However, this has to be confirmed by sensory analysis.

Antioxidant protection

Based on the polar paradox, the hypothesis that a hydrophilic antioxidant is more efficient in a w/o than an o/w emulsion, was successfully confirmed.

The improved protection provided by ascorbic acid in w/o compared to o/w emulsions was due to the higher concentration of ascorbic acid in the aqueous phase in w/o emulsion and hence closer location to the oil-water interface. However, the findings indicated that the polar paradox is not always valid even in simple emulsions, since both ascorbyl palmitate and ascorbic acid was equally efficient as antioxidants in o/w emulsions in the beginning of the storage period whereas ascorbyl CLA was practically inactive. The PV and volatile results measured in o/w emulsion with ascorbyl CLA compared to ascorbyl palmitate thus supported the cut-off phenomenon of lipophilized compounds in relation to the acyl chain length of the alcohol esterified to the phenolic compound. The cut-off effect means that when the hydrophobicity of the lipophilized compound increases above a certain level, the lipophilized compound is suggested to form micelles in the aqueous phase. The antioxidants that have formed micelles in the aqueous phase will not be located in the interface.

As hypothesized lipophilization changed the partitioning of the antioxidants into the different phases of an o/w emulsion compared with their parent compounds. Neither of the lipophilized compounds was detected in the aqueous phase of the o/w emulsions. These findings indicated that the lipophilized compounds were located in the oil-water interface or in the oil phase.

The hypothesis that lipophilization of phenolic compound will increase the oxidative stability of o/w emulsion compared with its parent compound was confirmed. Octyl dihydrocaffeate and oleyl dihydrocaffeate were stronger antioxidants than dihydrocaffeic acid, which acted as a prooxidant in the o/w emulsion. The differences in the antioxidative effect of octyl dihydrocaffeate and oleyl dihydrocaffeate supported a cut-off effect, where esterification with a C8 acyl chain resulted in a more efficient antioxidant than esterification with a C18 acyl chain. In fish oil enriched milk octyl dihydrocaffeate was also more efficient as antioxidant compared to oleyl dihydrocaffeate, however, the differences in their antioxidant efficacy were not as distinct as observed for the o/w emulsions.

Furthermore, rutin esters were better antioxidants than rutin in fish oil enriched milk and the same effect of the chain length as observed for dihydrocaffeate was also observed for rutin.

A very interesting conclusion based on findings in this PhD work was that the cut-off effect was not only specific for the individual lipophilized phenolic compounds, but also depends on the emulsion system e.g. simple emulsions and complex food emulsions. This was supported by earlier work reported by Lue (2009). However, to be able to conclude on the optimal alkyl chain esterified to dihydrocaffeate and rutin in relation to their strongest antioxidant protection, further research is needed with several different alkyl chain lengths and in a range of different emulsion systems.

Although the lipid oxidation data supported the cut-off phenomenon, the conclusions from the partitioning results were less clear, since the results just indicate that the lipophilized compounds were not detected in the aqueous phase. Therefore, it was assumed that the lipophilized compounds were located at the interface or in the oil phase. However, it is also possible that micelles were formed in the aqueous phase, and such micelles could be retained together with the oil phase in the filter of the centrifuge tube used to separate the emulsion phases before analysis. Further partitioning studies has to be performed in order to confirm the micelle formation in the aqueous phase.

Spices, spice and green tea extracts improved the oxidative stability of complex food systems such as fish oil enriched tuna salads and milk. Oregano, thyme and rosemary increased the oxidative stability of fish oil enriched tuna salads. Moreover, rosemary and green tea extracts also improved the oxidative stability of fish oil enriched milk. Although oregano had the strongest antioxidative activity, origanox (tasteless oregano based extract) did not improve the oxidative stability of fish oil enriched tuna salads. This may be explained by the change in oil from soy oil to a less unsaturated oil, rapeseed oil. The changes in type of oil might reduce the lipid oxidation and thereby result in the unclear effect of origanox. However, the effect of origanox should be evaluated further for its antioxidative effect in tuna salads with soy and fish oil.

The dry spices and rosemary and green tea extracts led to undesirable flavors in the traditional tuna salads and milk. Therefore, their use as antioxidant has to be considered for the specific product.

Overall, the PhD work has contributed with interesting findings regarding oxidative stabilization of fish oil enriched food systems. Amongst the alternatives investigated, the most promising way to reduce lipid oxidation of fish oil enriched food emulsions seems to be addition of lipophilized antioxidants and spice extracts, as long as they do not add additional flavor to the product. More work on lipophilization of other antioxidants than those investigated in the present thesis and investigations on their effects in real food systems is required to reveal the full potential of this strategy. Modifying the recipe by selecting alternative ingredients could also be a possible way to increase shelf-life of fish oil enriched food emulsions; however, this alternative is not easy to evaluate and requires extensive work. Substitution of ingredients may result in a different structure of the food matrix, and thereby change the release of volatile compounds. Thus, if this way is selected then a lot of efforts have to be put into studying the effect of the ingredients on volatile release.

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PAPERS AND APPENDICES

Paper I **Sørensen, A.-D.M.**, Nielsen, N.S. & Jacobsen, C. Oxidative stability of fish oil enriched mayonnaise based salads. *European Journal of Lipid Science and Technology,* 2010, 112, 476-487.

Paper II **Sørensen, A.-D.M.**, Nielsen, N.S., Hyldig, G. & Jacobsen, C. The influence of emulsifier type on lipid oxidation in fish-oil-enriched light mayonnaise. *European Journal of Lipid Science and Technology,* 2010, 112, 1012-1023.

Paper III **Sørensen, A.-D.M.**, Nielsen, N.S., Decker, E.A., Let, M.B, Xu, X. & Jacobsen, C. The efficacy of compounds with different polarities as antioxidant in emulsions with fish oil. *Journal of American Oil Chemists Society, Accepted 2010 (DOI: 10.1007/s11746-010-1696-5).*

Paper IV **Sørensen, A.-D.M.**, Nielsen, N.S., Yang, Z., Xu, X. & Jacobsen, C. The effect of lipophilization of dihydrocaffeic acid on its antioxidative properties in fish oil enriched emulsion. *European Journal of Lipid Science and Technology, Submitted 2011.*

Paper V **Sørensen, A.-D.M.**, de Diego, S., Petersen, L.K., Nielsen, N.S., Lue, B.-M., Yang, Z., Xu, X. & Jacobsen, C. The antioxidative effect of lipophilized dihydrocaffeic acid and rutin in fish oil enriched milk. *In preparation*.

Appendix I Oxidative stability of fish oil enriched tuna salads with origanox

Appendix II Effect of rosemary and green tea extracts on the oxidative stability of fish oil enriched milk

PAPER I

Sørensen, A.-D.M., Nielsen, N.S. & Jacobsen, C.

Oxidative stability of fish oil enriched mayonnaise based salads.

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Research Article

Oxidative stability of fish oil-enriched mayonnaise-based salads

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The oxidative stability of fish oil-enriched mayonnaise-based salads and the influence of different vegetables in shrimp and tuna salads were evaluated. Moreover, the lipid oxidation in the presence of 1% oregano, rosemary, or thyme in fish oil-enriched tuna salad was assessed. The results obtained showed that the mayonnaise itself was more oxidatively stable without vegetables and tuna or shrimp, in spite of the higher oil content in mayonnaise (63 and 6.3% fish oil, respectively) compared to salads (~24 and 2.4% fish oil, respectively). Surprisingly, the fish oil-enriched mayonnaise was only significantly different from the standard mayonnaise in the volatile concentration during the end of storage. In fish oil-enriched shrimp salad, asparagus had an anti-oxidative effect and shrimp a pro-oxidative effect, where the anti-oxidative effect of asparagus was strong enough to prevent the pro-oxidative effect of shrimp. The effect of ingredients in tuna salads was inconclusive, possibly due to a high content of volatiles in the vegetables themselves. However, the addition of spices increased the oxidative stability of tuna salad (oregano>rosemary>thyme).

Keywords: Fish oil / Ingredients / Lipid oxidation / Mayonnaise salads / Spices

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1 Introduction

Due to the low intake of fish in the Western countries and the beneficial health effects of $n-3$ long-chain polyunsaturated fatty acids (LC-PUFA), there is a growing market for $n-3$-enriched food products. However, addition of fish oil might introduce stability problems due to lipid oxidation of the highly unsaturated fatty acids (EPA and DHA). A way to overcome this problem could be to use antioxidants, either by adding antioxidants to the fish oil-enriched product or adding fish oil to a product that contains ingredients with natural compounds that possess anti-oxidative characteristics themselves, *e.g.*, phenols from vegetables or spices [1].

Earlier experiments with fish oil-enriched mayonnaise showed that it developed off-flavors much faster than mayonnaise without fish oil added. Several studies have been carried out to study the effect of different antioxidants (tocopherol, ascorbic acid, a mixture of ascorbic acid, lecithin and tocopherol, ascorbyl palmitate, gallic acid, propyl gallate, lactoferrin, phytic acid, and EDTA) in fish oil-enriched mayonnaise [2–6]. Among these antioxidants, only EDTA was shown to be an efficient antioxidant in mayonnaise. Thus, the fishy flavor formation was reduced up to 94% in fish oil-enriched mayonnaise due to the metal-chelating activity of EDTA [7]. However, EDTA is a synthetic compound and there is a trend in consumer preference for natural ingredients. As a consequence, there is a growing interest in the use of natural antioxidants. In addition, Jacobsen *et al.* [5] have proposed that iron from egg yolk, the emulsifier in mayonnaise, combined with low pH are the predominant factors responsible for lipid oxidation in fish oil-enriched mayonnaise. Thus, antioxidants for use in fish oil-enriched mayonnaise need to have metal-chelating activity in order to eliminate or reduce lipid oxidation in this type of food product.

Vegetables, fruits, and spices, all contain phenolic compounds, which are known to have antioxidant activity. However, their activity and mechanism of action depend on the structure of the phenols: the location and number of hydroxyl (–OH) groups and the nature of substitutions on

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Abbreviations: PCA, principal component analysis; **PLSR,** partial least squares regression; **PV,** peroxide value

the aromatic rings [8]. Several experiments have indicated that different vegetables and spices, such as sweet pepper (bell pepper), asparagus, snow peas, oregano, thyme, and rosemary, possess anti-oxidative activity [9–17]. Moreover, Zheng and Wang [13] observed a positive linear correlation between the phenolic content and the antioxidative capacity of the above-mentioned spices. Vegetables such as those mentioned above are often used in complex food systems like mayonnaise-based salads. However, the influence of such ingredients on lipid oxidation in complex food systems has not received much attention. Thus, there is a gap in knowledge in this area and research is needed for more successful development of *n*−3-enriched foods.

Due to the anti-oxidative effects observed for different vegetables and spices, we hypothesize that addition of vegetables to fish oil-enriched mayonnaise-based salads might increase their oxidative stability and that the oxidative stability might be further improved by adding spices to the mayonnaise-based salad. Therefore, the aims of the present study were to evaluate the oxidative stability of fish oil-enriched mayonnaise-based salads and to study the influence of different ingredients (vegetables) on lipid oxidation in shrimp and tuna salads enriched with fish oil. The oxidative stability of the salads was compared with that of mayonnaise. Furthermore, the objective was to study the anti-oxidative effects of three different types of commercial dried spices: oregano, rosemary, and thyme. These spices were tested at a concentration of 1% in fish oil-enriched tuna salad.

2 Material and methods

2.1 Materials

Cod liver oil was supplied by Maritex (Sortland, Norway). The fish oil had a peroxide value (PV) of 0.3 meq peroxides/kg oil, a tocopherol content of 202 mg α-tocopherol/kg, 9 mg β-tocopherol/kg, and 57 mg γ-tocopherol/kg, as measured according to the official AOCS method Ce 8-89 [18], and the fatty acid composition was as follows: 14:0, 3.4%; 16:0, 9.5%; 16:1, 6.0%; 18:0, 2.1%; 18:1, 25.1%; 18:2, 3.9%; 18:3, 2.0%; 18:4, 2.1%; 20:1, 11.3%; 20:5 (EPA), 7.0%; 22:1, 14.7%; and 22:6 (DHA), 10.5%, as measured according to the official AOCS methods Ce 2–66 [19] and Ce 1b–89 [20]. The total percentages of $n-3$ and $n-6$ in this oil were 23.6 and 4.3%, respectively. The soya oil had a PV of 0.2 meq peroxides/kg oil, a tocopherol content of 194 mg α-tocopherol/kg, 37 mg β-tocopherol/kg, 621 mg γ-tocopherol/kg and 101 mg δ-tocopherol/kg, and the fatty acid composition was as follows: 16:0, 11.0%; 18:0, 3.3%; 18:1, 24.7%; 18:2, 52.5%; and 18:3, 5.2%. The total percentages of $n-3$ and $n-6$ in this oil were 5.5 and 52.6%, respectively.

Soya oil, other ingredients for the mayonnaise, shrimp salad and tuna salad preparation, and dry spices were purchased from a local supermarket. The shrimps were cooked cold-water shrimps in brine (water, salt, acidifying agent: citric acid, preservatives: potassium sorbate and sodium benzoate) and the tuna used were tuna chunks in brine (water and salt). Furthermore, the manufacturer had added citric acid to the asparagus in brine (water, salt, and citric acid) and red bell pepper in brine (water, vinegar, sucrose, salt, citric acid, and calcium chloride).

The chemicals were from Merck (Darmstadt, Germany) or Amersham Biosciences (Uppsala, Sweden). The external standards for identification of volatile oxidation products were all from Sigma–Aldrich (Steinheim, Germany). All solvents were of HPLC grade (Lab-Scan, Dublin, Ireland).

2.2 Production of mayonnaise, shrimp, and tuna salads

2.2.1 Mayonnaise with or without fish oil added

Ingredients for the mayonnaise preparation were (all amounts are stated in wt%): water (30%), oil (soya oil 56.7% and cod liver oil 6.3% or soya oil 63%), pasteurized egg yolk (5%), tarragon vinegar, salt, and thickeners (modified starch, guar gum, xanthan gum). Sourness was adjusted to pH 3.5–4.1 with lactic acid, sodium acetate, and malic acid. Potassium sorbate and sodium benzoate were used for preservation. The ingredients were mixed under vacuum using a Frymakoruma (Romaco, Karlsruhe, Germany). Mayonnaise samples for the storage experiment were packed in transparent plastic trays (175 g) with lids. The mayonnaises used for the preparation of shrimp and tuna salads were prepared in the same way.

2.2.2 Shrimp salad with or without fish oil added

The full recipe for shrimp salads was: mayonnaise (with or without fish oil) 48%, shrimps 40%, and asparagus 12%. To adjust sourness to pH 4.4–5.3, citric acid was added. All the ingredients were carefully mixed and weighed into the same type of plastic trays (175 g each) with lids, as used for the mayonnaise.

2.2.3 Tuna salad with or without fish oil added

The full recipe for the tuna salads was: mayonnaise (with or without fish oil) 34%, tuna 32%, peas 10%, maize 10%, bell pepper (red) 10%, and onion 0.6%. To adjust sourness to pH 5.1–5.5, ascorbic acid and citric acid were added. All the ingredients were carefully mixed and weighted into plastic trays (175 g each) with lids, as described above. In the tuna salad with spices, 1% of the respective spice was added together with the other ingredients before mixing.

2.3 Storage experiment

Samples were stored at 2°C for 57 days in the dark. The length of the storage period was set according to the shelf life

of commercial mayonnaise salads of 8 weeks. Samples of mayonnaise, shrimp salad, and tuna salad were taken for analyses at fixed time points: 0, 10, 24, 36, 50, and 57 days of storage; however, the sensory analysis was performed at day 2 and not day 0. Droplet size measured on the mayonnaise samples and sensory evaluation of shrimp and tuna salads were performed on the respective days, whereas for other analyses the samples were kept at −40°C until use. Table 1 shows the experimental design including specification of the percentages of ingredients in the different salads. When an ingredient were kept out of the sample, it was just excluded from the recipe and no further adjustment of the amount of other ingredients was made. This resulted in different oil contents in the samples as shown in Table 1.

2.4 Droplet size

The droplet size was measured in the two types of mayonnaise by dissolving 1 g mayonnaise in 9 g SDS buffer (10 mM NaH_2PO_4, 5 mM SDS, pH 7) [21]. The solution was whirly mixed for 0.5 min; thereafter the samples were placed in an ultrasonic bath (30°C, 20 min). This mixing procedure was repeated. The droplet size was measured by laser diffraction with a Mastersizer2000 (Malvern Instruments, Worcestershire, UK). The instrument was set up with a refractive index of material of 1.4694 (sunflower oil) and of dispersant of 1.3333 (water). A few droplets of mayonnaise-SDS buffer solution were suspended directly in the recirculating water (3000 rpm, obscuration 12–14%). The results are given in surface area mean diameter $D[3,2] = \sum d^3/\sum d^2$. The analysis was performed in duplicate.

2.5 Extraction of lipids

Prior to the extraction, the mayonnaise salads were chopped in a mincer (Knifetec 1095 Sample Mill; FOSS, Sweden) until homogeneity was obtained (3 × 5 s). Lipids were extracted from the mayonnaise and mayonnaise salads according to the method described by Bligh and Dyer [22], using 10 g mayonnaise or mayonnaise salad for each extraction and a reduced amount of solvent [23]. The extracts were used for the determination of PV.

2.6 Analysis of primary oxidation products, PV

PV were determined in the lipid extract or oil by colorimetric determination of the ferric-thiocyanate complex at 500 nm (Shimadzu UV-160A spectrophotometer; Struers Chem A/S, Denmark) according to Shantha and Decker [24].

2.7 Analysis of volatile secondary oxidation products

Separation and identification of volatiles were performed by dynamic headspace GC-MS. The mayonnaise salads were chopped in a mincer for 3 × 5 s (Knifetec 1095 Sample Mill; FOSS, Sweden) prior to the analysis. Volatiles were collected

Table 1. Experimental design

Sample code	Oil content (%)	Ingredients[a)]
Mayonnaise		
M_STD	67.7 ± 2.2	Mayonnaise (produced with soya oil 63%)
M_STD_FO	64.5 ± 2.2	Mayonnaise FO (produced with soya oil 56.7% and fish oil 6.3%)
Shrimp salads		
S_STD	23.1 ± 2.0	Mayonnaise 40%, shrimp 39%, asparagus 20%
S_STD_FO	22.5 ± 1.8	Mayonnaise FO 40%, shrimp 39%, asparagus 20%
S_FO_S	29.7 ± 2.9	Mayonnaise FO 50%, shrimp 49%
S_FO_A	41.1 ± 1.4	Mayonnaise FO 65%, asparagus 34%
Tuna salads		
T_STD	22.2 ± 1.6	Mayonnaise 34%, tuna 32%, maize 10%, peas 10%, bell pepper 10%, onion 0.6%
T_STD_FO	22.8 ± 1.9	Mayonnaise FO 34%, tuna 32%, maize 10%, peas 10%, bell pepper 10%, onion 0.6%
T_FO_T	33.1 ± 2.0	Mayonnaise FO 52%, tuna 45%, onion 0.8%
T_FO_TMP	25.1 ± 1.9	Mayonnaise FO 41%, tuna 35%, maize 11%, peas 11%, onion 0.7%
T_FO_MPB	31.3 ± 1.7	Mayonnaise FO 53%, maize 14%, peas 14%, bell pepper 14%, onion 0.9%
T_FO_TB	28.9 ± 1.0	Mayonnaise FO 46%, tuna 39%, bell pepper 12%, onion 0.7%
Tuna salads + spices		
T_STD_FO + R	22.0 ± 1.0	T_STD_FO + 1% rosemary
T_STD_FO + O	21.4 ± 0.9	T_STD_FO + 1% oregano
T_STD_FO + T	22.9 ± 1.6	T_STD_FO + 1% thyme

a) The total percentage of the ingredients in the salads is not 100%, but around 97–99%. The remaining 1–3% was preservatives and acids to adjust the sourness of the salad.
Mayonnaise FO: mayonnaise with fish oil. The sample name, oil content (%) and ingredients (%) in the different codes are listed. For details on ingredients of standard mayonnaise, shrimp, and tuna salads refer to Section 2.

in Tenax^{TM} tubes (Perkin Elmer, Norwalk, CT, USA) by purging the mayonnaise or mayonnaise salads with nitrogen (150 mL/min, 30 min) at 60°C, and volatile acids were removed by KOH during the headspace collection as described by Hartvigsen *et al.* [25]. An ATD-400 automatic thermal desorber was used for thermally desorbing the collected volatiles. The transfer line of the ATD was connected to a 5890 IIA gas chromatograph (Hewlett-Packard, CA, USA) equipped with a DB wax column (length 30 m × 0.25 mm id × 0.5 μm film thickness; J&W Scientific, CA, USA) coupled to an HP 5972A mass-selective detector. The temperature program was as follows: 3 min at 35°C, 3°C/min from 35 to 120°C, 7°C/min from 120 to 160°C, 15°C/min from 160 to 200°C, and hold for 4 min at 200°C. To quantify the amount of different volatiles, a solution with external standards was prepared at different concentrations and 1 μL of these solutions was placed in Tenax tubes and analyzed. Standards used were 2-ethylfuran, 1-penten-3-one, pentanal, 2(*t*)-pentenal, hexanal, heptanal, 2-pentylfuran, 2(*t*)-heptenal, 4(*c*)-heptenal, 2,4(*t*,*t*)-heptadienal, 2,6(*t*,*c*)-nonadienal, 2-butenal, 1-penten-3-ol. Analyses were performed in triplicate and results are given as ng/g oil to compensate for the different oil contents in the mayonnaise salads; however, concentrations were also calculated as ng/g sample (not shown).

2.8 Sensory evaluation

Six of the samples were evaluated by descriptive analysis by eight panelists trained in descriptive analysis of fishy off-flavors in mayonnaise salads. The mayonnaise salads were chopped in a mincer (Knifetec 1095 Sample Mill; FOSS, Sweden) prior to sensory evaluation in order to obtain homogeneous samples. Descriptors used for flavor and taste assessment were: fish (tuna/shrimp), rancid, metallic, grass, mayonnaise, and miscellaneous. The scale ranged from zero intensity to a maximum intensity of 9 for each descriptor. Samples (25 g) were served randomized at 10°C with crisp bread and cold water after 2, 24, 36, 50, and 57 days of storage.

2.9 Statistical analysis

The obtained results were analyzed by two-way analysis of variance (GraphPad Prism, Version 4.03; GraphPad Software). As posttest, the Bonferroni multiple comparison test was used to test differences between samples or storage time, with $p<0.05$ as significant difference between samples or storage time.

Results from PV and volatile analysis were subjected to principal component analysis (PCA), and sensory and volatile results for a subset of shrimp salads (S_STD, S_STD_FO, and S_FO_S) were subjected to a partial least squares regression (PLSR) using Unscrambler version 9.0 (Camo, Oslo, Norway). The PCA model was built on the average of the measured data, and full cross-validation was used to validate the model. A PCA allows detecting similarities and dissimilarities between the different samples in a score plot, whereas correlation between the measured variables is visualized in a loadings plot. Connecting these plots in this case shows the degree of oxidation between the different samples. Furthermore, the PLSR model combines two datasets: *X*-variables (GC-MS data) and *Y*-variables (sensory profiling data). However, a preliminary treatment of the sensory results was necessary to remove the noise caused by scaling differences between the assessors. The noise was projected away by fitting a PLSR model to the sensory results for each sampling day. The resulting residuals from these models were pooled into three different means: two pseudo-duplicates obtained by separating the data randomly into two groups (rep 1 and rep 2) and an overall average from each shrimp salad [3]. Rep 1, rep 2, and the average were then used to compute the PLSR model with the volatile and sensory results.

3 Results

3.1 Droplet size

Droplets were largest for mayonnaise without fish oil. Thus, the droplets were between 3.1–3.4 and 2.9–3.2 μm for M_STD and M_STD_FO, respectively (data not shown). A slight increase in droplet size during the first half of the storage period (day 1 to day 28) was observed. Thereafter the droplet size was stable.

3.2 Primary lipid oxidation products

Generally, the mayonnaise samples had a low concentration of peroxides (0.18–0.65 meq peroxides/kg oil; Fig. 1A). There were no significant increases in PV during storage and no significant differences in PV between the two mayonnaise samples with and without fish oil at any time during storage.

The PV in the shrimp salads showed the same development as in the pure mayonnaise, except for the salad without asparagus, S_FO_S (Fig. 1B). After 24 days of storage, this sample had a significantly higher PV compared to the other three shrimp salads.

As regards the tuna salads, it was observed that most of them had a higher PV than the mayonnaise and shrimp salads, already from the start and throughout the storage period, except for two samples: the one without maize and peas (T_FO_TB) and the other without maize, peas and bell pepper (T_FO_T) (Fig. 1C), which had the same PV level as the mayonnaises and shrimp salads (except for S_FO_S). Interestingly, the combination of the different ingredients in the tuna salad influenced the starting level of PV in the salads. The order of PV was T_FO_TMP > T_FO > T_ FO_MPB > T_STD > T_FO_TB ≥ T_FO_T

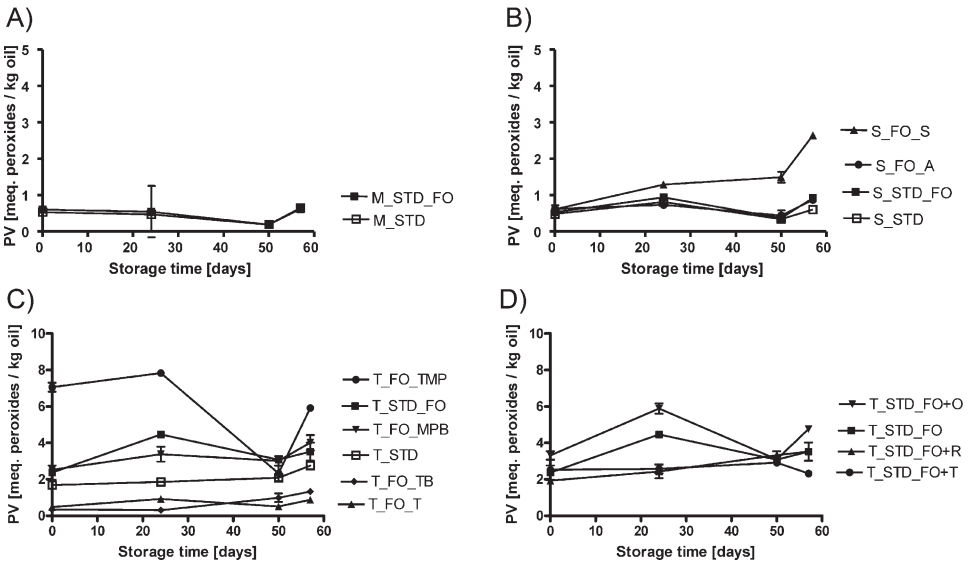

Figure 1. PV concentration during storage. (A) Mayonnaise, M_STD and M_STD_FO; (B) shrimp salads, S_STD, S_STD_FO, S_FO_S, and S_FO_A; (C) tuna salads, T_STD_FO, T_STD_FO, T_FO_T, T_FO_TMP, T_FO_MPB, and T_FO_TB; and (D) tuna salads with spices added, T_STD_FO, T_STD_FO + R, T_STD_FO + O, and T_STD_FO + T. For interpretation of the code names, refer to Table 1. The bars indicate the SD of two measurements.

(lowest content). Moreover, the PV level was unchanged in the different tuna salads during the storage period.

Addition of either rosemary or thyme tended to reduce the PV slightly during storage of the tuna salad with fish oil and all ingredients, whereas addition of oregano resulted in a significantly higher PV compared to tuna salad without spices, T_STD_FO (Fig. 1D), at all time points except for storage day 50. Notably, the concentration of lipid hydroperoxides was almost unchanged during storage in tuna salad with fish oil and thyme added.

Surprisingly, the concentration of lipid hydroperoxides only increased slightly during storage in mayonnaise and the different salads, irrespective of whether fish oil had been added or not and irrespective of the type of ingredient added to the salad. The only exception was the shrimp salad with shrimp only (S_FO_S), where the concentration increased six-fold during storage. For the other samples, this means either no development in lipid oxidation or that the decomposition to secondary oxidation products was just as fast as the formation of the hydroperoxides.

3.3 Secondary volatile lipid oxidation products

Nine volatiles (1-penten-3-one, pentanal, 2(*t*)-pentenal, hexanal, heptanal, 2-pentylfuran, 4(*c*)-heptenal, 2-butenal, and 1-penten-3-ol) were detected and quantified by dynamic headspace GC-MS. However, other volatiles previously detected in fish oil-enriched mayonnaise, such as 2-ethylfuran, 2(*t*)-heptenal, 2,4(*t*,*t*)-heptadienal, and 2,6(*t*,*c*)-nonadienal, could not be detected in the mayonnaises and mayonnaise salads in this experiment.

Mayonnaises with and without fish oil had similar concentrations throughout the storage period for 2-butenal, hexanal, heptanal, 2-pentylfuran, and 4-heptenal. For these volatiles, no significant increase in the concentration during storage was observed. Another pattern was observed for 1-penten-3-ol, 1-penten-3-one, pentanal, and 2-pentenal, where the concentration increased more during storage in mayonnaise with fish oil (M_STD_FO) than in mayonnaise without fish oil (M_STD). For 1-penten-3-ol, the concentration was significantly higher in the fish oil-enriched mayonnaise, already from the beginning, and this mayonnaise also had significantly higher levels of 1-penten-3-one after 24 days, after 50 days for pentanal, and after 57 days the level of 2-pentenal also became highest for this mayonnaise (Figs. 2A, B).

For shrimp salads, S_FO_S (only shrimp) had the highest concentration of 1-penten-3-one, 2-pentenal, 1-penten-3-ol, heptanal, and 4-heptenal (Fig. 2C). On the other hand, the lowest concentrations or among the lowest concentrations of 2-pentenal, 4-heptenal, 1-penten-3-ol, and 2-butenal were observed in S_FO_A (only asparagus) (Fig. 2C). However, the concentrations of hexanal and pentanal (Fig. 2D) in S_FO_A were generally higher than in the other types of shrimp salad, already from the beginning of the storage. For pentanal and hexanal, S_FO_A had a concentration of 303 and 1230 ng/g oil compared to 35–206 and 129–699 ng/g oil for the other samples. Interestingly, there was no significant difference between the shrimp salad with (S_FO) and without fish oil (S_STD) for any of the volatiles. When comparing the shrimp salads with pure mayonnaise, both with fish oil added, it seems that asparagus and shrimp as

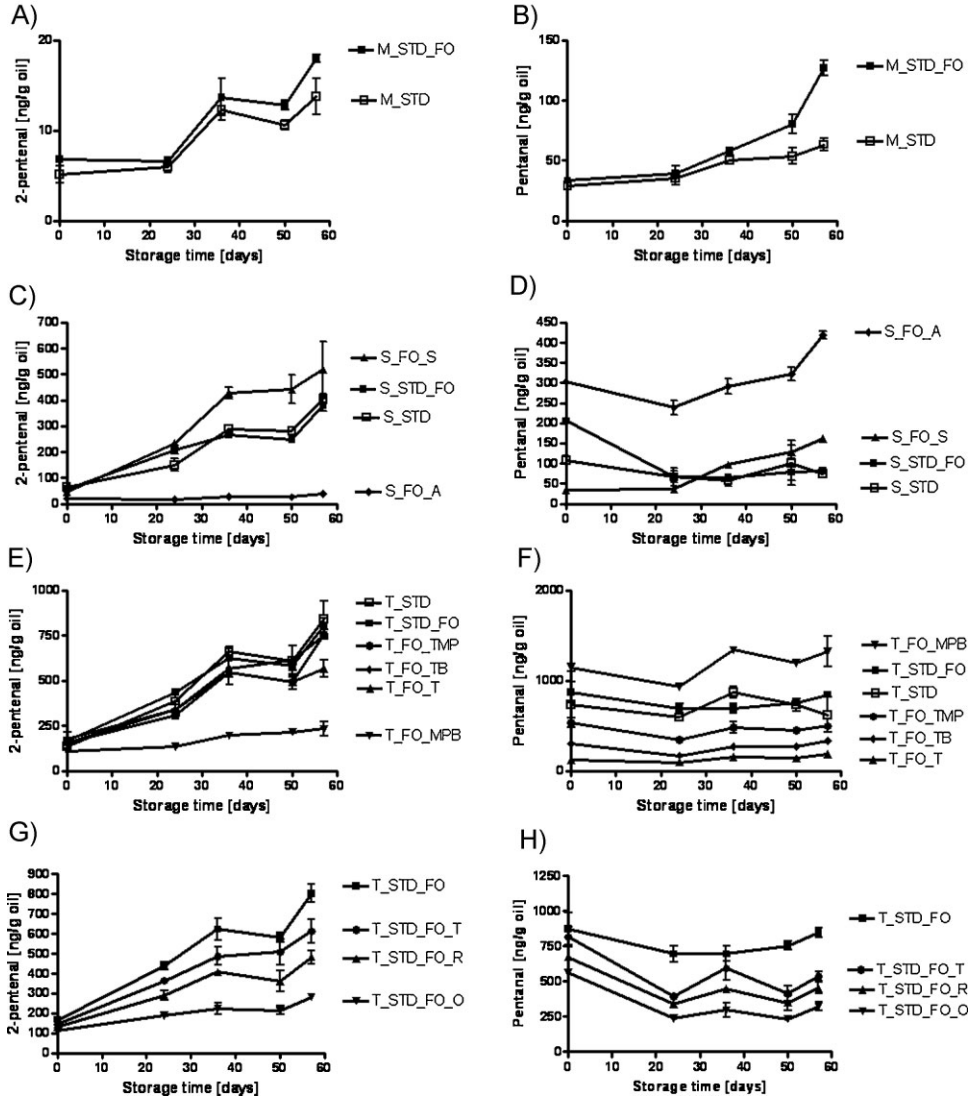

Figure 2. Concentrations of two selected volatiles (2-pentenal and pentanal) during storage in mayonnaise and different mayonnaise salads. (A) Concentration of 2-pentenal in mayonnaise, (B) concentration of pentanal in mayonnaise, (C) concentration of 2-pentenal in shrimp salads, (D) concentration of pentanal in shrimp salads, (E) concentration of 2-pentenal in tuna salads, (F) concentration of pentanal in tuna salads, (G) concentration of 2-pentenal in tuna salads with spices added, and (H) concentration of pentanal in tuna salads with spices added. For interpretation of the code names, refer to Table 1. The bars indicate the SD of three measurements.

ingredients had anti-oxidative and pro-oxidative effects, respectively.

In tuna salads, the development of the different volatiles was more complex than for the shrimp salads (Figs. 2E, F). Interestingly, most of the volatiles (4-heptenal, 2-pentylfuran, heptanal, 1-penten-3-ol, hexanal, 2-butenal, pentanal, and 1-penten-3-one) were found in different concentrations already at the beginning of the storage, depending on the ingredients in the tuna salads, as exemplified in Fig. 2F. Furthermore, in the tuna salads, only three of the detected volatiles increased in concentration during storage [2-butenal, 2-pentenal (Fig. 2E), and 4-heptanal], whereas the concentrations of the other volatiles were more or less constant (Fig. 2F). With regard to the secondary volatile oxidation products, one tuna salad, T_FO_MPB (without tuna), behaved differently from the rest of the tuna salad samples. Thus, this tuna salad in most cases had either a higher or a lower concentration of the different volatiles compared with the other tuna salads (Figs. 2E, F). To some extent, this behavior was the same as that observed for S_FO_A. However, in the cases where T_FO_MPB was found to have the lowest concentrations, this pattern was more distinct than for S_FO_A.

Addition of spices to the tuna salad with fish oil reduced the concentration of all volatiles during the entire storage

period (Figs. 2G, H), except for 1-penten-3-one. Thus, the tuna salad with fish oil and rosemary had a much higher concentration of 1-penten-3-one compared to the rest of the tuna salads with spices and T_STD_FO. For all other volatiles, oregano reduced the concentration of volatiles, most followed by rosemary and then thyme.

3.4 Sensory evaluation of lipid oxidation

Sensory evaluation was only performed on a subset of the salads (S_STD, S_STD_FO, S_FO_S, T_STD, T_STD_FO, and T_FO_T). Generally, the taste descriptors gave clearer results than the odor descriptors. Interestingly, there were no significant differences in rancid and metallic off-flavors between the standard salads without and with fish oil, as also observed for PV and volatiles.

For shrimp salads, the sample without asparagus (S_FO_S) was evaluated as having a significantly more rancid taste from day 24 (Fig. 3A) and metallic taste from day 36 to the end of storage (day 57) than S_STD and S_STD_FO. This was also the case for rancid and metallic odors; however, the differences were not significant before days 36–57 for rancid and day 57 for metallic odor (Figs. 3C, D). The sample without asparagus (S_FO_S) was evaluated slightly higher for grass than the two other shrimp samples. Grass, like rancid and metallic, is an indicator for lipid oxidation. In addition, to the simultaneous increase in the intensity of rancid, metallic and grass, a slight decrease in the intensity of fish (not significant) and mayonnaise (significant at day 57) for S_FO_S was observed.

In contrast to shrimp salads, it was more difficult to detect differences between the tuna salads. Thus, there were only significant differences in rancid taste at the end of the storage period, where T_FO_T was evaluated to be more rancid than the other two samples (Fig. 3B). As for the shrimp salads, the mayonnaise odor and taste decreased significantly at day 57 for T_FO_T when the rancid taste increased (Fig. 3B; rancid taste). Moreover, the metallic taste tended to be higher in T_FO_T from days 50–57 compared to the other tuna salads (T_STD and T_FO), although the differences were not significant (data not shown).

3.5 Multivariate data analysis

To give an overview of the results and to further evaluate the differences between mayonnaise, tuna salad and shrimp salad, a PCA was made on the chemical data. Two samples (S_FO_S and T_FO_MPB) were kept out of the PCA model since they dominated the model and made interpretation of the rest of the samples impossible. These samples were also described as being significantly different from the others by the univariate statistics. Moreover, a PLSR model was computed on the volatile and sensory results to explore the

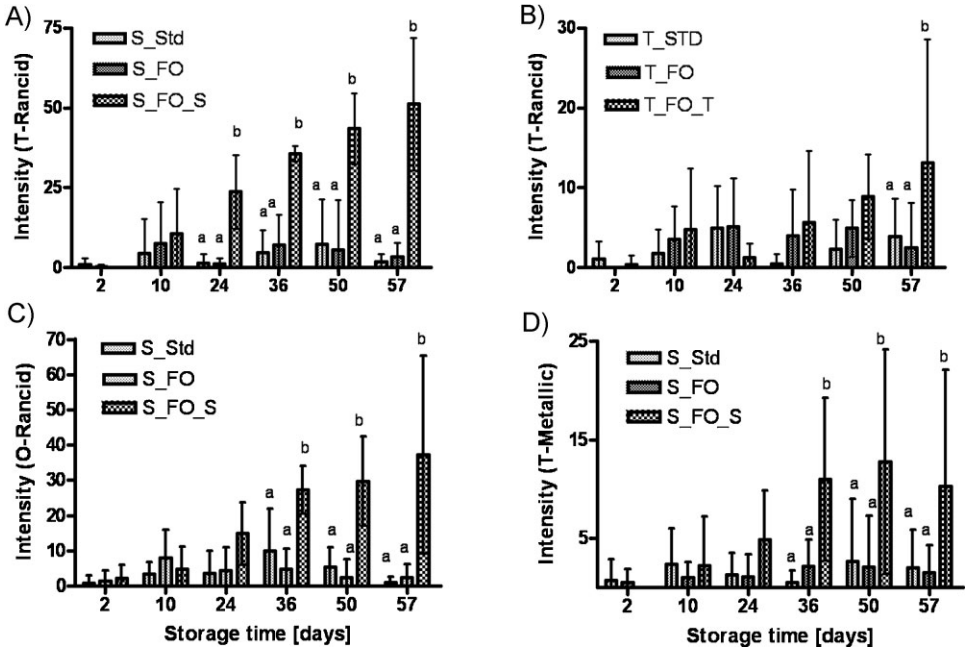

Figure 3. Sensory attributes determined by descriptive profiling, *Y*-axis showing intensity on a scale from 0 to 9 of the selected attributes. (A) Rancid taste in shrimp salads, (B) rancid taste in tuna salads, (C) rancid odor in shrimp salads, and (D) metallic taste in shrimp salad. The bars indicate the SD from all the panelists within each session. For interpretation of the code names, refer to Table 1. Different letters above the bars indicate significant differences between samples at that storage day; no letters above the bars equals to no significant differences between samples.

correlation between GC-MS data and the sensory profiling. However, the model was only computed from the sensory profiling of the shrimp salads since the differences were more easily detected in this salad type by the assessors.

3.5.1 PCA model (mayonnaise, shrimp salads, and tuna salads)

The first two principal components, PC 1 and PC 2, described 77% of the variation in the data-set (Fig. 4). The loadings plot visualizes correlations between the measured variables (PV and volatiles) (Fig. 4A). All the variables are located in the 1st and 4th quadrants in the loadings plot. Moreover, the plot indicates that all the unsaturated aldehydes (2-butenal, 2-pentenal, and 4-heptenal) and 1-penten-3-ol correlate in the 1st quadrant and the saturated aldehydes (pentanal, hexanal, and heptanal) and 2-pentylfuran correlate in the 4th quadrant. Variables such as PV and 1-penten-3-one are likewise located in the 4th quadrant, however not grouping with other variables. Thus, both

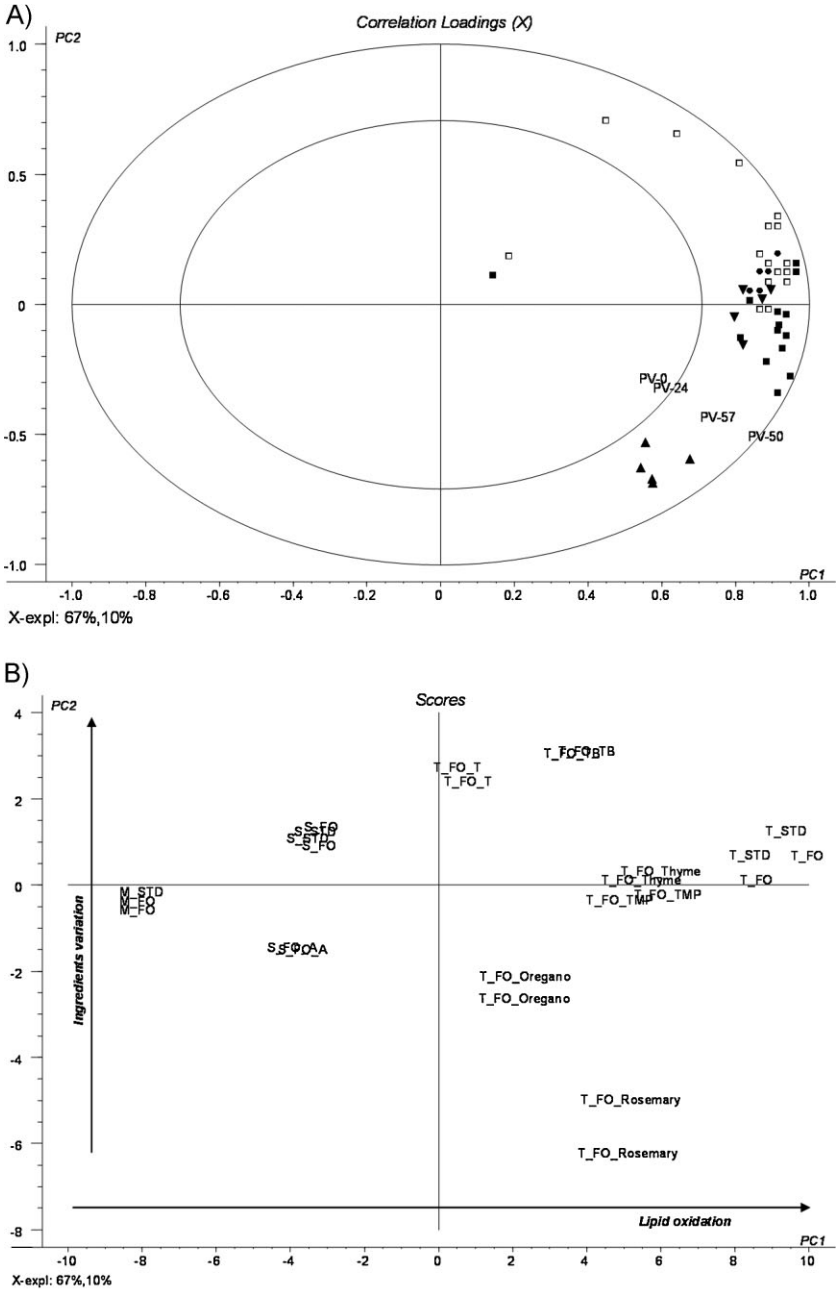

Figure 4. PCA model on PV and volatile results measured on all samples. T_FO_MPB and S_FO_S were kept out of the model. PC 1 and 2 explained 72% of the variation. (A) Loadings plot (■, saturated aldehhydes: pentanal, hexanal, and heptanal; □, unsaturated aldehydes: 2-butenal, 2-pentenal, and 4-heptenal; ▲, 1-penten-3-one; ▼, 2-pentyl furan; •, 1-penten-3-ol; PV at a specific storage day) and (B) score plot. For interpretation of the code names, refer to Table 1.

the first and the second principal components explain the variation in the lipid oxidation variables measured in the mayonnaise salads. PC 1 describes the degree of oxidation, whereas PC 2 mainly describes different development patterns between different volatiles. Furthermore, the scores plot from the PCA analysis gives information about similarities in PV and volatile profiles between the different sample codes (Fig. 4B), *i.e.*, clustering of samples indicates similarities. This score plot suggests that the standard products without and with fish oil were very alike, *i.e.*, M_STD and M_STD_FO were located close to each other and the same was the case for S_STD and S_STD_FO, T_STD and T_STD_FO, respectively.

The mayonnaise samples were located to the far left and close to the PC 1 axis, whereas the shrimp salads were located on each side of the PC 1 axis closer to the PC 2 axis than the mayonnaises. The different tuna salads were located to the right of the PC 2 axis in the 1st and 4th quadrants, with the standard tuna salads without and with fish oil to the far right. The two shrimp salads with fish oil and variation in ingredients were located on each side of the PC 1 axis, and the same was true for the tuna salads with fish oil and variation in ingredients or spices added.

Taken together, these findings indicate that the product type and ingredients had a greater influence on lipid oxidation than substitution of some of the soya oil with fish oil. Thus, the tuna salads were more oxidized than the mayonnaise and shrimp salads due to their closer location to the lipid oxidation variables (PV and volatiles), whereas mayonnaises were less oxidized followed by the shrimp salads. Furthermore, tuna salad with oregano and tuna salad with only tuna (T_FO_T) were located close to the PC 2 axis and were thus less oxidized than the standard tuna salad without and with fish oil added. The PCA model also suggests that the two other spices (rosemary and thyme) had an anti-oxidative effect as they were also located further to the left than the standard tuna salad. From the PCA model, it can thus be concluded that oregano had the highest anti-oxidative effect, followed by rosemary and thyme. This was also observed from the univariate data analysis.

3.5.2 PLSR model (shrimp salads: S_STD, S_STD_FO, S_FO_S)

The PLSR model was performed on results obtained from the shrimp salads only (data not shown). The results obtained from tuna salad by sensory evaluation were not included since many of the used attributes were not significantly different for these salads. The volatiles data as *X*-variables and the pretreated sensory data as *Y*-variables explained 83 and 76% of the variation in PC 1 and PC 2 , respectively, suggesting very strong correlations between sensory and volatile data. From the model it could thus be concluded that sensory attributes like rancid, metallic, and grass correlated positively with 1-penten-3-one, 1-penten-3-ol, and 4-heptenal, whereas 2-pentylfuran showed negative correlations (data not shown). These findings were in agreement with the conclusions from the univariate data analysis, which showed that shrimp salad with only shrimps, S_FO_S, had the highest concentration of those five volatiles compared to the other shrimp salads, and this salad was likewise evaluated as the most rancid sample by the sensory panel.

4 Discussion

Tuna salads generally oxidized faster than pure mayonnaise and shrimp salads, except for shrimp salad without asparagus (S_FO_S), which oxidized faster and developed more rancid, metallic and grassy off-flavors than the other shrimp salads. Interestingly, both chemical and sensory data showed that all the standard products with fish oil were not significantly different from the standard products without fish oil during storage, except for the tuna salads for which the salad enriched with fish oil had significantly higher PV during the entire storage period and which were slightly more rancid after 57 days.

4.1 Mayonnaise

The concentrations of hydroperoxides and the different volatiles were, in general, low throughout the whole storage period. The two samples differed in the concentration of four of the volatiles. Among these four volatiles, the concentrations of pentanal and 2-pentenal were only different at the end of the storage period. In contrast, the concentration of 1-penten-3-ol was significantly higher in the fish oil-enriched mayonnaise during the entire storage and 1-penten-3-one from day 24. 1-Penten-3-one has earlier been described to have rancid green flavor [25] and, in addition, the pure mayonnaise has no other ingredients such as vegetable and marine ingredients (tuna or shrimp) to mask the fishy off-flavor that might develop during storage. Hence, it cannot be ruled out that a sensory panel would be able to pick up a fishy note in the fish oil-enriched mayonnaise. However, the differences in concentrations of the volatiles between the two types of mayonnaise mostly occurred at the end of the storage, *i.e.*, after 8 weeks, and problems with fishy off-flavor might be eliminated by reducing the shelf life labeled on the product by 1–2 weeks. After all, sensory profiling is needed to confirm that reducing the shelf life of the fish oil-enriched mayonnaise will be enough to keep a good quality.

The results from this study are different from earlier results obtained with fish oil-enriched mayonnaise, where more pronounced lipid oxidation was observed. However, the mayonnaise in this experiment was designed for a mayonnaise-based salad, and therefore it had a lower total oil content than commercial mayonnaise. Thus, the higher oxidative stability in this mayonnaise type might be due to a combination of low storage temperature (2°C) and lower fish oil

(6.3%) and total oil content (63%) compared with earlier experiments with fish oil-enriched mayonnaise, which were conducted with a higher total oil content (80%) and higher fish oil contents (either 100% fish oil or 16% fish oil) [26, 27].

4.2 Shrimp salad

The concentrations of lipid hydroperoxides for the standard shrimp salads and the one with asparagus (S_STD, S_STD_FO, and S_FO_A) were similar to the mayonnaise during storage. However, the concentrations of volatiles were higher than for the mayonnaise, and for some of the compounds, the concentrations were even high from the beginning of the storage period. These data suggest that the ingredients are responsible for, or are contributing to, the higher concentrations of volatiles observed due to their flavor profile, or that oxidation already occurred in the processing of the salads. Moreover, the multivariate analysis showed that sensory attributes like rancid, metallic and grassy correlated with 1-penten-3-one, 1-penten-3-ol, and 4-heptenal, whereas 2-pentylfuran, which was one of the compounds found in high concentration in the salad with asparagus only (S_FO_A), correlated negatively with the attributes connected with oxidized flavor. Some of those compounds have also been reported in other studies to have an impact on the fishy and rancid flavor in mayonnaise and milk [25, 28].

The data also suggested that shrimp, as an ingredient alone in the mayonnaise salad, had a pro-oxidative effect on lipid oxidation, whereas asparagus could prevent the pro-oxidative effect of shrimps in shrimp salads. The reasons for the pro-oxidative effect of the shrimps is unknown, but might be ascribed to the presence of metals such as iron and copper in the shrimp or the brine. Iron and copper are known initiators of lipid oxidation. Several other studies have shown anti-oxidative effects of asparagus [11, 12, 29]. Rodriguez *et al.* [12] observed that extracts of asparagus had an anti-oxidative effect, but they only observed a low correlation between the anti-oxidative activity and the phenol content (including flavonoids and hydroxycinnamic acids), which led them to suggest that other compounds (ascorbic acid and saponins) in the asparagus might be implied in the inhibition mechanisms. In contrast, other studies on anti-oxidative activity in asparagus extracts have demonstrated significant correlation with the flavonoid concentration [11]. From the labeling of the asparagus used in the present study, it is known that citric acid was added. Whether, the anti-oxidative effect of the asparagus is due to the citric acid, the phenolics or other compounds in the asparagus, or a combination of these, is not known and needs further investigation. However, the citric acid is present in low concentration since it is present in the brine only and not in the asparagus itself. Thus, it is suggested that the phenolics, flavonoids, and maybe other compounds in the asparagus are responsible for the major part of the anti-oxidative effect.

4.3 Tuna salad

For all the tuna salads, there was more or less the same concentration of primary oxidation products during storage in each salad, but concentrations varied between the tuna salads. This means either no development in lipid oxidation or that the decomposition to secondary oxidation products was just as fast as the formation rates of the lipid hydroperoxides. However, from the concentration of volatiles, it is difficult to conclude if any of the ingredients influenced the oxidation of the lipids, since different ingredients resulted in different amounts of the volatiles already from the beginning and more or less no further development during storage. Thus, only three volatiles increased in the tuna salads during storage, and for those the concentrations in the different fish oil-enriched salads were not significantly different from the standard products. Although one tuna salad, T_MPB (maize, pea, and bell pepper), was found to be significantly different from the others, it is doubtful that it was more oxidized than the standards. This is due to the fact that this salad had the highest concentrations of some volatiles, but the lowest concentrations of other volatiles compared to the other salads. Moreover, the volatiles (1-penten-3-one, 1-penten-3-ol, and 4-heptenal) that correlate positively with the oxidized flavor in shrimp salad were lowest in this tuna salad. In addition, this salad had the lowest concentration of 2-pentylfuran, which correlated negatively with the oxidized flavor in shrimp salad.

The multivariate analysis indicated that a higher concentration of volatiles was found in the standard tuna salads with all ingredients compared to the tuna salad without vegetables. This finding suggests that addition of vegetables to the tuna salad promoted oxidation. However, this is in contrast to the findings from the sensory analysis, which suggested that the tuna salad without vegetables was more rancid. Hence, the location of the tuna salad without vegetables (T_FO_T) was far away from the standard tuna salads (Fig. 4). This is most likely due to a higher concentration of volatiles in the standard tuna salads, which may be caused by volatiles naturally present in the vegetables themselves and not by oxidation. Tuna salads with one or more vegetables removed from the recipe were located in between the standard tuna salads (T_STD and T_STD_FO) and the tuna salad without vegetables (T_FO_T) (Fig. 4), suggesting that their concentrations of volatiles and lipid hydroperoxides were somewhere in between those of T_STD/T_STD_FO and T_FO_T. Due to the same reasons as above, it is difficult to conclude whether this finding is due to the content of volatiles naturally present in the vegetables or to differences in degree of lipid oxidation.

Nevertheless, several other studies have found that bell pepper [15–17, 29] and snow peas [29] have anti-oxidative activity. As for the asparagus, the bell pepper was in brine with citric acid before use; thus, the citric acid may also contribute to a possible anti-oxidative activity.

4.4 Tuna salads with spices

Several studies have proved that spices, or extracts based on spices, possess anti-oxidative activity [9, 10, 13, 14, 30]; however, the studies have not been performed in complex food matrices such as tuna salads. In the present experiment, addition of 1% of one of the three spices to fish oil-enriched tuna salads reduced the volatile concentration compared to the standard (T_STD_FO). However, tuna salad with oregano had a significantly higher PV than the standard. The pro-oxidative effect of oregano on hydroperoxide formation was also observed by Jimenez-Alvarez *et al.* [9], who explained it by the ability of oregano to reduce Fe^{3+} to Fe^{2+}, which would then promote peroxide formation. The sample T_FO + O had lower concentrations of volatiles and, taken together, these findings thus indicate that the decomposition rate from PV to volatiles is slower in the salad with oregano than in the other tuna salads.

On the basis of the volatile data, it could thus be concluded that, in this experiment, oregano had the greatest anti-oxidative effect, followed by rosemary and then thyme. These findings are similar to the findings of Zheng and Wang [13] who found that oregano showed a higher antioxidant activity than seen in rosemary- and thyme-based oxygen radical absorbance capacity (ORAC) measurements. Moreover, Tsimidou *et al.* [14] observed a similar inhibition of PV with oregano and rosemary (0.5%) used as dry herbs in mackerel oil. Oregano was slightly more effective in the initial phase of the lipid oxidation in mackerel oil than rosemary. The difference is suggested to be due to the presence of the essential oil in oregano, which is rich in carvacrol and thymol. However, Schwarz *et al.* [31] observed only a relatively weak antioxidant activity of those compounds isolated from the nonpolar fraction of thyme.

Other authors have suggested that the anti-oxidative behavior of oregano can be attributed to the presence of phenolic compounds, *e.g.*, caffeic acid and rosmarinic acid. Under certain conditions, oregano can also act as a pro-oxidant [9], but this was not found in our experiment. Furthermore, oregano in a concentration of 0.5% was shown to be more efficient in reducing the concentration of lipid hydroperoxides compared to 200 mg/kg of the synthetic compound butylated hydroxyanisole (BHA) in mackerel oil [14].

5 Conclusions

In conclusion, this study did not confirm our hypothesis that vegetables in mayonnaise-based salads increase the oxidative stability of fish oil-enriched mayonnaise, although some vegetables showed anti-oxidative activity in the system. Surprisingly, the fish oil-enriched mayonnaise (S_STD_FO) was the most oxidatively stable compared to fish oil-enriched salads, and almost as oxidatively stable as the standard mayonnaise without fish oil added (M_STD), except for the last 2 weeks of storage. The high oxidative stability observed for this fish oil-enriched mayonnaise is suggested to be due to the lower fish oil concentration and total oil content as well as storage temperature compared to earlier experiments with fish oil-enriched mayonnaise. Moreover, by reducing the storage time from 8 to 6 weeks, the lower oxidative stability of fish oil-enriched mayonnaise compared with the standard mayonnaise might be overcome, but this has to be further confirmed by sensory analysis.

Shrimp in the fish oil-enriched salad had a pro-oxidative effect, whereas asparagus had an anti-oxidative effect, which was efficient enough to prevent the pro-oxidative effect of the shrimps in this type of salads. For the tuna salad, the influence of ingredients herein seemed more complex, and it was not possible to draw clear conclusions on the effect of the ingredients. This might be due to an already high concentration of volatiles in the ingredients. However, the use of oregano, rosemary or thyme increased the oxidative stability of the tuna salad compared to fish oil-enriched tuna salad without added spice. Oregano was the most efficient spice evaluated, followed by rosemary and then thyme. Although addition of spices had an anti-oxidative effect, the taste introduced to the product by the addition might be unappreciated in this type of products. In addition to these findings, it might be worthwhile to evaluate the anti-oxidative effect of extracts based on especially oregano in fish oil-enriched mayonnaise-based products.

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PAPER II

Sørensen, A.-D.M., Nielsen, N.S., Hyldig, G. & Jacobsen, C.

The influence of emulsifier type on lipid oxidation in fish-oil-enriched light mayonnaise.

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Research Article

Influence of emulsifier type on lipid oxidation in fish oil-enriched light mayonnaise

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The oxidative stability of fish oil-enriched light mayonnaise (40% oil) and the influence of two different emulsifiers, egg yolk and milk protein-based emulsifier, were evaluated. Moreover, the effects of different fish oil concentrations (4, 10 and 14%) and storage temperatures (2 and 20°C) were investigated. As expected, the results showed that lipid oxidation increased with storage temperature, and at 20°C with increasing fish oil concentrations. On the basis of the findings in this study, a storage temperature of 20°C for 4 months cannot be recommended for light mayonnaise due to significant lipid oxidation even in mayonnaises without fish oil. However, enrichment of light mayonnaises with 4% fish oil without adding antioxidant did not result in increased oxidation when stored at 2°C, and thus seems feasible; however, this has to be confirmed by sensory analysis. Surprisingly, our hypothesis that substitution of egg yolk with a less iron-containing emulsifier (milk protein-based emulsifier) could increase the oxidative stability of fish oil-enriched mayonnaises was not confirmed. These findings suggest that the initial quality of the emulsifiers was more important than its iron content in terms of lipid oxidation.

Keywords: Egg yolk / Fish oil / Light mayonnaise / Lipid oxidation / Milk protein

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1 Introduction

Long chain n-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to have several health beneficial effects such as protective effects against cardiovascular disease [1]. Even though EPA and DHA can be synthesized in the human body, these PUFAs are needed through the diet since in most cases they are not synthesized to a sufficient level [2]. The major source of EPA and DHA is fish and fish products, and several investigations indicate that the intake of fish and fish products in Western countries is too low to ensure adequate amounts of EPA and DHA in the human body [3–5]. Different recommendations exist regarding EPA and DHA intake. The Danish Veterinary and Food Administration recommend to eat fatty fish at least twice a week [6], whereas other countries such as Canada, Sweden, United Kingdom and Japan recommend a daily intake of EPA and DHA in the level around 0.3–0.5 g [7].

Due to the relatively low intake of fish in the Western countries, there is a growing market for n-3 PUFA-enriched food products. However, incorporation of fish oil into foods might introduce stability problems due to oxidation of EPA and DHA, which will lead to an unpleasant fishy off-flavour. By understanding the oxidation mechanisms in each particular food product intended for fish oil enrichment it could be possible to overcome this problem, *e.g.* by adding appropriate antioxidants or by substituting prooxidative ingredients with other ingredients.

In previous work with fish oil-enriched mayonnaise, iron from egg yolk used as the emulsifier in mayonnaise combined

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Abbreviations: DHA, docosahexaenoic acid; **EDTA,** calcium disodium ethylenediaminetetraacetate; **EPA,** eicosapentaenoic acid; **PCs,** principal components; **PCA,** principal component analysis; **PUFAs,** polyunsaturated fatty acids; **PV,** peroxide value; **WPI,** whey protein isolate

with low pH were found to be the predominant factors responsible for lipid oxidation [8]. Therefore, Jacobsen *et al.* [8] proposed the following mechanism for lipid oxidation in this food system; lowering the pH from neutral to around four in fish oil-enriched mayonnaise will break iron bridges between phosvitin, low density lipoproteins (LDLs) and lipovitellins thereby releasing iron from the egg yolk. Subsequently, the released iron can catalyze lipid oxidation in mayonnaise. Hence, a prerequisite for antioxidants to be efficient in fish oil-enriched mayonnaise seems to be that they should have metal chelating activity in order to eliminate or reduce lipid oxidation in this type of food product. Among the evaluated metal chelating compounds (calcium disodium ethylenediaminetetraacetate (EDTA), lactoferin and phytic acid), EDTA was the only compound acting as an efficient antioxidant in fish oil-enriched mayonnaise [9, 10]. However, there is a trend in consumer preference for natural ingredients rather than synthetic compounds. Moreover, based on our experience with the European food industry it seems that EDTA is amongst the least preferred synthetic compounds. Thus, there is a need for finding other solutions in order to increase the oxidation stability of fish oil-enriched mayonnaise.

Based on the above-mentioned findings we hypothesize that substituting egg yolk as an emulsifier with an emulsifier with a lower iron content could increase the oxidative stability of the PUFAs in fish oil-enriched mayonnaise. An appropriate alternative to egg yolk might be milk protein. Earlier results with fish oil-enriched dressing in which denatured whey protein was used as emulsifier and a mixture of guar gum, xanthan gum and acetylated distarch (modified starch) as stabilizing agents showed good oxidative stability of this product [11].

A recent study compared the oxidative stability of mayonnaise with that of mayonnaise-based tuna and shrimp salads. The results showed that mayonnaise was more oxidative stable than mayonnaise-based salads with different vegetables, tuna or shrimp even though the amount of oil was higher in the pure mayonnaise [12]. Interestingly, this study also indicated that fish oil-enriched mayonnaise [12] had a higher oxidative stability compared to fish oil-enriched mayonnaises used in earlier experiments without antioxidants added [9, 10]. However, the storage temperature, oil concentration and even the fish oil concentration were higher in earlier experiments (20°C, 80% total oil, 16% fish oil [9] compared to 2°C, 63% total oil, 6.3% fish oil [12] in the most recent experiment).

Due to the general demand for healthier products, such as low calorie products, the industry wishes to produce products with reduced fat content such as light mayonnaise. As mentioned above previous experiments with fish oil-enriched mayonnaise indicated less lipid oxidation with lower oil content and fish oil concentration, however this finding might also be partly a result of different storage temperatures [13]. Other studies with different food products indicate contradicting results regarding the effect of reduced fat content on lipid oxidation. Lipid oxidation in liver pâté (35, 40 and 45% fat) decreased with decreasing fat content [14], whereas the total volatile content was higher with low fat content for irradiated grounded beef (10, 15 and 20% fat) [15]. Hence, the rate of lipid oxidation seems to be product specific when the fat content in the product is reduced. Thus, knowledge about the effect of a reduced oil content in fish oil-enriched mayonnaise and the influence of fish oil concentration and storage condition is required.

According to the above-mentioned needs regarding knowledge about the effect of reduced fish oil concentration and storage condition on lipid oxidation and the hypothesis that an emulsifier with a lower iron content than egg yolk might increase the oxidative stability of light mayonnaise, the aim of the present work was to evaluate the effect of fish oil concentration (4, 10 and 14% fish oil), storage temperature (2 and 20°C) and influence of emulsifier (low and high iron content emulsifier) on lipid oxidation in fish oil-enriched light mayonnaise during storage. A milk protein-based emulsifier was evaluated as the low iron containing emulsifier, whereas egg yolk, normally used as emulsifier in commercial mayonnaise, was evaluated as the high iron containing emulsifier. The different fish oil concentrations were selected according to earlier results and recommended daily intake of EPA and DHA. Thus, the high fish oil concentration (14% fish oil) was selected in order to obtain an intake of EPA and DHA of 0.5 g by consumption of 20 g light mayonnaise. Furthermore, the low and high temperatures were selected according to earlier results with fish oil-enriched mayonnaise and mayonnaise-based salads. The high temperature storage simulates the storage condition for mayonnaise in the supermarket and the lower temperature was selected as it was expected to be the optimal storage condition.

2 Materials and methods

2.1 Materials

Cod liver oil was supplied by Maritex Norway (subsidiary of TINE BA, Norway). This oil had an initial peroxide value (PV) of 0.7 meq peroxides/kg oil, tocopherol content of 252 mg α-tocopherol/kg, 37 mg γ-tocopherol/kg and 14 mg δ-tocopherol/kg, and the fatty acid composition was as follows: 14:0, 3.0%; 16:0, 9.4%; 16:1, 8.0%; 18:0, 2.0%; 18:1, 23.9%; 18:2, 2.8%; 18:3, 1.4%; 18:4, 2.1%; 20:1, 10.3%; 20:5 (EPA), 8.4%; 22:1, 5.4%; 22:5, 1.1% and 22:6 (DHA), 10.6%. The total percentages of n-3 and n-6 in the fish oil were 24.5 and 3.5%, respectively. The rape seed oil had an initial PV of 0.2 meq peroxides/kg oil, tocopherol content of 249 mg α-tocopherol/kg, 52 mg β-tocopherol/kg, 259 mg γ-tocopherol/kg and 13 mg δ-tocopherol/kg, and the fatty acid composition was as follows: 16:0, 4.7%; 18:0, 1.6%; 18:1,

59.5%; 18:2, 19.6%; 18:3, 10.0% and 20:1, 1.5%. The total percentages of n-3 and n-6 in the rape seed oil were 10.0 and 19.7%, respectively.

Egg yolk (PV of < 0.1 meq peroxides/kg oil) and commercial milk protein-based emulsifier (milk protein, starch, guar gum, sodium alginate and xanthan gum, PV of 9.8 meq peroxides/kg oil) were obtained from Eiproducten Wulro B.V. (Weert, Netherlands) and Palsgaard A/S (Juelsminde, Denmark), respectively. Chemicals were from Merck (Darmstadt, Germany) or Amersham Biosciences (Uppsala, Sweden). External standards for identification of secondary volatile oxidation products were all from Sigma Aldrich (Steinheim, Germany). All solvents were of HPLC grade (Lab-Scan, Dublin, Ireland).

2.2 Production of light mayonnaises

2.2.1 Mayonnaise with or without added fish oil

The mayonnaises consisted of water, oil (neat rape seed oil or rape seed oil and fish oil, Table 1), tarragon vinegar, sugar, salt and egg yolk 4% or milk protein-based emulsifier 4%. The applied milk protein-based mixture as emulsifier contained thickernes (as described above) and mayonnaise with egg yolk as emulsifier modified starch, guar gum and xanthan gum were added as thickernes. Total oil % for both types of mayonnaise was 40%. Sourness was adjusted to pH 3.5–4.1 with malic acid. Potassium sorbate and sodium benzoate were used for preservation. The ingredients were mixed using a Robot coupe Blixer® 4v.v. (Vincennes, France) for a total time of 3.5 min. The content of oil, different types of oil and emulsifier used is shown in Table 1.

2.3 Storage experiment

Mayonnaise samples were stored at 20 or 2°C for around 4 months (113 days) in the dark. The selected storage temperature at 20°C and the length of storage were set according to the shelf life of commercial light mayonnaise. Samples were taken at time = 0 and for mayonnaises stored at 20°C samples were taken for analyses at fixed time points after: 29, 58, 85 and 113 days, whereas for mayonnaises stored at 2°C samples were only taken for analyses after 58 and 113 days of storage. For droplet size measurements and sensory analysis, the first sampling was day 1 instead of day 0 and for the viscosity measurements samples were only taken at storage day 92 (20°C), day 113 (20°C) and day 115 (2°C). Moreover, droplet size plus viscosity measurements and sensory evaluation were performed on the respective sampling days, whereas for other analyses, mayonnaise samples were kept at −40°C until the analyses were carried out.

Table 1. Experimental design

Code	Emulsifier	Rape seed oil (%)	Fish oil (%)
EY_STD	Egg yolk	40	0
EY_4%FO	Egg yolk	36	4
EY_10%FO	Egg yolk	30	10
EY_14%FO	Egg yolk	26	14
MP_STD	Milk protein[a)]	40	0
MP_14%FO	Milk protein[a)]	26	14

All codes were stored at 2 and 20°C.
[a)] Milk protein refers to a commercial mixture of emulsifier and stabilizers (starch, skim milk powder, milk protein, sodium caseinate, guar gum, sodium alginate and xanthan gum) with milk protein as emulsifier.

2.4 Droplet size

Mayonnaise was dissolved in SDS buffer (10 mM NaH_2PO_4, pH 7) in a ratio 1:9 and sonicated (2×20 min, 30°C). The droplet size was measured by laser diffraction (Mastersizer2000, Malvern Instruments Ltd., Worcestershire, UK). Results are given in surface area mean diameter: $D_{3,2} = \sum d^3 / \sum d^2$ [16]. The analysis was performed in duplicate.

2.5 Viscosity

A Stresstech® HR (Reologica Instruments, Lund, Sweden) with both upper and lower plate serrated (P30 serrated polycarbonate plate) was used to measure the viscosity of the mayonnaise samples. The instrument was operating at 2 mm gaps. Other settings were as follows: the stress increased at oscillations of 1 Hz from 50–200 Pa in 30 logarithmic steps, continuous shear, delay time 11 s, integration time 11 s and initial equilibrium time 300 s. This analysis was performed in triplicate. An estimate of the viscosity was then calculated by use of a viscosity analysis programme (Analyse, Version 3.60, Reological Instruments, Lund, Sweden). All three viscosity measurements were used to generate one model for each mayonnaise sample. High values indicate that the mayonnaise sample is viscous and when the value decrease, the mayonnaise is less viscous.

2.6 Iron concentration

Egg yolk, milk proteins and mayonnaise samples were digested in nitric acid (65%) and destroyed in a microwave oven at 1400 W (Anton Paar multiwave 3000, Graz, Austria). Thereafter, the iron concentration was measured by an atomic absorption spectrophotometer (AAS 3300, Perkin Elmer, MA, USA). The mayonnaise samples were measured twice and the egg yolk and milk protein-based mixtures were measured four times.

2.7 Extraction of lipids

Lipids were extracted from the mayonnaise samples (10 g) according to the method described by Bligh and Dyer [17] with reduced amount of solvent applied [18]. The analysis was done in duplicate and further used for determination of PV, fatty acid composition and tocopherol concentration.

2.8 Primary oxidation products, peroxide value (PV)

Peroxide value in the lipid extracts were determined by a colorimetric method based on formation of an iron-thiocyanate complex measured according to the method described by Shantha and Decker [19]. One determination was made on each of the two extracts prepared from the same mayonnaise sample.

2.9 Fatty acid composition

Lipid extract was evaporated under nitrogen. Firstly, the glycerol bound fatty acids were trans esterified with methanolic NaOH (0.5 M). Then, hydrolytic released and free fatty acids were methylated by a boron trifluoride reagent (20%) catalyzed process. Methyl esters were extracted with heptane followed by separation on GC (HP 5890A, Hewlett Packard, CA, USA). The procedure was according to the AOCS methods [20, 21]. One determination was made on each of the two extracts prepared from the same mayonnaise sample.

2.10 Tocopherol concentration

Lipid extract was evaporated under nitrogen, re-dissolved in heptane and analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) according to the AOCS method [22] to determine tocopherol concentration in the different mayonnaise samples. The reported tocopherol concentrations are averages of duplicate lipid extractions and duplicate analysis on the HPLC.

2.11 Volatile secondary oxidation products

Collection, separation and identification of volatiles was performed by dynamic headspace GC-MS (GC: 5890 IIA, Hewlett-Packard, CA, USA and MS: HP 5972A mass selective detector) equipped with a DB wax column (length 30 m × ID 0.25 mm × 0.5 μm film thickness, J&W Scientific, CA, USA). Solutions with external standards at different concentrations were prepared and used to quantify the amount of different volatiles. For further details refers to Sørensen *et al.* [12]. Analyses were preformed in triplicate and results given as ng/g mayonnaise.

2.12 Sensory evaluation of mayonnaise samples

Descriptive analysis was performed on the mayonnaises stored at 20°C by nine tested assessors who were trained during 3 × 2 sessions on profiling fish oil-enriched mayonnaise. Moreover, the assessors were trained 4 × 2 sessions, *i.e.* two sessions the day before profiling mayonnaise from the storage experiment. Before each profiling session, the assessors were served a fresh mayonnaise reference (egg yolk as emulsifier and without fish oil) for calibration. For each profiling the following attributes were evaluated: odour (acidulous, sweet, tarragon vinegar, oily, rancid and other), flavour (acidulous, sweet, tarragon vinegar, egg yolk, oily, rancid and other) and texture (sticky, airy, oily and other). The panel rated all attributes for each sample on separated 15 cm scales to indicate intensities (0 indicated no intensity and 15 cm high intensity) and data were recorded on computers by use of the *FIZZ* programme (Biosystems, Couternon, France). Mayonnaise was served to the panelists in small transparent plastic cups with white plastic lids. Beside the reference sample, ten samples were served per session. The order of serving was randomized to minimize carry-over effects between mayonnaise samples. The mayonnaises were evaluated in red light due to colour differences between the mayonnaises. Distilled water (room temperature), crisp bread and peeled cucumber were provided for oral rinsing between each mayonnaise sample. Mayonnaise containing fish oil was evaluated twice by the assessors and the standards (without fish oil) only once.

2.13 Data treatments

2.13.1 Statistics

ANOVA followed by Bonferroni multiple comparison test were applied to evaluate significant differences between storage time (progress) and differences between samples at a specific time point. The applied significance level was $p < 0.05$. When a significant difference was observed between two samples, they are denoted with different superscripts in the text, tables or graphs.

2.13.2 Assessor/Panel performance

For checking the performance of the panel or individual assessors PanelCheck (MATFORSK (Norwegian Food Research Institute, Norway) and Consonance analysis (Unscrambler version 9.0, Camo, Oslo, Norway) were used [23, 24]. One assessor's evaluation at day 113 was substituted by missing values, since these results were not reliable because of hay fever.

2.13.3 Assessor corrections

Preliminary treatment of the sensory results was necessary to remove the noise caused by scaling differences between the assessors. The noise was projected away by fitting a PLSR model to the sensory results for each sampling day (Unscrambler version 9.0, Camo, Oslo, Norway). The

resulting residuals from these models were pooled into an overall average from each mayonnaise sample [25]. These averages were used for the principal component analysis (PCA) on the sensory results.

2.13.4 Principal component analysis (PCA)

Results from sensory analysis were subjected to PCA using Unscrambler version 9.0 (Camo, Oslo, Norway). Full cross validation was used to validate the model. A PCA allows detection of similarities and dissimilarities between the different samples in a score plot, whereas correlations between the measured variables are visualized in a loadings plot. Connecting these plots in this case shows the degree of rancidity in the different mayonnaises.

Attributes that changed over time and/or between samples in spider plots were selected for the PCA. The selected attributes were: for the odour only rancid, for the flavour: egg yolk, sweet and rancid and for the texture: airy and sticky.

3 Results and discussion

3.1 Physical stability of the mayonnaises

Figure 1 shows results from droplet size and viscosity measurements, which provide information about the physical stability of the mayonnaises. The droplet size ($D_{3,2}$) increased during storage at 20°C except for the mayonnaise with milk protein as emulsifier and no fish oil added (Fig. 1A). Moreover, the droplet size tended to increase with increasing fish oil concentration. At 2°C storage there were no changes in the droplet size not even with higher fish oil concentrations (data not shown). The droplet size data indicate that the mayonnaises were not physically stable when stored at 20°C. Moreover, mayonnaise with milk protein-based emulsifier resulted in a more viscous mayonnaise than when egg yolk was used as emulsifier (Fig. 1B). The mayonnaise stored at 20°C with egg yolk as emulsifier tended to be less viscous when fish oil was added. This tendency was not observed for mayonnaise stored at 2°C. Moreover, in contrast to the findings for mayonnaises with egg yolk, addition of fish oil to mayonnaise produced with milk protein and stored at 20°C increased viscosity. This got more pronounced after 113 days of storage. Furthermore, sensory data showed that this mayonnaise (MP_14%FO) became gritty over time. Hence, increased grittiness correlated with the increase in viscosity. Interestingly, the large increase in viscosity which was observed for MP_14%FO mayonnaise when stored at 20°C was not observed at 2°C.

Both mayonnaise with egg yolk and with milk protein contained thickeners and stabilizers such as starch, guar gum and xanthan gum and the mayonnaise with milk protein also contained sodium alginate. These compounds are polysaccharides, which usually are added to enhance viscosity of the aqueous phase [26]. However, the higher viscosity of mayonnaise with milk protein as emulsifier might not only be due to its polysaccharide content, but also due to some interactions between polysaccharides and the milk protein.

3.2 Fatty acid composition

As expected the content of n-3 PUFAs increased from 0.2 to 7.5%, whereas n-6 PUFAs decreased from 19.5 to 14.2% when increasing amounts of rape seed oil was substituted with fish oil (data not shown). In most of the samples there was no indication of reduced concentration of n-3 PUFA during 113 days of storage. However, mayonnaises with 14% fish oil tended to have slightly reduced n-3 PUFA content (6.9%) at the end of the storage. This was only observed for the two mayonnaises (EY_14%FO and MP_14%FO) stored at 20°C and not in the mayonnaises stored at 2°C.

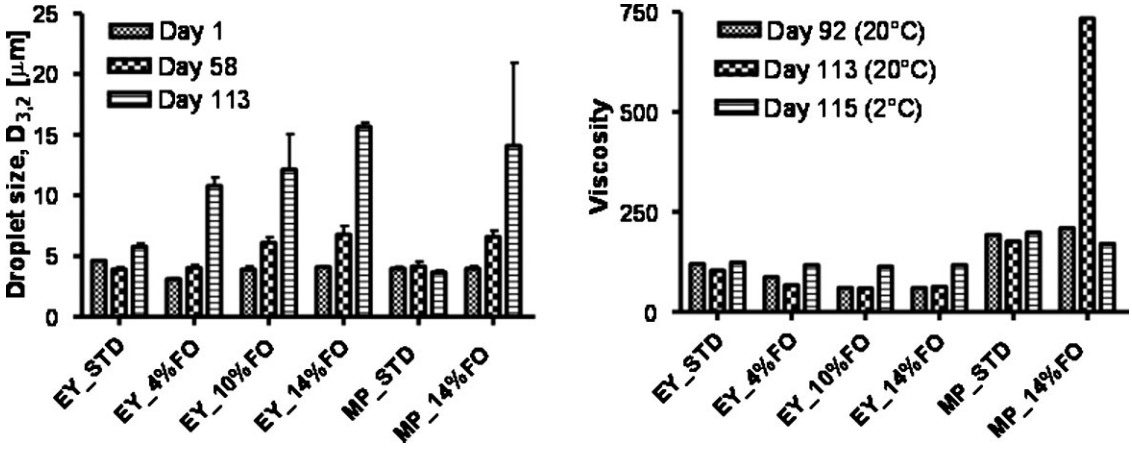

Figure 1. Physical stability of the mayonnaises during storage. (A) Droplet size, $D_{3,2}$ (µm) of the oil droplets in the mayonnaise stored at 20°C. The error bars indicate the SD ($n = 3$). (B) Viscosity of the mayonnaise stored at 20 and 2°C. Sample codes are explained in Table 1.

Table 2. The concentration of iron (μg/g) in the emulsifiers and the different mayonnaises (average ± SD, $n = 2$)

Sample	Iron (μg/g)
Egg yolk	51 ± 0.33
Milk protein mixture	7.3 ± 0.39
EY_STD	4.4 ± 0.05
EY_4%FO	4.4 ± 0.00
EY_10%FO	4.0 ± 0.34
EY_14%FO	4.2 ± 0.07
MP_STD	0.9 ± 0.02
MP_14%FO	0.9 ± 0.15

Sample codes are explained in Table 1.

3.3 Iron concentration

The iron concentration was measured in the emulsifiers to 51 and 7.2 μg iron per g emulsifier for egg yolk and milk protein-based emulsifier, respectively. Iron content in the different emulsifiers and mayonnaises is shown in Table 2 where it can be observed that mayonnaises with egg yolk had more than a fourfold higher iron content than mayonnaises with milk protein-based emulsifier.

3.4 Lipid oxidation in the mayonnaises

All four tocopherol homologues (α, β, γ and δ) were detected in the different mayonnaise samples and the concentrations of γ-tocopherol in the mayonnaises are shown in Table 3. Generally, for both storage temperatures, the concentration decreased significantly in the mayonnaise from day 1 to day 113, and at the end of storage concentrations were significantly lower for mayonnaise stored at 20°C compared to 2°C.

For α- and δ-tocopherol concentrations were similar in the different mayonnaises at day 0 (data not shown), and this was also the case for δ-tocopherol at the end of storage. After 113 days of storage, α-tocopherol concentrations decreased with increasing amount of fish oil in the mayonnaise stored at 2°C, except for EY_4%FO which had the same concentration as EY_STD (without fish oil). The same tendency was observed at 20°C although differences were more pronounced between the different samples for the mayonnaise with egg yolk as emulsifier. These observations were a clear indication on increased oxidation with increasing fish oil concentration. For mayonnaise with milk protein, the opposite was observed; MP_STD < MP_14%FO (data not shown).

For β- and γ-tocopherol, concentrations depended on the fish oil concentration throughout the storage period (Table 3). Thus, a higher fish oil concentration resulted in lower β- and γ-tocopherol concentrations due to the higher amount of these tocopherols in rape seed oil compared to fish oil. At the end of the storage, the differences in tocopherol concentrations with different fish oil concentrations were even more pronounced. However, in the mayonnaises with milk protein-based emulsifier, the concentrations of β- and γ-tocopherol was not significantly different after storage at 20°C for 113 days (MP_STD and MP_14%FO). Compared to the initial concentrations, the concentrations were reduced more in MP_STD (without fish oil) than MP_14%FO indicating severe oxidation of MP_STD.

Figure 2 shows results from the PV measurements. Generally, PV increased during storage at both temperatures. However, the PV increased much faster and to a higher level when the mayonnaises were stored at 20°C than at 2°C. Throughout the storage period PV tended to be highest in mayonnaises with highest fish oil concentration. For mayonnaises stored at 20°C for 113 days (Fig. 2A), the ranking was; $MP\_14\%FO^{d} \geq EY\_10\%FO^{cd} \geq EY\_14\%FO^{bc} \geq EY\_4\%FO^{ab} \geq EY\_STD^{ab} \geq MP\_STD^{a}$ (different superscripts indicate significantly different PV levels). A slightly different pattern was observed for mayonnaises stored at low temperature (2°C) (Fig. 2B). At the end of the storage period, PV levels were as follows: $MP\_14\%FO^{d} > EY\_14\%FO^{c} \geq EY\_10\%FO^{bc} \geq EY\_4\%FO^{ab} \geq MP\_STD^{a} = EY\_STD^{a}$. Nevertheless, the results from the PV analysis showed that substituting egg yolk with milk protein and adding high levels of fish oil to the mayonnaise raised the PV level at both storage temperatures.

Table 3. The concentration of γ-tocopherol (mg/kg mayonnaise) after production and after storage with indication of significant differences in concentration between different mayonnaises[a)]

	EY_STD	EY_4%FO	EY_10%FO	EY_14%FO	MP_STD	MP_14%FO
Day 0 (mg/kg)	100 ± 1.1^{e}	93 ± 1.1^{d}	79 ± 0.7^{c}	70 ± 0.3^{a}	108 ± 0.8^{f}	73 ± 1.4^{b}
20°C: day 113 (mg/kg)	45.5 ± 1.2^{e}	34.7 ± 0.2^{d}	25.7 ± 0.4^{c}	13.6 ± 0.1^{a}	19.1 ± 0.2^{b}	18.7 ± 0.1^{b}
20°C: Δ (mg/kg)	55 ↓	59 ↓	53 ↓	56 ↓	88 ↓	55 ↓
2°C: day 113 (mg/kg)	67.6 ± 5.0^{d}	61.0 ± 0.1^{c}	50.0 ± 0.5^{b}	43.8 ± 0.7^{a}	69.6 ± 1.2^{d}	47.1 ± 2.8^{ab}
2°C: Δ (mg/kg)	33 ↓	32 ↓	29 ↓	26 ↓	38 ↓	26 ↓

Different letters in same row indicate that the concentration in these samples was significant different. The changes in concentration during storage at 20 and 2°C after 113 days are also given. The arrows behind the number indicate if the changes are increased or decreased. Sample codes are explained in Table 1.

[a)]Different letters in the same row indicate significant differences in the concentration between mayonnaise samples at that specific day and temperature.

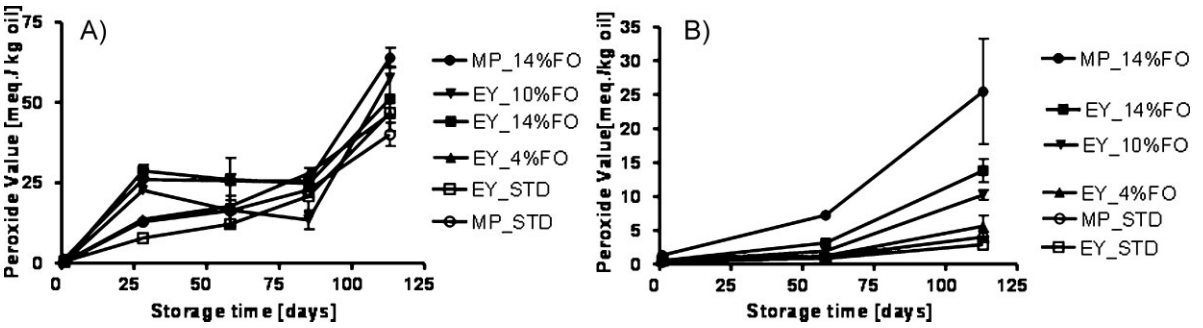

Figure 2. The concentration of lipid hydroperoxides expressed in PV in the mayonnaises during storage. (A) Mayonnaises stored at 20°C and (B) Mayonnaises stored at 2°C. The error bars indicate the SD of two measurements. Codes are explained in Table 1.

Several volatiles were detected and quantified in the mayonnaises during storage: 2-ethyl furan, pentanal, 2(t)-pentenal, 1-penten-3-one, 2(t)-butenal, hexanal, 1-penten-3-ol, heptanal, 2(t)-hexenal, 2-pentyl furan, 4(c)-heptenal, nonanal, 2,4(t,t)-heptadienal and 2,6(t,c)-nonadienal. Similar to the findings for PV, the concentration of the different volatiles increased during storage and a faster increase was observed for mayonnaises stored at 20°C as exemplified in Fig. 3 by the increase in concentrations of 1-penten-3-one (Fig. 3A and B) and 2,6(t,c)-nonadienal (Fig. 3C and D) in the mayonnaises during storage at 20 and 2°C, respectively. Significant differences in concentrations of 1-penten-3-one and 2,6(t,c)-nonadienal between the mayonnaise samples at day 113 are indicated in Fig. 3. A higher fish oil concentration generally resulted in increased volatile concentrations compared to mayonnaises with low or no fish oil added. Either, the mayonnaise with milk protein and high fish oil level (MP_14%FO) or the mayonnaise with egg yolk and high fish oil level (EY_14%FO) had the highest concentration of the different volatiles. Concentrations of 1-penten-3-one, 2(t)-butenal, 1-penten-3-ol and 2,4(t,t)-heptadienal, were significantly higher in mayonnaise with milk protein (MP_14%FO). In contrast, concentrations of pentanal, nonanal, heptanal, hexanal and pentyl furan were significantly higher in mayonnaises with egg yolk (EY_14%FO) at the end of the storage period. The reason for the increased formation of pentyl furan and saturated volatiles in mayonnaise with egg yolk could be that these volatiles were released

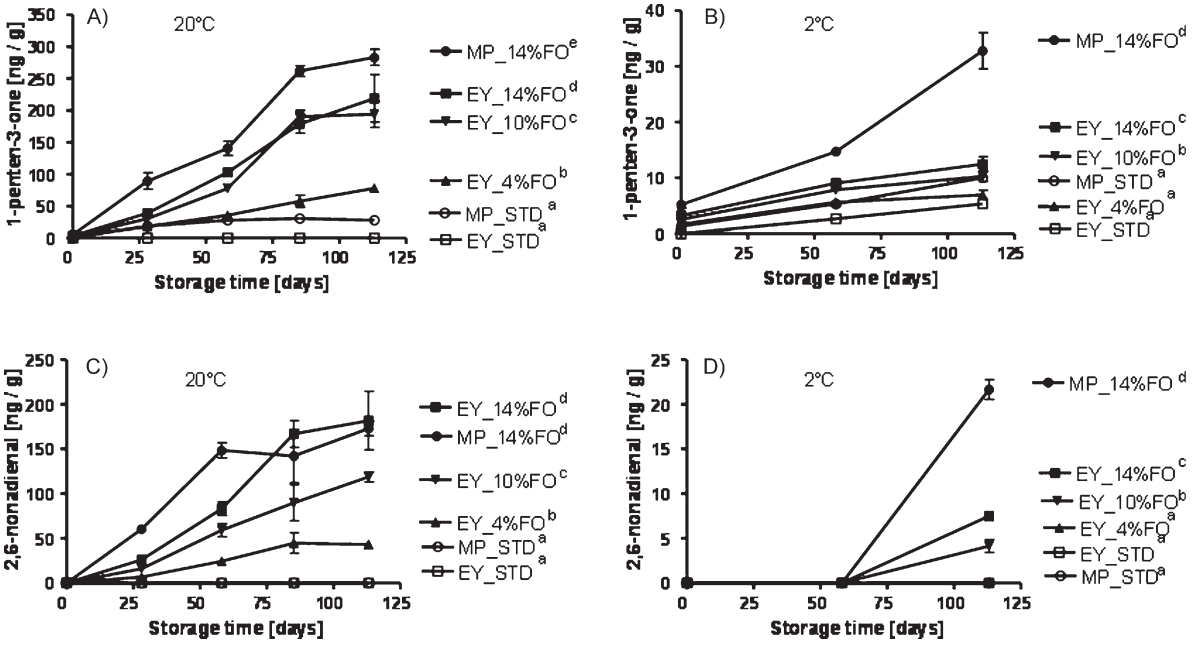

Figure 3. The concentration of 1-penten-3-one and 2,6(t,c)-nonadienal quantified in the mayonnaises during storage. (A) 1-Penten-3-one, mayonnaises stored at 20°C, (B) 1-penten-3-one, mayonnaises stored at 2°C, (C) 2,6-nonadienal, mayonnaises stored at 20°C and (D) 2,6-nonadienal, mayonnaises stored at 2°C. The error bars indicate the SD of three measurements. Codes are explained in Table 1. A significant difference between two codes is denoted with different superscripts after the codes (significance level $p < 0.05$).

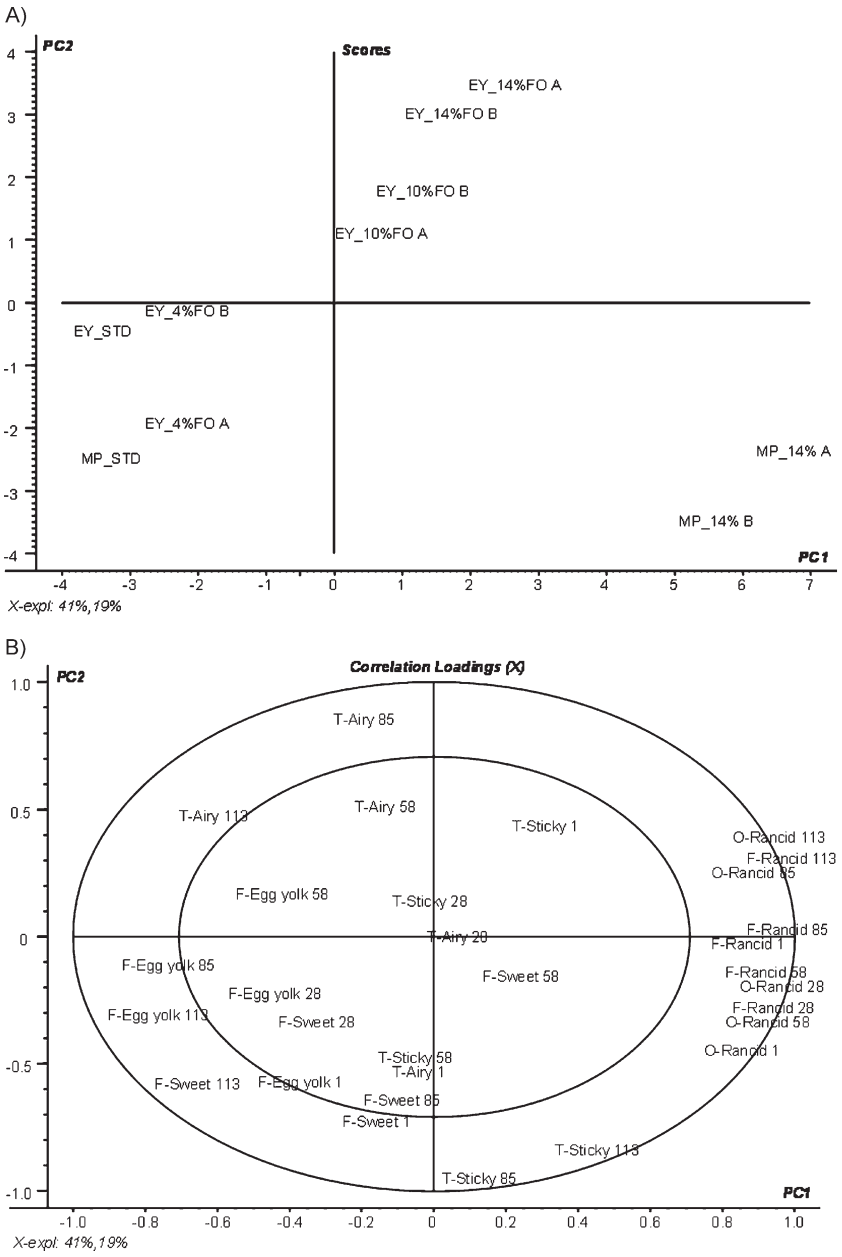

Figure 4. PCA model with sensory results (mayonnaise, 20°C) build on selected attributes (PC 1 *vs.* PC 2, 60% explained variance). (A) Score plot, codes are explained in Table 1 plus A and B after code name denotes double evaluation, (B) correlation loadings (attributes). Numbers refer to storage day and letters in front of the attributes (O, F and T) are abbreviations for odour, flavour and texture, respectively.

more easily from the matrix in the egg yolk stabilised mayonnaise, when lipid oxidation proceeds. The pattern for the remaining volatiles at the two different temperatures was either similar to that of 1-penten-3-one or to that of 2,6-nonadienal. For a few volatiles (hexanal, heptanal and nonanal), the standard mayonnaises (without fish oil) had higher concentrations at the end of the storage at 2°C compared with the other mayonnaises (data not shown).

Sensory results were analyzed by PCA, which explained 60% of the variance in the two first principal components (PCs) (Fig. 4). The score plot clearly shows grouping of the mayonnaises depending on fish oil concentration and type of emulsifier added. Thus, mayonnaises without fish oil (EY_STD and MP_STD) and the mayonnaise with low fish oil concentration and egg yolk as emulsifier (EY_4%FO) constituted one group, mayonnaises with medium and high fish oil concentration and egg yolk as emulsifier (EY_10%FO and EY_14%FO) a second group and mayonnaises with high fish oil concentration and milk protein as emulsifier (MP_14%FO) the third group. From the loading plot a clear pattern in the grouping of the sensory attributes could be observed (Fig. 4B). The first PC thus mainly explained the variation in the attributes sweet and egg yolk flavours with negative PC 1 values and rancid odour and flavour with

positive PC 1 values, which indicated negative correlations between egg yolk plus sweet flavour and rancid odour and flavour. PC 2 mainly described the differences between airy and sticky, which moved further up and down this axis during storage, respectively. Thus, mayonnaises without fish oil and the mayonnaise with 4% fish oil were characterised by a higher intensity in sweet and egg yolk flavour even though MP_STD did not contain egg yolk. This group was located opposite to the attributes for rancidity, and this finding thus indicates less lipid oxidation in these mayonnaises. The other two groups, namely milk protein mayonnaise with high fish oil levels and egg yolk mayonnaise with medium plus high fish oil were located in the same side of the plot as rancidity, however separated from each other by airy and sticky. Hence, mayonnaise with higher fish oil concentration was characterized by higher rancid intensity. For egg yolk mayonnaises with 10 or 14% fish oil, the mayonnaise got a more airy texture during storage, whereas milk protein mayonnaises with fish oil became stickier during storage. These findings are in accordance with the results from the viscosity measurements.

3.5 Effect of storage temperature and fish oil concentration

It is well known from the lipid oxidation theory that increased temperature leads to increased lipid oxidation [27] and other experiments with mayonnaise and milk enriched with fish oil have shown the same [13, 28, 29]. Hence, the increased oxidation at higher temperature was in agreement with these findings. Results from both lipid hydroperoxides and volatiles measurements showed significant oxidation at 20°C after 4 months in all mayonnaises. Hence, it is not recommended to store mayonnaise at 20°C for 4 months, even when fish oil is not added. This is common practice in many supermarkets today.

Comparison of the oxidation after approximately 60 days of storage, fish oil-enriched mayonnaise produced for mayonnaise salads (63% oil, 6.3% fish oil, storage: 57 days at 2°C) in an earlier study [12] with similar data for mayonnaise containing 40% oil and 4% fish oil in the present study, showed that the level (%, calculated based on their corresponding controls without fish oil) of some volatile compounds like 1-penten-3-one, 2-butenal, 2-pentenal, 1-penten-3-ol, 2-pentyl furan, nonanal and 2,4-heptadienal were higher in 40% light mayonnaise than in 63% mayonnaise. Moreover, 80% mayonnaise (16% fish oil, storage: 14 wk at 5°C) had a similar relative increase in lipid oxidation products (5–14 wk of storage at 5°C [25]) as observed for 40% light mayonnaise between 8 and 16 wk of storage at 2°C (10% fish oil). In spite of the lower total oil content in the light mayonnaise it oxidized faster than 63% mayonnaise and at same rate as full fat mayonnaise (80%). Thus, it seems that other factors than the oil content have an impact on the oxidation rate in such light products and this has to be investigated further.

To the best of our knowledge there are no published results of the effect of different fish oil concentrations in mayonnaises or other products. However, it is expected that increasing the amount of polyunsaturated fatty acids will increase the level of lipid oxidation, since the oxidation rate is much faster for highly unsaturated fatty acids [27].

Taken together, the data in the present study show that addition of 4% fish oil to mayonnaise with egg yolk result in higher concentration of volatiles, but did not result in increased sensory scores for rancidity compared to mayonnaise without fish oil when stored at 20°C. However, mayonnaise enriched with 4% fish oil and stored at 2°C was not more oxidized than mayonnaise without fish oil as concluded from PV and volatiles. Therefore, it seems possible to enrich light mayonnaises with 4% fish oil without adding antioxidant, if stored at 2°C, but this has to be confirmed by sensory analysis. At such a substitution level, one portion of mayonnaise (20 g) would provide 0.16 g EPA + DHA.

3.6 Effect of emulsifier on lipid oxidation

As previously mentioned, earlier experiments with fish oil-enriched mayonnaise led to the conclusion, that iron from the egg yolk and low pH were responsible for the oxidative deterioration of fish oil-enriched mayonnaise [8, 30]. Surprisingly, the present study showed that mayonnaises with milk protein oxidized faster than mayonnaises with egg yolk as emulsifier, even though the iron content was much higher in egg yolk stabilized mayonnaise.

The pH of the produced mayonnaises were between 3.5 and 4.1, and depending of pI (isoelectronic point) of the applied proteins, the surface of the lipid droplet can be either positively (pH < pI) or negatively (pH > pI) charged. Repulsive forces between positively charged lipid droplets and iron would repel the iron from the oil–water interface where interactions between iron and lipids or lipid hydroperoxides otherwise would promote oxidation. Hence, less lipid oxidation might be expected when the lipid droplet is positivity charged. In contrast, attractive forces between negatively charged lipid droplets and iron could lead to increased oxidation [31]. Several studies have confirmed that repulsive forces reduce lipid oxidation in emulsions [32–34]. Both milk proteins (4.6–5.2 [35]) and egg yolk proteins (~5.5 [36]) have pI values higher than pH of the mayonnaises. Thereby, the net charge of the oil droplets in both types of mayonnaises might be expected to be positive. Thus, the differences observed in the oxidative stability of mayonnaises prepared with the two types of emulsifier were most likely not due to different charges of the lipid droplets. Moreover, the milk protein-based emulsifier contains hydrocolloids with pK_a lower than pH in the mayonnaises (alginate pK_a: 1.5–3.5). Hence, then the hydrocolloids will be negatively charged, which gives the possibility for formation of a

multilayer between milk proteins and the hydrocolloids [37]. Such a multilayer can protect the lipids from prooxidants by acting as a physical barrier [31]. According to our findings, a possible multilayer in the mayonnaise with milk protein did not improve the oxidative stability compared to mayonnaise with egg yolk as emulsifier. However, the charge of the lipid droplets and thickness are not the only factors of importance, since food emulsions are complex matrixes where several factors might influence oxidation such as other ingredients, their quality, anti- and prooxidative properties, metals and viscosity [31, 38].

Emulsifier dependant changes observed in droplet sizes and viscosity were observed during storage at 20°C, but not at 2°C of storage, but still differences in the oxidative stability between the different mayonnaises were observed at both temperatures. Therefore, it is not likely that the differences in the microstructure contributed significantly to the differences in oxidative stability.

An increased viscosity might result in reduced diffusion rate of prooxidants to the oil droplet surface, which will decrease the rate of lipid oxidation [39]. Paraskevopoulou *et al.* have confirmed this in a study with olive oil–lemon juice salad dressings stabilized with polysaccharides. They found that the presence of xanthan gum together with gum arabic or alginate decreased oxidation and that this could partly be due to an increase in viscosity [26]. In addition, Shimada *et al.* have evaluated the antioxidative effect of xanthan gum and other polysaccharides and concluded that the antioxidative mechanism of xanthan gum primarily was due to its high metal-binding ability and secondly to its viscous behaviour. In contrast, guar gum was not able to form a complex with Fe^{2+} in acidic or neutral media [40]. The mayonnaise with egg yolk also contain xanthan gum, however the iron level in this mayonnaise was fourfold higher than in the mayonnaise with milk protein. Thus, previous data suggest that, in contrast to our findings, mayonnaise with milk protein may be expected to be more oxidative stable than mayonnaise with egg yolk.

As already mentioned xanthan gum may have antioxidative properties due to its metal chelating properties [26, 39, 40]. However, studies have also indicated that it can have prooxidative properties by interacting with unadsorbed whey protein isolate (WPI) in the continuous phase in WPI stabilized o/w emulsions. Thereby, xanthan gum prevents WPI from acting as antioxidant and it might also loose its metal binding properties itself [39]. This might at least partly explain our findings that milk protein stabilized mayonnaise was more oxidized than egg yolk stabilized mayonnaise.

Another issue in fish oil-enriched food products is the quality of the oil and ingredients. The importance of initial oil quality was confirmed by Let *et al.* [13] who showed that oil quality significantly affected the onset of lipid oxidation. The only difference between the two fish oil-enriched mayonnaises in the present study was the emulsifiers. To investigate whether the quality of the emulsifiers could have influenced the oxidative stability of the mayonnaises, their PV was measured. The milk protein emulsifier had more than a 100-fold higher PV than the egg yolk emulsifier based on per kilogram oil (9.8 *vs.* < 0.1 meq peroxides/kg oil) and even higher based on per kilogram emulsifier. This means that the milk protein mixture was not of high quality from the start. This might explain the increased lipid oxidation observed in fish oil-enriched mayonnaise with milk protein compared with the mayonnaise emulsified with egg yolk.

4 Conclusion

There was a significant increase in lipid oxidation when even small amounts of the rape seed oil were substituted with fish oil at 20°C. Thus, it is not recommended to store mayonnaises at 20°C. Mayonnaise enriched with 4% fish oil and stored at 2°C was not more oxidized than mayonnaises without fish oil. Therefore, it seems possible to enrich light mayonnaises with 4% fish oil without adding antioxidant, but this has to be confirmed by sensory analysis. Moreover, it might be possible to further increase the fish oil concentration slightly, since earlier experiment showed good oxidative stability with 6.3% fish oil in mayonnaises containing 63% oil.

Our hypothesis that substituting egg yolk with a less iron-containing emulsifier might increase the oxidative stability of fish oil-enriched mayonnaises, was not confirmed. Moreover, the possible formation of a multilayer or a cationic surface around the oil together with a more viscous mayonnaise did not increase the oxidative stability compared to mayonnaise with egg yolk as emulsifier. However, the PV level for milk protein-based emulsifier was around 100-fold higher than the PV level in egg yolk. Therefore, the poorer oxidative stability of the milk protein-based mayonnaise is suggested to be due to the initial quality of the emulsifier. In this experiment the initial quality of the emulsifiers seemed to be more important than their iron content. However, the effect of the initial quality on the subsequent lipid oxidation in the product has to be further investigated.

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PAPER III

Sørensen, A.-D.M., Nielsen, N.S., Decker, E.A., Let, M.B, Xu, X. & Jacobsen, C.

The efficacy of compounds with different polarities as antioxidant in emulsions with fish oil.

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The efficacy of compounds with different polarities as antioxidant in emulsions with omega-3 lipids

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Running title: Efficacy of compounds with different polarities as antioxidant

Abstract

According to the so-called polar paradox hypothesis, the efficacy of an antioxidant in emulsions is highly affected by its polarity and thereby location in the different phases. However, other factors also affect the efficacy of antioxidants in multiphase systems. The aim of this study was to evaluate the efficacy of antioxidants (ascorbic acid, ascorbyl palmitate, ascorbyl CLA and CLA) with different polarities in two different emulsion systems: o/w emulsion (5% oil) and w/o emulsion (98% oil) stabilized with citrem and PGPR, respectively. The efficacy of the antioxidants was compared to their partitioning in an o/w emulsion system and to results obtained from different antioxidant assays: iron reducing power, chelating activity and radical scavenging activity. For the w/o emulsions the efficacy of the antioxidants followed the polar paradox hypothesis: ascorbyl palmitate = ascorbyl CLA > ascorbic acid > CLA > reference. For the o/w emulsion the antioxidative effects were not in accordance with the polar paradox. In the beginning of the storage, ascorbyl palmitate and ascorbic acid were most efficient, however in the end they acted as prooxidants. Ascorbyl CLA was located at the interface but was inactive as an antioxidant. This may be due to impurities or interaction with citrem.

Introduction

Healthy n-3 long chain polyunsaturated fatty acids (PUFA) in fish oil, EPA and DHA, are currently added to a number of different food systems as functional ingredients. These lipids are highly susceptible to lipid oxidation and therefore protection against oxidation, e.g. by antioxidant addition is necessary.

According to the so-called polar paradox hypothesis, the efficacy of antioxidants in multiphase systems are highly affected by the polarity of the antioxidants and thereby the distribution in the different phases of multiphase systems [1, 2]. Therefore, amphiphilic antioxidants have previously been suggested to have better efficacy than lipophilic or hydrophilic antioxidants in multiphase systems [2]. In addition, earlier work of Frankel and co-workers [1] on the antioxidant effect in bulk oil versus emulsion showed that ascorbyl palmitate was more efficient in 10% o/w emulsions than ascorbic acid. The reverse trend was evident in bulk oil [1]. Thus, we hypothesize that the antioxidative properties of hydrophilic compounds such as ascorbic acid in emulsions may also be improved by esterifying hydrophilic antioxidants with other fatty acids such as CLA (conjugated linoleic acid).

Ascorbyl palmitate is an antioxidant, which has been tested and used in several food systems [3] whereas the antioxidative effect of ascorbyl CLA has not previously been investigated. Earlier studies have shown a weight reducing effect of CLA as well as antioxidative [4, 5] and anticarcinogenic effects in vitro and in animal studies [6]. However, the antioxidative properties reported for CLA conflicted with other reported results, which showed prooxidative effects of CLA [7].

Several reports on lipophilized ascorbic acid have indicated antioxidative effects of the esters in different systems [8]. Thus, a combination of ascorbic acid and CLA might have a similar antioxidative activity compared with ascorbyl palmitate.

Emulsions can either be o/w or w/o emulsions. The initial step in lipid oxidation in emulsions has been suggested to take place at the interface between the oil and water phases [9]. Differences in the efficacy of amphiphilic antioxidants in the two systems are hard to anticipate. A few reports are available on the effects of antioxidants in w/o emulsions and to our knowledge no direct comparison of the efficacy of the same antioxidant in o/w and w/o emulsions has been reported.

Lipid hydroperoxides are more polar than lipids and therefore more water soluble than lipids. Especially lipid hydroperoxides from EPA and DHA are more polar [10], since more than one peroxyl group can be attached to the same fatty acids. Hence, it is expected that peroxyl radicals to some degree will be present at the water-lipid interface. Moreover, hydroxyl radicals might be present in the water phase. Therefore, it may be anticipated that hydrophilic antioxidants will offer better protection in the w/o system than in the o/w system. Moreover, in the w/o emulsion hydrophilic antioxidants will be present in a relatively higher concentration in the disperse water phase than in the continuous water phase in the o/w emulsion provided that the disperse phase constitutes approximately the same volume in the two systems. The higher concentration of hydrophilic antioxidants in the water phase in the w/o emulsion may imply that antioxidant will be located closer to the interface in the w/o emulsion than in the o/w emulsion and this could in turn lead to a better effect.

To investigate our hypothesis and to improve our understanding about antioxidant effects in w/o versus o/w emulsions, the effect of four different antioxidants with different polarities was evaluated in two model systems: o/w emulsion (5% oil) with citrem as an emulsifier and w/o emulsion (98% oil) with PGPR (polyglycerol polyricinoleat) as emulsifier, both at pH 7. The development of hydroperoxides and secondary volatile oxidation products were measured. In addition the antioxidative effects of the antioxidants (ascorbyl palmitate, ascorbyl CLA and ascorbic acid) were compared with their partioning in a system resembling the o/w emulsions. Furthermore, the antioxidative mechanism was characterised by different antioxidant assays: iron reducing power, chelating activity and radical scavenging activity (DPPH).

Material and methods

Materials. The mixture of fish oil and rapeseed oil (1:1) without antioxidant added was supplied by Maritex Norway (subsidiary of TINE BA, Norway). This oil had an initial PV < 0.1 meq peroxides.kg-1 oil, tocopherol content of 274 mg α-tocopherol, 30 mg β-tocopherol, 172 mg γ-tocopherol and 9 mg δ-tocopherol.kg oil-1, and the fatty acid composition was as follows: 14:0, 1.5%; 16:0, 7.0%; 16:1, 4.2%; 18:0, 1.9%; 18:1, 42.0%; 18:2, 10.2%; 18:3, 4.6%; 18:4, 1.1%; 20:1, 6.6%; 20:5 (EPA), 4.5%; 22:1, 3.2% and 22:6 (DHA), 5.9%. The total percentages of n-3 and n-6 in the oil were 11.5% and 14.6%, respectively.

The emulsifiers Polyglycerol polyricinoleate (PGPR) and Citrem LR 10 Extra (citric acid ester of mono- and diglyceride) without antioxidants were supplied by Palsgaard A/S (Juelsminde, Denmark) and Danisco A/S (Grindsted, Denmark), respectively. Ascorbic acid was obtained from Merck (Darmstadt, Germany), ascorbyl palmitate was obtained from Sigma Aldrich (Steinheim, Germany), CLA (Tonalin® FF80) was obtained from Cognis (Monheim, Germany). Ascorbyl CLA was synthesized at the Department of Molecular Biology, Faculty of Science, Aarhus University. Chemicals were purchased from Merck (Darmstadt, Germany) and external standards for identification and quantification of secondary volatile oxidation products were all from Sigma Aldrich (Steinheim, Germany). All solvents were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland) or Fisher Scientific (Fair Lawn, USA).

Antioxidant activity assays.

Iron chelating activity. Ferrozine is an iron chelating compound, which develops an intensively coloured complex with an absorption maximum at 562 nm when free ferrous iron is chelated by the ferrozine molecule. Briefly, antioxidant solutions (0-200 μmol.L-1) were made up to a volume of

3.7 mL with distilled water. Due to solubilization problems with CLA in water, CLA was dissolved in small amounts of methanol and then diluted with water. Ferrous chloride (2 mmol.L-1, 0.1 mL) was added and after 3 min the reaction was inhibited by the addition of a ferrozine solution (5 mmol.L-1, 0.2 mL). The mixture was shaken vigorously and left at room temperature for 10 min, whereafter the absorbance was measured at 562 nm (UV mini 1240 Shimadzu, Kyoto, Japan). The chelating activities (%) of the antioxidants were calculated as follows: Fe^{2+} chelating activity $=\left(\frac{A_0 - A_s}{A_0}\right)\times 100$, where A_0 is absorbance of control (only iron and ferrozine) and A_s is absorbance of sample minus absorbance of sample blank. Triplicate measurements were performed and EDTA was included as a positive control.

Radical scavenging effect. DPPH is a free radical with a purple colour with an absorption maximum at 517 nm. When the odd electron of DPPH becomes paired the colour changes to yellow. The antioxidant (0-200 μmol.L-1 in methanol) was mixed with a 0.1 mmol.L-1 methanolic solution of DPPH (1:4, v/v). Absorbance was measured after 30 min (ambient temperature, darkness) at 517 nm (UV mini 1240 Shimadzu, Kyoto, Japan). The results was reported as percent inhibition: $Inhibition\,[\%]=\left(1-\frac{A_s}{A_0}\right)\times 100$, where A_s is absorbance of DPPH after reaction with antioxidant minus absorbance of antioxidant in methanol and A_0 is absorbance of DPPH in methanolic solution. Triplicate measurements were performed and BHT was included as a positive control.

Reducing power. Reduction of Fe^{3+} to Fe^{2+} can be measured spectrophotometrically at 700 nm, since Fe^{2+} forms a coloured complex with cyanide. An aliquot of sample (1 mL, 0-200 μmol.L-1 antioxidant) was mixed with 0.2 mol.L-1 phosphate buffer (pH 6.6, 2.5 mL) and 30 mmol.L-1 potassium ferricyanide (2.5 mL). The mixture was incubated for 20 min at 50°C. A 0.6 mol.L-1 trichloroacetic acid solution (2.5 mL) was added and thereafter the mixture was centrifuged (10 min,

2000 rpm). The upper layer of the solution (2.0 mL) was mixed with an equal amount of distilled water and 0.4 mL 6 mmol.L-1 $FeCl_3$, and the absorbance (700 nm) was measured after 10 min. High absorbance equals high reducing power. Triplicate measurements were performed.

Partitioning of antioxidants. Partitioning of the three different antioxidants (ascorbic acid, ascorbyl palmitate and ascorbyl CLA) in buffer/oil, emulsifier/buffer and in o/w emulsion was measured according to the method described below [11]. Antioxidants were dissolved in methanol and the methanol was evaporated by nitrogen before the different phases were added into the centrifuge tube in the buffer/oil and emulsifier/buffer system.

Water (buffer) /Oil. Antioxidant (100 μmol.L-1), 10 mmol.L-1 acetate-imidazol buffer (95%) and oil (5%) to a total amount of 40 g were vigorously mixed with a vortex mixer and thereafter placed overnight at 5°C in dark and sealed centrifuge tubes for equilibration of antioxidants between the phases. The next day the oil and water phase was separated by centrifugation (10 min, 210*g*). The oil phases were discarded and water phase centrifuged once more before analysis of antioxidant concentration. This experiment was performed in triplicate for each antioxidant.

Emulsifier/Water (buffer). Antioxidant (100 μmol.L-1), 10 mmol.L-1 acetate-imidazol buffer (99%) and citrem (1%) to a total amount of 40 g were vigorously mixed with a vortex mixer and thereafter placed overnight at 5°C in dark in sealed centrifuge tubes for equilibration of antioxidants between the phases. Thereafter, the buffer was separated from citrem by centrifugation (3 mL, 10 min, 210*g*) in Amicon® Ultra centrifugal filter devices (regenerated cellulose 3,000 MW cut off, Millipore, Carrigtwohill, Ireland). The buffer in the bottom of the tube was collected and the rest of the buffer-citrem solution at the top was discarded every time and the aqueous phase in the bottom was discarded the first 3 times to saturate the filter. This procedure was repeated 6 times and the last

3 buffer fractions were collected for measuring the concentration of antioxidant in the buffer phase. This experiment was performed in triplicate for each antioxidant.

Emulsions (o/w). Antioxidant (100 μmol.L-1), 10 mM acetate-imidazol buffer (94%), oil (5%) and citrem (1%) to a total amount of 200 g were mixed for 3 min with an Ultra-Turrax (Janke & Kunkel IKA-Labortechnik, Staufen, Germany) and thereafter placed overnight at 5°C in dark, capped bottles for equilibration of antioxidants between the phases. The procedure described for the emulsifier/buffer system was also used here to separate the buffer from the emulsion. Two emulsions were performed for each antioxidant with duplicate separation of the aqueous phase from each emulsion.

Concentration of antioxidants.

Ascorbic acid. Ascorbic acid in the buffer was quantified using an enzymatic kit (R-BIOPHARM AG, Darmstadt, Germany). In two of the partitioning studies: emulsifier/water and the whole emulsion, the buffer fractions obtained by repeated centrifugations after saturation of the filter, were pooled prior to analysis.

Ascorbyl palmitate and Ascorbyl CLA. These compounds were analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) with a C18 Thermo Hypersil ODS (250, 4.6 mm) column and using a gradient elution at a flow rate of 1 mL.min-1. Solvent A was 1% phosphoric acid (v/v) and solvent B was acetonitrile:methanol, 1:1 (v/v). Gradient condition: 0-20 min 80-100% B and 20-25 min 100-80% B. Injection volume was 20 μL. Ascorbyl palmitate and Ascorbyl CLA were measured spectrophotometrically at 250 nm. Ascorbyl palmitate and ascorbyl CLA were quantified by calibration curves of these compounds dissolved in methanol and acetonitrile, respectively.

Emulsion production for storage experiments.

It is not possible to use the same emulsifier for production of o/w and w/o emulsions, but the emulsifiers used in the present study were selected to have similarities in their structure (Figure 1). Since both have a glycerol structure with either citric acid (citrem) and 1 or 2 fatty alkyl group substituted or different degrees of fatty alkyl groups substituted to an n degree polymerized glycerol (PGPR, n average > 1). The antioxidants ascorbic acid, ascorbic palmitate, ascorbic CLA and CLA (Figure 2), were added separately in a concentration of 100 μM in the emulsion. The antioxidative effects were evaluated during storage of both o/w and w/o emulsions (37°C)

W/O emulsions. The water-in-oil emulsion systems consisted of 98% oil (fish oil and rapeseed oil, 1:1), 1% 10 mmol.L-1 acetate-imidazole buffer (pH 7), 1% PGPR as emulsifier and 100 μmol.L-1 antioxidant (CLA only 68 μmol.L-1 due to deviation in purity). Oil with PGPR was heated in a water bath (50°C, 15 min). Buffer was added within the first min of 2 mins prehomogenization with a hand-held homogenizer (M133/1281-0, Biospec Products, Inc., Bartlesville, OK). For emulsification a microfluidizer (Microfluidics, Newton, MA) was used with the settings as follows, a 75 micron chamber, pressure at 9,000 psi and 3 circulations of the emulsions. During homogenization, ice was used to cover the homogenizer chamber and coil in order to maintain the emulsion temperature around 25°C. Ascorbic acid, ascorbyl palmitate or ascorbyl CLA was added directly to the continuous phase (oil), whereas CLA was dissolved in methanol and then added to the oil (0.5 mL). The same amount of methanol was added to all the other emulsions. The final emulsion was pipetted into 10 mL GC vials (volume: 1.1 mL equals 1 g of emulsion) capped with aluminium caps with PTFE/silicone septa and stored in the dark at 37°C. For each sampling day three vials were analyzed once (triplicates) for each type of analysis. For the w/o emulsions, one reference without antioxidant was included.

O/W emulsions. The oil-in-water emulsions consisted of 94% 10 mmol.L-1 acetate-imidazole buffer (pH 7), 5% oil (fish oil and rapeseed oil, 1:1), 1% citrem as emulsifier and 100 μmol.L-1 antioxidant (CLA only 68 μmol.L-1 due to deviation in purity). Similar to the w/o emulsions, the o/w emulsions were pre-emulsified prior to homogenization. For pre-emulsification, the buffer was stirred with an Ultra-Turrax (Janke & Kunkel IKA-Labortechnik, Staufen, Germany) and oil-citrem solution was added during the first min of the 2 mins total mixing. The pre-emulsion was then homogenized at a pressure of 80 and 800 bar with 4 circulations of the emulsion at room temperature using a 2 stage homogenizer (GEA Niro Soavi Spa, Parma, Italy). Ascorbic acid, ascorbyl palmitate or ascorbyl CLA was added directly to the continuous phase (buffer), whereas CLA was dissolved in methanol and then added to the buffer (0.5 mL). The same amount of methanol was added to all the other emulsions. The emulsions (100 g) were stored in 100 mL blue cap bottles on stirring plates in the dark (37°C). One bottle per emulsion was taken at days 0, 4, 8, 12 and 15 and divided in brown glass bottles and stored at -40°C until analyses of peroxides and volatiles were performed. The droplet size was measured at day 1, 8 and 15 without prefreezing. For the o/w emulsions, two references without antioxidants were included and the average of these two samples was used as a reference.

Droplet size.

W/O emulsions. Droplet sizes were measured by a Zetasizer Nano-25 (Malvern Instruments Ltd., Worcestershire, UK). The emulsion was mixed with hexadecane (1:2 v/v). The following settings were used; refractive index (RI) for the oil was 1.434, viscosity of the disperse phase 5.5 mPaS and RI_{water} was 1.330. The droplet size was calculated from viscosity of the continuous phase and the measured back scattering from particle movements. Triplicate measurements were performed.

O/W emulsions. The size of the lipid droplets in the o/w emulsion was determined by laser diffraction using a Mastersizer2000 (Malvern Instruments Ltd., Worcestershire, UK). The o/w emulsion was diluted directly in recirculating water (3000 rpm) reaching an obscuration of 12-14%. Sunflower oil (RI_{oil} = 1.469) and water (RI_{water} = 1.330) were used as particle and dispersant, respectively. Duplicate measurements were performed. Results are given in surface area mean diameter: $D_{3,2}=\frac{\sum d^3}{\sum d^2}$.

Primary oxidation products, Peroxide value (PV).

W/O emulsions. Lipid hydroperoxides were extracted by a mixture of methanol and 1-butanol (2:1, v/v). After centrifugation for 3 min (2000*g*), the clear upper layer (lipid phase) was used directly to quantify hydroperoxides by a modified method of Shanta and Decker [12]. Hydroperoxide concentrations were determined using a cumene hydroperoxide standard curve.

O/W emulsions. Lipids were extracted from emulsion according to a modified Bligh and Dyer method [13] using a reduced amount of methanol:chloroform, 1:1 v/v [14]. Hydroperoxides were measured as described for the lipid extracts from w/o emulsions, but the concentrations were calculated using a ferrichloride standard curve and a conversion factor of 2 to transform the unit from meq O_2.kg oil-1 to meq peroxides.kg oil-1. Duplicate measurements were performed.

Secondary oxidation products.

W/O emulsions. Propanal and hexanal were determined directly in the stored GC vials using a GC 2014 Shimadzu gas chromatograph equipped with an autosampler (Shimadzu, Kyoto, Japan) and a DB-1 column (30 m x ID 0.32 mm x 1 mm film thickness, Supelco, Bellefonte, PA). Each sample was heated at 55°C in the autosampler heating block for 15 mins and headspace volatiles were

adsorbed on a solid phase microextraction (SPME) fiber needle (50/30 μm DVB/Carboxen/PDMS, Supelco, Bellefonte, PA) over 1 min. Volatiles were desorbed in the injector port at 250°C for 3 mins and passed through a DB-1 column in 10 min at 65°C (FID temperature was 250°C). Concentrations were determined from peak areas using propanal and hexanal standard curves prepared from propanal and hexanal in a w/o emulsion using MCT oil (medium chain triglyceride) in the emulsion. Results are given in ng.g emulsion-1.

O/W emulsions. Collection, separation and identification of volatiles was performed by dynamic headspace GC-MS (GC: 5890 IIA, Hewlett-Packard, CA, USA and MS: HP 5972 mass selective detector) equipped with a DB wax column (30 m x ID 0.25 mm x 0.5 μm film thickness, J&W Scientific, CA, USA). Volatiles from the emulsions (4 g) with 2-4 mL of synperonic (antifoam) were purged from the emulsion (45°C) with nitrogen for 30 min and trapped on Tenax tubes. The trapped volatiles were desorbed and analyzed on GC-MC. Settings for the GC temperature program were as follows: 45°C for 4 min, 45-55°C by increasing temperature at 1.5°C/min, 55-90°C by increasing temperature at 2.5°C/min, 90-220°C by increasing temperature at 12°C/min and hold for 4 min. The temperature of the detector was 280°C. For quantification of the different volatiles, solutions with external standards at different concentrations were prepared and analyzed directly on Tenax tubes. The analysis was performed in triplicate and results given in ng.g emulsion-1.

Tocopherol concentration.

O/W emulsion. Approximately 3 g of the same lipid extract as used for PV analysis, was evaporated under nitrogen, re-dissolved in heptane and analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) according to the AOCS method [15] to determine tocopherol concentration in all emulsion samples. The reported tocopherol concentrations are averages of duplicate lipid extractions and duplicate analysis on the HPLC.

Data analysis.

The obtained results were analyzed by two way analysis of variance (GraphPad Prism, Version 4.03, GraphPad Software, Inc). The Bonferroni multiple comparison was used to test differences between samples or storage time (significance level $p < 0.05$). When a significant difference was observed between two samples, they are denoted with different superscripts in the text, figure or table. To compare the efficacy of the antioxidants in the two different emulsion systems, inhibition percentages ($Inhibition[\%] = \left(1 - \frac{C_{Volatile\ Sample,\ Antioxidant}}{C_{Volatile\ Sample,\ Control}}\right) \times 100$) were calculated.

Results and Discussion

Antioxidant properties of the compounds.

Radical scavenging, iron chelation and reducing power activities were measured for ascorbyl palmitate, ascorbic acid, ascorbyl CLA and CLA.

The radical scavenging activity (Figure 3A), measured by the DPPH assay, indicates whether the compound is able to act as a free radical scavenger. It is very clear that CLA has no scavenging effect at all, whereas ascorbic acid, ascorbyl palmitate and ascorbyl CLA showed significantly higher scavenging effects than BHT (positive control). Their effects were concentration dependent and they had equal scavenging effects at each concentration.

Reducing power indicates the ability of a compound to donate electrons to reactive free radicals. Similar to the radical scavenging activity, CLA did not exert any reducing power (Figure 3B). A concentration dependent effect was observed for ascorbic acid, ascorbyl CLA and ascorbyl palmitate, where higher concentrations resulted in higher reducing power. High reducing power was observed for ascorbic acid, while for ascorbyl palmitate and ascorbyl CLA it was very low. All three antioxidants had significantly different reducing power, and the ranking was as follows: ascorbic acid^{d} > ascorbyl palmitate^{c} > ascorbyl CLA^{b} > CLA^{a}.

EDTA was used as positive control in the metal chelating activity assay, and showed a concentration dependent chelation between 50-100% in the tested concentration area. EDTA was observed to bind 50% ferrous ion at 25 μmol.L-1 and 99.7% was bound at both 102 and 205 μmol.L-1 EDTA concentrations (data not shown). The results showed that CLA had very limited

chelation between 4-7% and for the remaining compounds the chelation was observed to be 0% using this assay (data not shown). Thus, the results indicate that the chelating acitivity was not as strong as ferrozine for these compounds.

Ascorbic acid is known to have multifunctional effects as a radical scavenger of hydrophilic radicals and reducing ability due to its ability to donate an electron to reactive free radicals. However, reducing power can result in prooxidative effects, if Fe^{3+} is reduced to Fe^{2+}, especially in the presence of already existing lipid hydroperoxides [16]. In addition, ascorbic acid is known to act as a synergist with tocopherol by regenerating tocopherol from the tocopheroxyl radicals, formed after reaction between tocopherol and lipophilic radicals [16]. Due to the molecular structure of ascorbyl palmitate and ascorbyl CLA (Figure 2), their antioxidative properties most likely rely on the ascorbyl group in these molecules.

The measured radical scavenging activities for ascorbic acid and its esters were similar to values earlier reported for ascorbic acid by Yen et al. [17]. Thus, the radical scavenging activity seemed independent of the esterification of ascorbic acid. In contrast, the reducing power was reduced by esterification of ascorbic acid (ascorbic acid > ascorbyl palmitate > ascorbyl CLA). These data indicate that the OH-group, which is changed by esterification, plays a role in the reducing power of ascorbic acid. This is in accordance with findings obtained with dihydrocaffeic acid esters by Sabally et al. [18], who suggested that the acid group in dihydrocaffeic acid contributed in the donation of H-atoms. The properties of ascorbic acid and ascorbyl palmitate were in accordance with earlier reports, which also observed radical scavenging activity and reducing properties of ascorbic acid and ascorbyl palmitate [19]. Moreover, the significant difference between ascorbyl

palmitate and ascorbyl CLA might indicate that the degree of saturation and perhaps the chain length of the fatty acid had an impact on the reducing power.

The finding that CLA had no antioxidative properties except for very limited metal chelating activity conflicts with results obtained by Ha et al. [5] and Yu [20], but confirms with results obtained by van den Berg et al. [7]. The antioxidative properties of CLA reported in the study by Ha et al. [5] were based on PV measurements in an ethanol-buffer system containing linoleic acid, but the mechanism for the antioxidative properties of CLA was not explained. Moreover, Yu [20] showed radical scavenging activity of CLA at concentrations between 5-80 mg.L-1 and in accordance with our study, ascorbic acid was shown to be more efficient than CLA [20]. In the study by van den Berg et al. [7] neither radical scavenging nor metal chelating activity of CLA in radical or iron initiated lipid oxidation were observed. It is unclear why CLA had limited chelating activity in the present study, whereas ascorbyl CLA showed no activity. Either, the activity can be ascribed to the acid group or it might be due to solubility problems, since the assay is normally conducted with compounds solubilized in water. However, CLA was not soluble in water and therefore it was solubilized in small amounts of methanol and then diluted with water. The result obtained by van den Berg et al. [7] and the molecular structure of CLA together suggest that the limited metal chelating properties of CLA observed in the present study was most likely due to interaction with methanol used for solubilization of CLA in our experiment.

Physical stability of emulsions.

The average droplet diameter of the aqueous droplets in the oil phase (w/o emulsion) was measured to 240-395 nm (Table 1). The droplet size in all these emulsions increased significantly from day 1 to day 4 or 8, thereafter the emulsion droplets did not change significantly (Table 1). However, the

droplet size for the reference emulsion decreased from day 8 to 16, but this decrease in size cannot be explained by the present data. An exception was the emulsion with ascorbic acid for which the droplet size was stable through out the storage period. Thus, the w/o emulsions seemed to be physically unstable during storage, except for the emulsion with ascorbic acid added. However, no creaming was observed in the emulsions.

In contrast to the w/o emulsions, droplets in the o/w emulsions were stable during the entire storage period (data not shown). Average diameter of the oil droplets in the o/w emulsion was around 110 nm. The emulsion with ascorbic acid had slightly smaller droplets compared to the other emulsions.

Partitioning of compounds.

All three compounds, ascorbic acid, ascorbyl palmitate and ascorbyl CLA, were detected in the aqueous buffer phase in the oil-buffer system. The concentration in the aqueous phase was highest for ascorbic acid followed by ascorbyl CLA and ascorbyl palmitate (Table 2). However, in the other two systems, buffer-emulsifier and emulsion, only ascorbic acid was detected in the aqueous phase and no ascorbyl palmitate or ascorbyl CLA could be detected in the aqueous phase (Table 2). This may partly be explained by the interaction of ascorbyl palmitate and ascorbyl CLA with surfactant.

The smaller amount of ascorbic acid detected in the aqueous phase in the buffer-emulsifier system compared with the oil-buffer system could indicate an interaction with citrem. However, in the emulsion system all ascorbic acid was detected in the aqueous phase, which indicates no interactions between ascorbic acid and citrem in this system. Moreover, at pH 7 both citrem and ascorbic acid is expected to be negatively charged, hence repulsive forces existed between the interface and ascorbic acid [21]. Thus, it might be that ascorbic acid was located away from the

interface due to repulsive forces. In contrast, the emulsifier in the w/o emulsion, PGPR, is a non-ionic emulsifier, which enables ascorbic acid to be close to the interface in this emulsion system.

The partitioning data for the o/w system showed that in the buffer-oil system a little less than half of the amount of added antioxidant was detected in the buffer phase. However, when emulsifier (citrem) was present ascorbyl palmitate and ascorbyl CLA were not detected in the aqueous phase. This might indicate that ascorbyl CLA and ascorbyl palmitate partly interacted with citrem located at the interface or participated in micelles formed with citrem in the aqueous phase, but also that some ascorbyl CLA and ascorbyl palmitate partitioned into the oil phase. The critical micelle concentration (cmc) of citrem (≈ 15 mg.L-1 [22]) is higher than the concentration of citrem (10 mg.L-1) in our experiment, hence it might generally be assumed that citrem is mainly located at the interface.

Lipid oxidation in emulsions.

The primary oxidation products, lipid hydroperoxides, were measured in both emulsion systems. Headspace propanal and hexanal were determined in the w/o emulsions, whereas 1-penten-3-one, 2(t)-pentenal, 1-penten-3-ol, hexanal, 2(t)-hexenal, 4(c)-heptenal, nonanal, 2(t)-octenal and 2,4(t,t)-heptadienal were determined in the o/w emulsions.

For the w/o emulsion a clear lag phase was observed for lipid hydroperoxide concentrations in all samples (Figure 4A). The lag phase was shortest for the reference emulsion (no antioxidant added) followed by CLA and ascorbic acid and the longest lag phase was observed for emulsions with ascorbyl CLA or ascorbyl palmitate added. Similarly, in the middle of the storage period, the reference had the highest concentration of lipid hydroperoxides followed by CLA and ascorbic acid

and lowest concentration was observed in the emulsions with ascorbyl CLA and ascorbyl palmitate. During the entire storage period there was a lower concentration of lipid hydroperoxides in the emulsions with antioxidants compared to the reference emulsion.

Similar to the lipid hydroperoxides, a lag phase for headspace propanal was observed (Figure 4B). The ranking was the same as for the lipid hydroperoxides: reference (shortest lag phase) < CLA = ascorbic acid = ascorbyl CLA = ascorbyl palmitate. All the compounds acted as antioxidants throughout the storage period. However, at the end of the storage period the efficacy of the compounds in retarding lipid oxidation was reduced as shown in Table 3. The concentration of propanal at day 16 was as follows in the different w/o emulsions: ascorbyl palmitate^{a} = ascorbyl CLA^{a} < ascorbic acid^{b} ≤ CLA^{b} < reference^{c} (different superscripts indicate significantly different concentrations). The reduced efficacy of the applied compounds towards the end of the storage period might indicate depletion of them.

For concentration of hexanal, no significant differences between the efficacies of the different compounds were observed in the beginning and middle of the storage period (Table 3). At the end of the storage period the hexanal concentration in the emulsion was as follows: ascorbic acid^{a} = ascorbyl CLA^{a} = ascorbyl palmitate^{a} ≤ CLA^{ab} ≤ reference^{b} (highest concentration), data not shown. A tendency that CLA resulted in a higher concentration of hexanal than the other antioxidants was observed, however the hexanal concentration in the CLA emulsion was not significantly different from the reference.

In contrast to the findings for w/o emulsions, no lag phase was observed for lipid hydroperoxides in the o/w emulsions (Table 4). However, there was a lower concentration of lipid hydroperoxides at

day 4 in emulsions with ascorbyl CLA, ascorbic acid and ascorbyl palmitate added compared to the reference emulsion (no antioxidant added). The pattern in the PV data became more complex. For CLA and ascorbic acid emulsions, PV continued to increase and these two emulsions had the highest PV after 15 days. In contrast, PV in emulsions with ascorbyl palmitate, ascorbyl CLA or without antioxidants decreased towards the end of the storage period. After 15 days, the reference and the ascorbyl CLA emulsions had the lowest PV.

The development of the different volatiles was significantly affected in o/w emulsions by the antioxidant addition as shown by inhibition percentages of the applied antioxidants compared with reference emulsion (Table 3). No lag phase for any of the measured volatiles was observed (Table 4). In the beginning of the storage period ascorbic acid and ascorbyl palmitate reduced the formation of hexanal, 1-penten-3-one, 1-penten-3-ol and 4-heptenal and had no effect on the development of 2-hexenal and 2-octenal (Table 3, Table 4). In contrast, ascorbyl CLA and CLA had no or prooxidative effect on all volatiles. At the end of the storage period all the compounds generally resulted in a significantly increased formation of volatiles compared to the reference, except for 2,4-heptadienal (Table 4). For this compound the concentration was higher in the reference emulsion than in the emulsions with antioxidants throughout the storage period and at day 15 the ranking was as follows: ascorbic acid^{a} = CLA^{a} ≤ ascorbyl palmitate^{ab} ≤ ascorbyl CLA^{bc} ≤ reference^{c}. Interestingly, the data for ascorbyl CLA and ascorbyl palmitate showed that despite their similar molecular structures, they behaved differently. Ascorbyl palmitate was most efficient in reducing the development of volatiles in the beginning of the storage period, but at the end of the storage it was the most prooxidative compound tested seen from the concentration in this emulsion of most of the volatiles measured. The observation that antioxidants changed from being antioxidative at day 4 to being prooxidative at day 15 might indicate depletion of them during

storage. A decrease in PV towards the end of the storage period in the o/w emulsion with ascorbyl palmitate, ascorbyl CLA or without antioxidants could indicate an increased formation of volatiles in these samples or formation of polymers. However, the increased formation of volatiles could only be confirmed for the emulsion with ascorbyl palmitate.

In addition to PV and volatiles, the tocopherol concentration in the different o/w emulsions was measured. Results showed that almost the same amount of α-tocopherol was consumed during storage in all emulsions. The consumption varied from 13 (ascorbyl CLA) to 15 μg.kg-1 (ascorbyl palmitate). In contrast, the amount of consumed γ-tocopherol depended upon the antioxidant added to the emulsion, and the ranking of the concentrations was as follows at the end of the storage period: reference = ascorbyl CLA > ascorbyl palmitate = ascorbic acid = CLA (Figure 5). This ranking could indicate that the reference and the ascorbyl CLA emulsions oxidized less than the other emulsions. This finding was in accordance with PV data and most of the volatiles data at day 15, which also showed that ascorbyl CLA and the reference were less oxidized in the end of the storage.

In summary our results showed that in the w/o emulsion all 4 compounds acted as antioxidants and for lipid hydroperoxide and propanal measurements their activity was best in the middle of the storage period. The best antioxidative activity was observed for ascorbyl palmiate and ascorbyl CLA. In the o/w emulsion all 4 compounds had different effects on the development of the different volatiles. Generally, ascorbic acid and ascorbyl palmitate were most efficient in the beginning of the storage period. However, all the tested compounds acted as prooxidants at the end of the storage period, although ascorbyl CLA was less prooxidative than the other antioxidants.

Lipid oxidation in w/o vs. o/w emulsions

The results clearly showed that lipid oxidation was initiated much faster in the o/w emulsion than the w/o emulsion. To the best of our knowledge, comparison of lipid oxidation in o/w versus w/o emulsion has not previously been reported. Instead, lipid oxidation rates for bulk oil and o/w emulsions have been compared [1, 23, 24]. All these studies indicated, that lipid oxidation was faster in o/w emulsions. In this study, the w/o emulsion contained only 1% water and 1% emulsifier and it thus resembles bulk oil to some extent as bulk oil also contains small amounts of water and surface active compounds such as free fatty acid, monoacylglycerols and diacylglycerols [25].

The different lag phases in the two systems might be due to the differences in droplet size i.e. total surface area, as the initial step in lipid oxidation takes place at the interface [9]. In the present study, the droplet size was smallest and the total surface area thus largest in the o/w emulsions. Some studies on emulsions support this interpretation of the data [26], *w*hereas other studies have found the opposite. In studies with fish oil enriched milk, lipid oxidation decreased with decreasing droplet sizes [27]. These findings were explained by a more favourable protein composition at the interface in emulsions with small droplets. A similar change in the composition of the interface most likely did not take place in the present study due to the much simpler emulsifier system applied. However, the interface between the droplets and continuous phase in the o/w emulsion with citrem, as emulsifier, are negatively charged and thus attract metals. Therefore, the reason for the faster lipid oxidation in o/w emulsion could be due to the larger surface and negatively charged droplets in this emulsion system compared to the w/o emulsion.

In addition, factors such as different emulsifiers, production processes and especially storage conditions might also be a reason for the different oxidative stability observed for the o/w vs. w/o

emulsions. The o/w emulsions were continuously stirred during storage, which means that headspace oxygen was distributed in the emulsion. This is suggested to be an important reason for the faster initiation of oxidation in the o/w emulsion.

Antioxidant effects in w/o vs. o/w emulsions.

The efficacy of the different compounds tested as antioxidants in the two emulsions systems was different. Both systems were multiphase systems and according to the polar paradox hypothesis, amphiphilic antioxidants are more efficient compared to hydrophilic and lipophilic antioxidants in protecting against lipid oxidation in emulsions [1, 2]. In this study, the polar paradox hypothesis was partly confirmed, since ascorbyl palmitate was a better antioxidant than ascorbic acid in the beginning of the storage period in the o/w emulsions. However, ascorbyl CLA was practically inactive, and at the end of the storage period all antioxidants seemed to promote formation of most of the measured volatile oxidation products. Moreover, ascorbyl palmitate became more prooxidative than ascorbic acid towards the end of the storage period. Earlier studies have also shown antioxidant effects that differed from the polar paradox [28], and it was concluded that the polar paradox was too simple to explain antioxidant effects in multiphase systems as emulsions, most likely because of interactions between iron, emulsifiers and antioxidants [28].

The ascorbyl CLA was not a completely purified product after esterification and contained free fatty acids, which can have a prooxidative effect in o/w emulsions together with iron [29]. However, this might be more important in the w/o emulsions, where the droplets were not negatively charged. Moreover, iron was not added to the emulsions in this study, however it is expected to be present in the oil in trace amounts. Thus, the antioxidative activity of ascorbyl CLA may be neutralized due to its content of free fatty acids and this could explain its poor effect in the o/w emulsion. Furthermore,

different chain lengths of ascorbic acid esters have been shown to influence their efficacy as antioxidant in copper and metal initiated LDL oxidation as follows: ascorbyl laurate > ascorbyl palmitate > ascorbyl caprylate [8]. Thus, the chain length and saturation may influence their efficacy as antioxidant.

In the w/o emulsions ascorbyl palmitate and ascorbyl CLA were more efficient than ascorbic acid and CLA, which is in accordance with the polar paradox for emulsions. As described, the w/o emulsion resembled bulk oil to some extent although the structure of the two systems is different. In bulk oil, water is located in micelles, whereas in w/o emulsions the aqueous phase is surrounded by emulsifier. Our findings may therefore indicate that the type of interface has an influence. In addition, CLA acted as an antioxidant in the w/o emulsion, whereas in the o/w emulsion CLA was inactive or had prooxidative effect on the development of the different volatiles.

Previously we hypothesized that hydrophilic antioxidants might offer better protection in a w/o emulsion compared to the reverse o/w emulsions if the concentration of the disperse phase was equal. In our experiment the amount of disperse phase was less in the w/o emulsions (1 %) than in the o/w emulsions (5 %). Accordingly, it was observed that ascorbic acid inhibited oxidation in the w/o emulsion more than in the o/w emulsion. We suggest that ascorbic acid is closer to the interface in a w/o emulsion due to its higher concentration in the aqueous phase compared to an o/w emulsion and therefore exhibits higher antioxidative efficacy. In addition, the concentration of the disperse phase in our w/o emulsion was smaller than the disperse phase in our o/w emulsion with even higher concentration of ascorbic acid in the aqueous phase of the w/o emulsions; 10,000 μmol.L-1 vs 106 μmol.L-1 in the aqueous phase in w/o and o/w emulsions, respectively. However, interactions with other compounds such as emulsifier might also be considered [29].

Partitioning of the antioxidants related to their efficacy

The negative charge of citrem and neutral charge of PGPR, might also be a reason for the higher efficiency of ascorbic acid in the w/o emulsions. However, partitioning studies with PGPR and ascorbic acid are required to conclude further.

The structure of ascorbyl palmitate and ascorbyl CLA only differs in the fatty acyl group by the chain length and degree of saturation (Figure 2). Hence, it might be speculated that the structural differences result in different interactions between citrem and ascorbyl CLA than between citrem and ascorbyl palmitate, which reduces the possibility of ascorbyl CLA to reduce lipid oxidation. After all, the data from the oil/buffer system indicated a higher concentration of ascorbyl palmitate in the oil phase compared to ascorbyl CLA. This may partly explain why it was more efficient than ascorbyl CLA in the beginning of the storage period.

Although ascorbyl CLA was located closer to the lipids than ascorbic acid and therefore supposed to be more efficient than ascorbic acid, it was ascorbic acid that was more efficient as antioxidant compared to ascorbyl CLA in o/w emulsion in the beginning of the storage. However, both ascorbyl CLA and ascorbyl palmitate had less reducing and radical scavenging activity compared to ascorbic acid. Ascorbic acid was suggested to be repelled from the interface by citrem, however the repulsive forces may not be that strong.

For further specification of mechanism that results in different antioxidative action of ascorbyl CLA and ascorbyl palmitate and better protection with ascorbic acid than ascorbyl CLA in o/w emulsions, studies on interaction of these compounds with citrem are required.

Conclusion

Initiation of lipid oxidation was influenced by the emulsion system. Oxidation occured faster in the o/w emulsions compared to w/o emulsions and this was suggested to be due to smaller droplets and continuous stirring during storage of the o/w emulsions. As hypothesized ascorbic acid was more efficient in preventing the initial lipid oxidation in the w/o emulsions compared to o/w emulsions. This is explained by the higher concentration of ascorbic acid in the aqueous phase in w/o emulsions and hence a closer location to the interface. However, the polar paradox hypothesis was only partly confirmed. In the w/o emulsion system the amphiphilic antioxidants, ascorbyl palmitate and ascorbyl CLA, were more efficient than the hydrophilic antioxidant, ascorbic acid.

The efficacy of the antioxidants in the o/w emulsions was more complex, since ascorbyl palmitate and ascorbic acid were most efficient in the beginning of the storage and towards the end they acted as prooxidants. In addition, ascorbyl CLA was practically inactive. Since ascorbyl CLA was located at the interface or in the oil phase in o/w emulsions and thus was supposed to work efficiently as an antioxidant, the inactivity of this compound as antioxidant may be due to impurities such as free fatty acids or interaction with citrem that prevent it from acting as antioxidant.

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Figure legends

Figure 1 Structure of the two emulsifiers applied in two different emulsions system: w/o and o/w. A) Polyglycerol polyricinoleate (PGPR), R = H or a fatty acyl group, n = degree of polymerization of glycerol (average > 1). B) Citrem (citric acid ester of mono- and diglyceride), citric acid ester of monoglyceride (-OH) and diglyceride (fatty acid).

Figure 2 The structure of the compounds evaluated as antioxidants: A) Ascorbic acid, B) Ascorbyl palmitate, C) Ascorbyl CLA and D) CLA.

Figure 3 Antioxidant activity of ascorbic acid, ascorbyl palmitate, ascorbyl CLA and CLA. A) Radical scavenging activity (* BHT included as a positive control) and B) Reducing power. Legends: ● Ascorbic acid, ■ Ascorbyl palmitate, ▲ Ascorbyl CLA and ▼ CLA, the error bars indicate the standard deviation of 3 measurements. Different superscripts after sample code indicate significant differences in the concentration range of 25-200 μmol.L-1.

Figure 4 Concentration of lipid hydroperoxides [μmol.g oil-1] (A) and propanal [ng.g-1] in w/o emulsions during storage [hours]. Legends: * Reference, ● Ascorbic acid, ■ Ascorbyl palmitate, ▲ Ascorbyl CLA and ▼ CLA, the error bars indicate the standard deviation of 3 measurements.

Figure 5 Concentration of A) α- and B) γ-tocoherol [μg.g-1] in o/w emulsions measured at day 0 and 15. The error bars indicate the standard deviation of 4 measurements for emulsion with antioxidants and 8 measurements for the reference emulsion. Different subscriptions at the same day indicate a significant difference in the concentrations between these emulsions.

Table 1 Droplet size of aqueous droplets [nm] measured after storage. Different superscripts in the same row indicate that droplet size changed significantly during storage for that specific emulsion. Standard deviation is based on 3 measurements (n = 3).

Droplet size, Diameter [nm]	**Day 1**	**Day 4**	**Day 8**	**Day 16**
Reference	265 ± 7^{a}	341 ± 8^{bc}	355 ± 23^{c}	297 ± 24^{ab}
Ascorbic acid	254 ± 1^{a}	291 ± 19^{a}	304 ± 13^{a}	281 ± 21^{a}
Ascorbyl palmitate	293 ± 12^{a}	327 ± 5^{ab}	395 ± 54^{c}	349 ± 54^{bc}
Ascorbyl CLA	260 ± 5^{a}	317 ± 11^{b}	304 ± 18^{ab}	300 ± 17^{ab}
CLA	241 ± 1^{a}	303 ± 23^{b}	345 ± 47^{b}	329 ± 10^{b}

Table 2 Concentration [%] of antioxidant measured in the aqueous phase (aq).

Antioxidants	Oil / Buffer [% in aq]	Citrem / Buffer [% in aq]	Emulsion (o/w) [% in aq]
Ascorbic acid	87 ± 10	70 ± 7	96 ± 1
Ascorbyl palmitate	48 ± 3	-	-
Ascorbyl CLA	57 ± 2	-	-

- indicates that the compound was not detected in the aqueous phase in the particular system.

Table 3 Inhibition percentages of the development of volatiles in w/o and o/w emulsions during storage depended on the compound added. A straight line (-) indicates that the amount was below the detection limit for both the emulsion with antioxidant and without antioxidant (reference), whereas 100 indicates that the concentration of volatiles in emulsion with antioxidants was below detection limit but not the reference emulsion. Negative values indicate prooxidative effect of the respective antioxidant that particular day.

Volatiles	**Storage [Days]**	**AA [%]**	**AP [%]**	**ACLA [%]**	**CLA [%]**
W/O Emulsion					
Propanal	5	60	46	75	53
	12	32	44	45	5
	15	12	24	20	10
Hexanal	5	86	85	76	66
	12	16	24	48	-57
	15	41	34	35	26
O/W Emulsion					
Hexanal	4	25	100	-46	-2
	8	4	26	-9	25
	16	-172	-212	-57	-222
1-penten-3-one	4	64	90	23	-28
	8	7	24	-10	-3
	16	-209	-239	30	-188
2-pentenal	4	21	100	21	-14
	8	30	53	17	20
	16	-144	-248	-48	-220
1-penten-3-ol	4	62	70	37	38
	8	30	-36	-7	23
	16	-69	-118	-5	-83
2-hexenal	4	-	-	-	-

	8	-	-	-	-
	16	-62	-131	-46	-77
4-heptenal	4	100	100	100	-80
	8	47	32	26	21
	16	-25	-67	-8	-42
Nonanal	4	-3	-91	-73	-6
	8	16	-52	-20	34
	16	-66	-168	-52	-70
2-octenal	4	-100	100	-400	100
	8	100	13	-50	25
	16	-85	-162	-100	-154
2,4-heptadienal	4	68	89	12	15
	8	50	41	9	23
	16	30	15	6	30

1 Abbreviations: AA: ascorbic acid, AP: ascorbyl palmitate, ACLA: ascorbyl CLA and CLA.

2

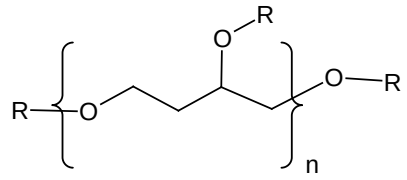
3

Table 4 Concentration (ng.g-1 emulsion) of primary (PV) and two secondary (1-penten-3-one and 2,4-heptadienal) oxidation products in o/w emulsions measured at different time points [Days]. Different superscripts in a column within the same oxidation product indicate significant different concentrations in the two emulsions.

	Storage time [Days]				
	0	**4**	**9**	**12**	**15**
PV [meq peroxides / kg oil]					
Reference	0.2 ± 0.05^{a}	27.8 ± 1.60^{c}	19.8 ± 2.74^{a}	23.0 ± 10.5^{b}	8.6 ± 1.24^{a}
Ascorbic acid	0.1 ± 0.03^{a}	16.5 ± 0.09^{ab}	33.2 ± 0.98^{c}	22.0 ± 0.56^{b}	36.5 ± 0.20^{c}
Ascorbyl palmitate	0.0 ± 0.04^{a}	10.0 ± 0.05^{a}	28.4 ± 0.41^{bc}	11.0 ± 0.09^{a}	22.6 ± 0.19^{b}
Ascorbyl CLA	0.0 ± 0.00^{a}	23.8 ± 0.25^{bc}	22.2 ± 1.20^{ab}	6.1 ± 0.06^{a}	6.3 ± 0.10^{a}
CLA	0.0 ± 0.30^{a}	27.0 ± 0.20^{c}	28.1 ± 1.27^{bc}	34.5 ± 0.23^{c}	37.1 ± 0.31^{c}
1-penten-3-one [ng / g emulsion]					
Reference	0	64.0 ± 27.7^{cd}	90.6 ± 22.3^{ab}	93.9 ± 24.6^{cd}	32.9 ± 6.3^{a}
Ascorbic acid	0	23.0 ± 1.1^{ab}	85.4 ± 12.0^{ab}	74.4 ± 3.0^{bc}	102 ± 12.0^{b}
Ascorbyl palmitate	0	6.1 ± 0.5^{a}	69.0 ± 5.8^{a}	60.4 ± 7.7^{ab}	112 ± 8.7^{b}
Ascorbyl CLA	0	48.5 ± 6.7^{bc}	99.3 ± 7.8^{b}	40.8 ± 8.3^{a}	23.7 ± 5.2^{a}
CLA	0	82.1 ± 4.4^{d}	93.8 ± 7.0^{ab}	107 ± 5.9^{d}	95.4 ± 2.4^{b}
2,4-heptadienal [ng / g emulsion]					
Reference	0	1646 ± 5^{c}	4469 ± 549^{d}	4588 ± 1592^{b}	5019 ± 282^{b}
Ascorbic acid	0	524 ± 54^{ab}	2256 ± 297^{a}	4305 ± 130^{ab}	3517 ± 127^{a}
Ascorbyl palmitate	0	190 ± 27^{a}	2653 ± 310^{ab}	5296 ± 292^{bc}	4255 ± 156^{ab}
Ascorbyl CLA	0	1454 ± 29^{bc}	4057 ± 401^{cd}	5790 ± 86^{c}	4713 ± 88^{b}
CLA	0	1406 ± 437^{bc}	3449 ± 261^{bc}	3558 ± 165^{a}	3521 ± 256^{a}

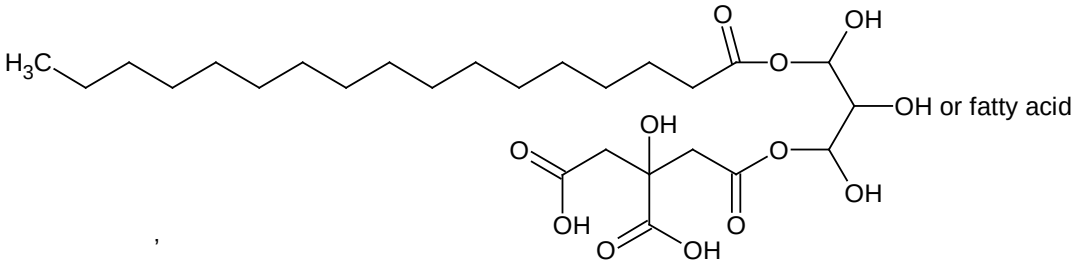
1

A



Polyglycerol polyricinoleate (PGPR)

B



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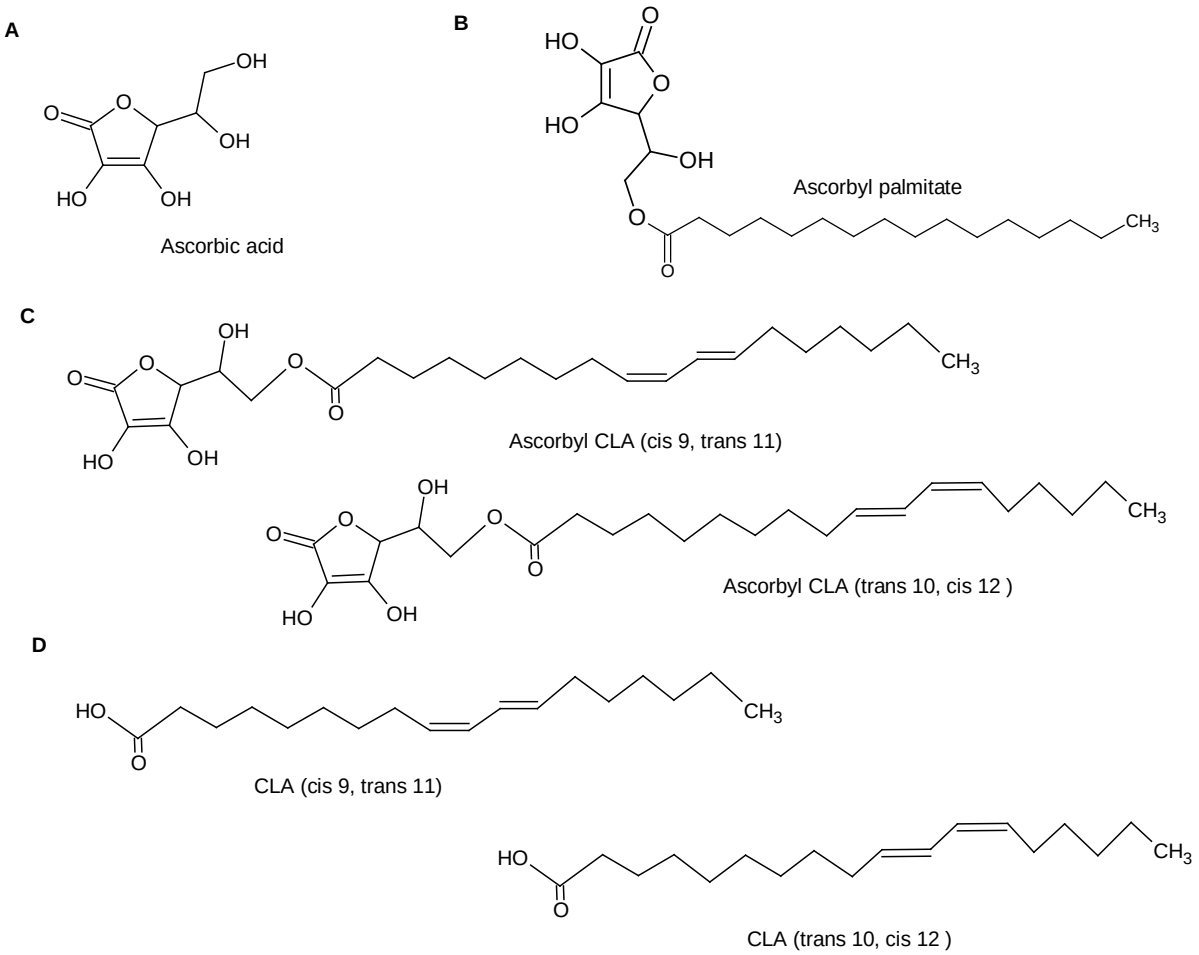
Citrem (citric acid ester of mono-and diglyceride)

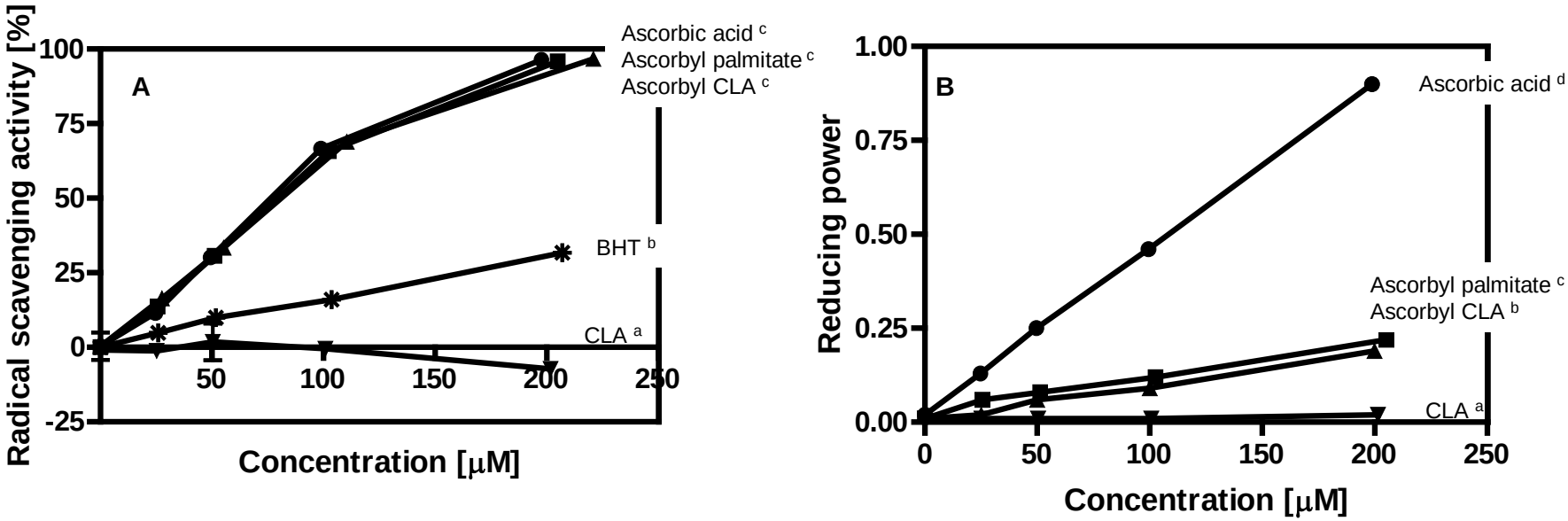
2

3 Figure 1


A
Ascorbic acid
B
Ascorbyl palmitate
C
Ascorbyl CLA (cis 9, trans 11)
Ascorbyl CLA (trans 10, cis 12)
D
CLA (cis 9, trans 11)
CLA (trans 10, cis 12)

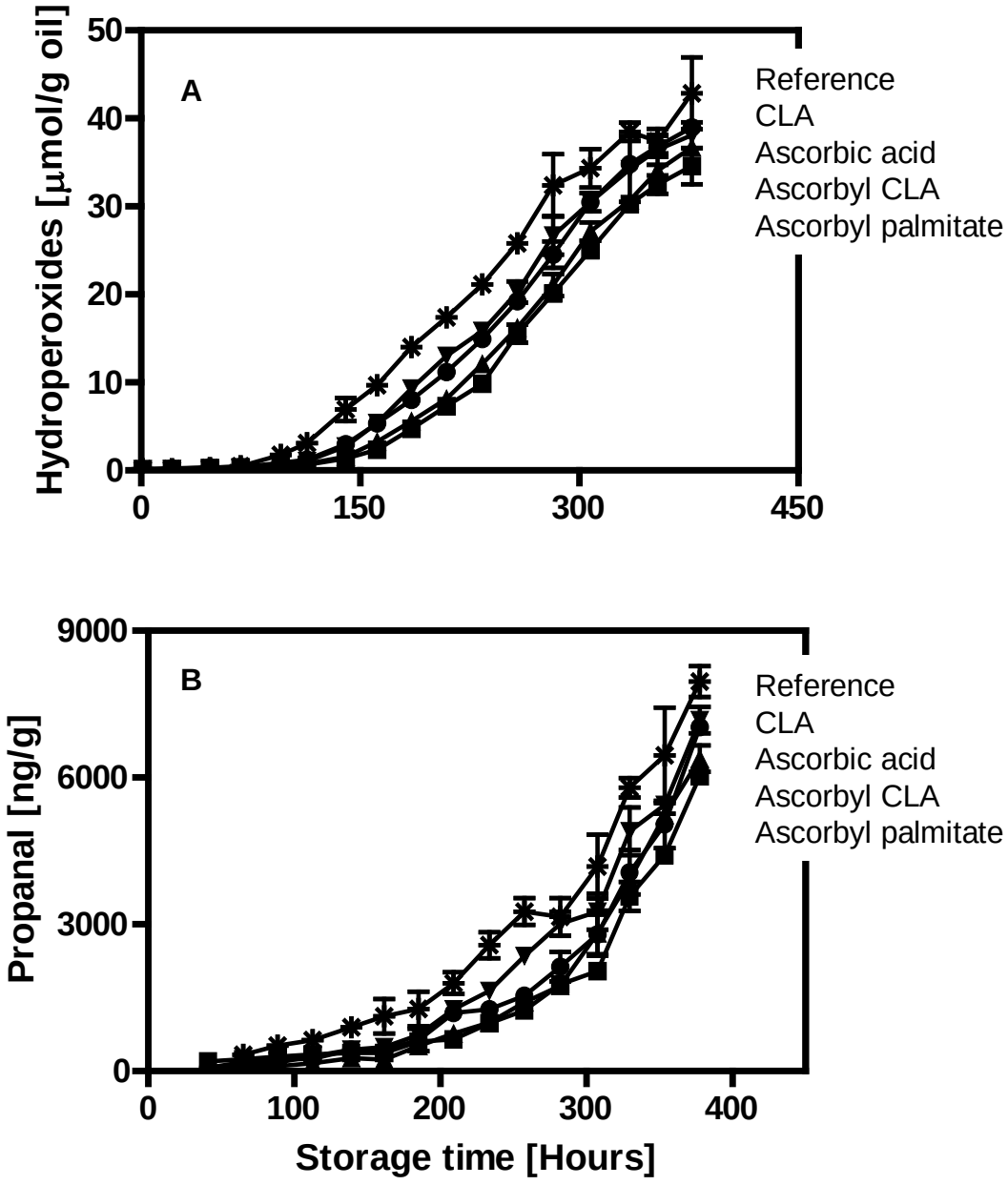
1

2 Figure 2


A
Radical scavenging activity [%]
100
75
50
25
0
-25
50
100
150
200
250
Concentration [μM]
Ascorbic acid c
Ascorbyl palmitate c
Ascorbyl CLA c
BHT b
CLA a
B
Reducing power
1.00
0.75
0.50
0.25
0.00
0
50
100
150
200
250
Concentration [μM]
Ascorbic acid d
Ascorbyl palmitate c
Ascorbyl CLA b
CLA a

1

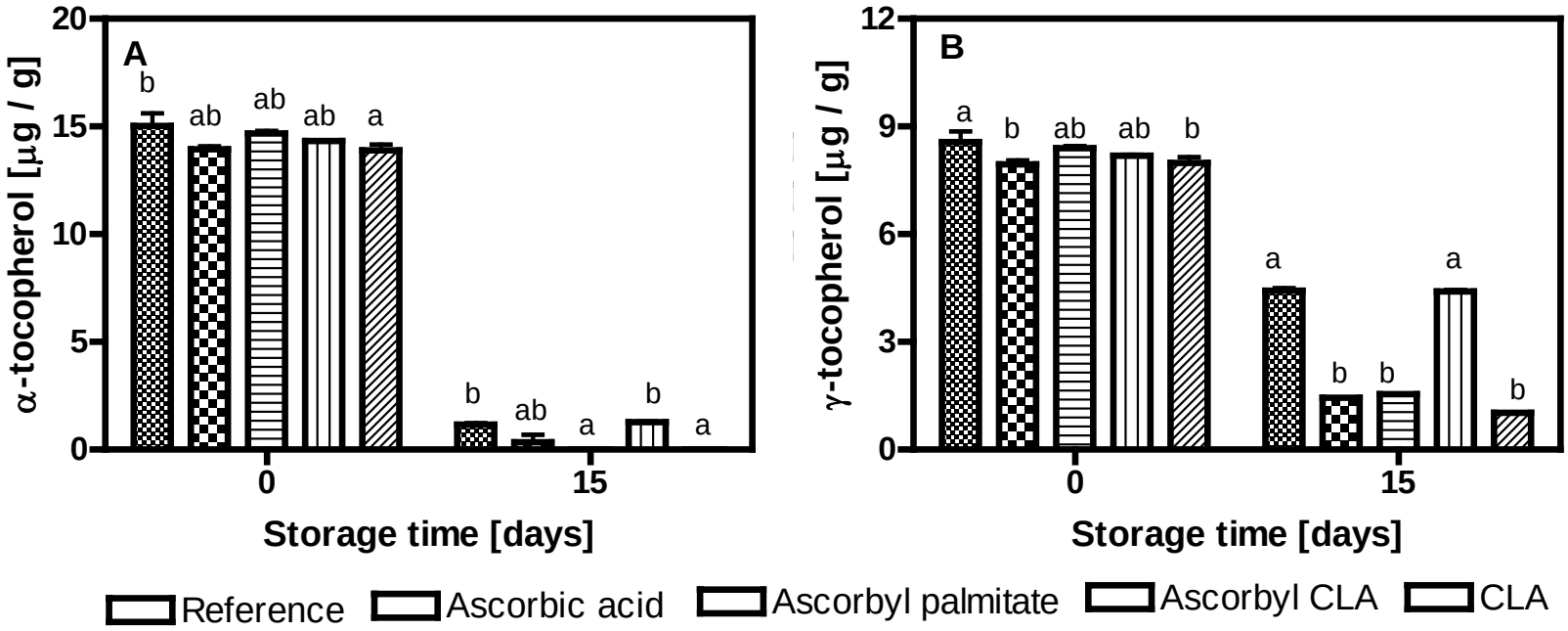
2 Figure 3


A
Hydroperoxides [µmol/g oil]
50
40
30
20
10
0
0
150
300
450
Reference
CLA
Ascorbic acid
Ascorbyl CLA
Ascorbyl palmitate
B
Propanal [ng/g]
9000
6000
3000
0
0
100
200
300
400
Storage time [Hours]
Reference
CLA
Ascorbic acid
Ascorbyl CLA
Ascorbyl palmitate

1

2 Figure 4

3


A
B
α-tocopherol [µg / g]
γ-tocopherol [µg / g]
Storage time [days]
Storage time [days]
Reference
Ascorbic acid
Ascorbyl palmitate
Ascorbyl CLA
CLA

1

2 Figure 5

3

PAPER IV

Sørensen, A.-D.M., Nielsen, N.S., Yang, Z., Xu, X. & Jacobsen, C.

The effect of lipophilization of dihydrocaffeic acid on its antioxidative properties in fish oil enriched emulsion.

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Lipophilization of dihydrocaffeic acid affects its antioxidative properties in fish-oil-enriched emulsions

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Keywords: o/w emulsion, lipid oxidation, partitioning, caffeic acid, *in vitro* antioxidant assays

Running title: Antioxidant efficacy of lipophilized phenolics

Abbreviations: BHT: Butylated hydroxytoluene, CAF: Caffeic acid, CMC: Critical micelle concentration, CON: Control, DCA: Dihydrocaffeic acid, DC:C8: Octyl dihydrocaffeate, DC:C18: Oleyl dihydrocaffeate, DPPH: 2,2-diphenyl-1-picrylhydrazyl, LC: Long chain, OLAL: Oleyl alcohol, PC: Principal Component, PCA: Principal Component Analysis, PV: Peroxide Value, RI: Refractive Index

Abstract

The aim of the present study was to evaluate the antioxidative effect of lipophilized dihydrocaffeic acid, i.e. octyl dihydrocaffeate and oleyl dihydrocaffeate. Furthermore, the relationship between the measured efficacy of the antioxidants in emulsions and their partitioning into different phases of an emulsion system and their *in vitro* antioxidant properties was also evaluated. Lipid oxidation in the emulsions was affected by the antioxidants applied. Thus, despite a reduced antioxidant activity of lipophilized dihydrocaffeic acid in the antioxidant assays, lipophilized dihydrocaffeic acid was more efficient than caffeic and dihydrocaffeic acids. Octyl dihydrocaffeate had a significantly higher antioxidative effect than oleyl dihydrocaffeate in emulsions. The results partly supported the polar paradox hypothesis, since lipophilized compounds resulted in increased oxidative stability. However, the decreased antioxidative efficacy with increasing alkyl chain length esterified to dihydrocaffeic acid supported a newly suggested "cut-off effect" hypothesis. This hypothesis suggests that when a certain level of hydrophobicity is obtained for lipophilized phenolic acids, the ester forms micelles in the aqueous phase rather than being located at the interface or oil phase. This phenomenon is suggested to explain the reduced antioxidant activity of oleyl dihydrocaffeate compared with octyl dihydrocaffeate.

Practical application: The finding that lipophilization of phenolic compounds increase their efficacy opens up new possibilities for producing new and more efficient antioxidants for food systems. However, the results also show that optimisation of the chain length for each type of phenolic compound may be necessary.

1 Introduction

During the last decade substantial efforts have been put into enriching foods with the healthy n-3 long chain (LC) polyunsaturated fatty acids (PUFAs) from fish oil as reviewed by Jacobsen et al. *(1)*. These efforts are carried out in order to increase the populations' intake of especially EPA and DHA. However, the shelf-life of such fish oil enriched products is in general limited due to the high susceptibility of these healthy PUFAs to oxidation. Thus, protection e.g. by antioxidants is necessary to obtain a fish oil enriched product with a satisfactory shelf-life.

Phenolic compounds have several different antioxidative properties such as radical scavenging, iron chelating and reducing activities depending on their structure i.e. number and location of hydroxyl groups primarily in the phenolic ring structure *(2)*. Caffeic acid is a phenolic acid belonging to the group of hydrocinnamic acids, and dihydrocaffeic acid is a degradation product of caffeic acid. The molecular structures of these phenolics are illustrated in Figure 1. Both these phenolic acids contain a catechol moiety in the phenolic structure. This catechol moiety is to a large extent responsible for the antioxidative properties of phenolic compounds e.g. chelation and reducing activity *(3, 4)*. Caffeic and dihydrocaffeic acids are reported to be located primarily in the aqueous phase of either an octanol-water (1:1) or oil-water (1:9) system *(5-7)*. Due to the so-called polar paradox phenomenon, hydrophilic compounds may in many cases be better antioxidants in bulk oil than lipophilic compounds, whereas lipophilic compounds are better at increasing the oxidative stability in emulsions compared to hydrophilic compounds. This phenomenon has been explained by the affinity of the compounds towards the different phases in bulk oil and emulsions *(8, 9)*.

Many food systems are emulsions. Thus, the hydrophilic character of phenolics may reduce their efficacy in inhibiting lipid oxidation in the food emulsions. However, lipophilization of phenolics with a fatty alcohol may improve the antioxidant efficacy of these compounds in emulsions by altering their location in the emulsion matrix. To the best of our knowledge only limited work has been carried out regarding the antioxidative effect of lipophilized phenolics in model emulsions or real food products. Mostly, the focus has been on the production of these new antioxidants rather than evaluation of their effects. Lipophilized dihydrocaffeic acid has only been evaluated in the *in vitro* DPPH assay *(10, 11)* or by the Rancimat method *(12)*. Although, these data may give an indication of their efficacy, the results may not be comparable with their effect in emulsions or real food products.

Laguerre et al. *(13)* have evaluated, the effect of the alkyl chain length attached to lipophilized chlorogenic acid on the resulting antioxidative activity connected with partitioning of these synthesized antioxidants. They observed that antioxidant capacity increased as the added alkyl chain was increased from 1 carbon atom to 12 carbon atoms, whereas further increase of the alkyl chain length resulted in a drastic decrease in the antioxidant capacity of lipophilized chlorogenic acid. On the basis of these results, Laguerre et al. *(13)* suggested a so-called cut-off effect related to the alkyl chain length esterified to chlorogenic acid. Thus, when the antioxidant hydrophobicity increases to above a certain level - in the case of chlorogenic acid alkyl chain length > 12 carbon atoms *(13)* - the lipophilized chlorogenic acid was suggested to form micelles in the aqueous phase. This hypothesis is supported by the fact that the critical micelle concentration (CMC) decreases with increased alkyl chain length *(14)*.

Although, Laguerre et al. *(13, 15)* has only reported a cut-off effect for lipophilized chlorogenic acid with longer alkyl chain than 12 carbon atom and rosmarinic acid with longer alkyl chain than 8 carbon atom, similar cut-off effects may exist for other phenolic compounds e.g. dihydrocaffeic acid. Therefore, the aim of this study was to evaluate the antioxidative effect of dihydrocaffeic acid lipophilized with either octyl or oleyl alcohol in an o/w emulsion system (5 % fish oil). A second aim was to relate the antioxidative efficacy of the lipophilized compounds with their partitioning into the different phases in an emulsion system and with their *in vitro* antioxidant properties evaluated by their ability to 1) donate H-atoms, 2) donate electrons and 3) chelate metal ions. Besides the two lipophilized dihydrocaffeic acids and their parent compound, dihydrocaffeic acid, caffeic acid was included in this study. The purpose was to evaluate similarities or dissimilarities between the effect of caffeic and dihydrocaffeic acids, and to relate these findings to earlier work on caffeic acid in different systems. We also intended to include lipophilized caffeic acid in the present study. However, the enzyme based process developed for synthesising lipophilized phenolicsdid not end up with significant conversion when caffeic acid was used and it was only possible to obtain a satisfactory yield when dihydrocaffeic acid was used. New approach is now under development which will be communicated in future publications.

2 Materials and methods

2.1 Materials.

Fish oil was supplied by Maritex Norway (subsidiary of TINE BA, Norway). This oil had an initial PV of 0.1 meq peroxides / kg oil, tocopherol content of 243 mg α-tocopherol, 115 mg γ-tocopherol and 48 mg δ-tocopherol / kg oil, and the fatty acid composition was as follows: 14:0, 3.0%; 16:0, 8.8%; 16:1, 8.2%; 18:0, 1.9%; 18:1, 20.7%; 18:2, 2.0%; 18:4, 2.6%; 20:1, 11.6%; 20:5 (EPA), 9.4%; 22:1, 6.0%; 22:5, 1.1% and 22:6 (DHA), 11.7%. The total percentages of n-3 and n-6 in the oil were 27.0% and 2.5%, respectively.

Citrem LR 10 Extra (citric acid ester of mono- and diglyceride) without antioxidants was supplied by Danisco A/S (Grindsted, Denmark). Caffeic acid (purity ≥ 98%), dihydrocaffeic acid (purity ≥ 98%) and oleyl alcohol (purity 85% contain around 15% of cetyl alcohol, myristal alcohol and arachidyl alcohol mixture) were from Sigma Aldrich (Steinheim, Germany). Lipophilized dihydrocaffeic acid with octyl alcohol (C8) with a purity of 80% (contain 1-2% of dihydrocaffeic acid and 18% of unremoved octyl alcohol) or lipophilized dihydrocaffeic acid with oleyl alcohol (C18:1) with a purity of 60% (contain 1-2% of dihydrocaffeic acid and 38% of oleyl alcohol, cetyl alcohol, myristal alcohol and arachidyl alcohol mixtute) were synthesized at the Department of Molecular Biology, Faculty of Science, Aarhus University. The synthesis was conducted according to a modified method developed by Sabally et al. *(16)*. Dihydrocaffeic acid (5 mmol) was mixed with octyl or oleyl alcohol (25 mmol) in 100 ml of a mixture of organic solvent of hexane/2-butanone (75/25) in a batch reactor. The reactor was kept at 60˚C in a water bath and the mixture was stirred at 300 rpm. The reaction was initiated by adding 2% Novozym 435 (*Candida antarctica* lipase B immobilized on resin, Novozymes, Bagsvaerd, Denmark) and molecular sizes (3 Å). At the end of reaction (approximate 5 days), enzyme and molecular sieves

were removed through vacuum filtration to terminate the reaction. The solvent phase was washed three times by equal volume of salt water (0.5M NaCl) to remove unreacted dihydrocaffeic acid. The solvent phase was collected and evaporated to concentrate the products. Chemicals were from Merck (Darmstadt, Germany) and external standards for identification and quantification of secondary volatile oxidation products were all from sigma Aldrich (Steinheim, Germany). All solvents were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland).

2.2 Emulsion (o/w) production for storage experiments.

The oil-in-water emulsions consisted of 94% 10 mM acetate-imidazole buffer (pH 7), 5% fish oil, 1% citrem as emulsifier and 100 μM antioxidant. These emulsions were prepared in two steps: pre-emulsification and homogenization. During pre-emulsification, the buffer was stirred with an Ultra-Turrax (Janke & Kunkel IKA-Labortechnik, Staufen, Germany) for a few seconds, and thereafter the oil-citrem solution was added during 1 min followed by 2 min of mixing. The pre-emulsion was then homogenized at a pressure of 80 and 800 bar with 4 circulations of the emulsion at room temperature using a table homogenizer from GEA Niro Soavi Spa (Parma, Italy). The emulsions (100 g) were stored in 100 mL blue cap bottles at 25°C ± 2°C. Samples, one flask pr. code, were taken at day 0, 2, 4, 6, 9, 12 and 15 and divided into brown glass bottles and stored at -40°C until analyses of peroxides, volatiles, fatty acid compositions and tocopherols were performed. The droplet size was measured at day 1, 9 and 15 without pre-freezing.

2.3 Experimental design.

Four different antioxidants were evaluated: caffeic acid, dihydrocaffeic acid, octyl dihydrocaffeate and oleyl dihydrocaffeate. Antioxidant concentration tested in the o/w emulsion was 100 μM. As previously mentioned the synthesized oleyl dihydrocaffeate was only 60 % pure,

and contained ≤ 40% free oleyl alcohol. Therefore, an emulsion with oleyl alcohol with the same amount of oleyl alcohol (29 mg/kg) as in the emulsion with oleyl dihydrocaffeate was included to evaluate the effect of oleyl alcohol on lipid oxidation in the o/w emulsion. For the *in vitro* antioxidant assay 4 concentrations of the antioxidants were evaluated: 25, 50, 100 and 200 μM.

2.4 Droplet size.

The size of the lipid droplets in the o/w emulsion was determined by laser diffraction using a Mastersizer2000 (Malvern Instruments Ltd., Worcestershire, UK). The o/w emulsion was diluted directly in recirculating water (3000 rpm) reaching an obscuration of 12-14%. The RI (refractive index) of sunflower oil at 1.469 and water at 1.330 were used as particle and dispersant, respectively. Duplicate measurements were performed. Results are given as surface area mean diameter $D_{(3,2)}$ *(17)*.

2.5 Fatty acid composition (FAME).

Lipids were extracted from the emulsion according to a modified Bligh and Dyer method *(18)* using a reduced amount of methanol:chloroform, 1:1 v/v *(19)*. Lipid extract was evaporated under nitrogen. Firstly, the glycerol bound fatty acids were trans esterified with methanolic NaOH (0.5 M). Then, hydrolytic released and free fatty acids were methylated by a boron trifluoride reagent (20%) catalyzed process. Methyl esters were extracted with heptane followed by separation on GC (HP 5890A, Hewlett Packard, CA, USA). The procedure was according to the AOCS methods *(20, 21)*. One determination was made on each of the two extracts prepared from the same o/w emulsion sample.

2.6 Measuring lipid oxidation.

Lipid hydroperoxides. Peroxide Value (PV) were measured in lipid extracts, the same as used for FAME analysis, or oil and determined based on colorimetric measurement of a ferric-thiocyanate complex (Shimadzu UV-160A spectrophotometer, Struers Chem A/S, DK) according to Shanta and Decker *(22)*. The PV was calculated by using a ferrichloride standard curve and a conversion factor on 2 to transform the unit to meq peroxides / kg oil. Duplicate extractions were performed.

Secondary volatile oxidation products. Volatiles were collected on Tenax GR packed tubes by dynamic headspace (45°C, 150 mL N_2 / min, 30 mins). The trapped volatiles were desorbed by use of an ATD-400 automatic thermal desorber and the transfer line of the ATD was connected to a GC-MS (GC: 5890 IIA, Hewlett-Packard, CA, USA and MS: HP 5972 mass selective detector) equipped with a DB wax column (length 30 m x ID 0.25 mm x 0.5 μm film thickness, J&W Scientific, CA, USA). The temperature programme used was as follows: 45°C for 5 mins, 45-55°C increased by 1.5°C/min, 55-90°C increased by 2.5°C/min. 90-220°C increased by 12.0°C/min and hold at 220°C for 4 mins. For quantification of the different volatiles, solutions with external standards at different concentrations were prepared and analyzed directly on Tenax tubes. The analysis was performed in triplicate and results given in ng / g emulsion.

Tocopherol concentration. Lipid extract, the same as used for FAME analysis, was evaporated under nitrogen, re-dissolved in heptane and analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) according to the AOCS method *(23)* to determine tocopherol concentration in the different emulsion samples. The reported tocopherol concentrations are averages of duplicate lipid extractions and duplicate analysis on the HPLC.

2.7 Antioxidant activity assays.

Iron chelating activity. Antioxidant solutions with different concentrations of antioxidants (0-200 μM) were made up to a volume of 3.7 mL with distilled water. Due to solubilisation problems with octyl dihydrocaffeate and oleyl dihydrocaffeate, these compounds were dissolved in small amounts of acetone nitrile and then diluted with water. Ferrous chloride (2 mM, 0.1 mL) was added and after 3 min the reaction was inhibited by the addition of a ferrozine solution (5 mM, 0.2 mL). The mixture was shaken vigorously and left at room temperature for 10 min, where after the absorbance was measured at 562 nm (UV mini 1240 Shimadzu, Kyoto, Japan). The chelating activity (%) of the antioxidants was calculated as follows: Fe^{2+} chelating activity = $\left(\frac{A_0 - A_s}{A_0}\right) \times 100$, where A_0 is absorbance of control (only iron and ferrozine) and A_s is absorbance of sample minus absorbance of sample blank. Triplicate measurements were performed and EDTA was included as a positive control.

Radical scavenging effect. Sample (different concentrations (0-200 μM) of antioxidants in methanol) was mixed with a 0.1 mM methanolic solution of DPPH (1:4, v/v). Absorbance was measured after 30 min (ambient temperature, darkness) at 517 nm (UV mini 1240 Shimadzu, Kyoto, Japan). The results was reported as percent inhibition: $Inhibition\,[\%] = \left(1 - \frac{A_s}{A_0}\right) \times 100$, where A_s is absorbance of DPPH after reaction with antioxidant minus absorbance of antioxidant in methanol and A_0 is absorbance of DPPH in methanolic solution. Triplicate measurements were performed and BHT was included as a positive control.

Reducing power. An aliquot of sample (1 mL) with different concentrations of antioxidants (0-200 μM) was mixed with 0.2 M phosphate buffer (pH 6.6, 2.5 mL) and 30 mM potassium ferricyanide (2.5 mL). The mixture was incubated for 20 min at 50°C. A 0.6 M TCA solution (2.5 mL) was added and thereafter the mixture was centrifuged (10 min, 2000 rpm). The upper layer of the solution (2.0 mL) was mixed with an equal amount of distilled water and 0.4 mL 6 mM

$FeCl_3$, and the absorbance (700 nm) was measured after 10 min. High absorbance equals high reducing power. Triplicate measurements were performed and ascorbic acid was included as a positive control.

2.8 Partitioning of antioxidants.

Partitioning of the different phenolics and dihydrocaffeates in buffer/oil, emulsifier/buffer and in o/w emulsion was measured according to the method described by Schwarz et al. *(24)* with modifications as described in the following. All antioxidants were dissolved in methanol before addition to the respective systems. However, the methanol was evaporated by nitrogen before the remaining reagents were added. The procedures are briefly described below. For further details refer to Sørensen et al. *(25).*

Water (buffer) / Oil. 40 g of a mixture containing antioxidant (100 µM), 10 mM acetate-imidazol buffer (95%) and fish oil (5%) was mixed. Separation of the two phases was done the next day to allow for equilibration of antioxidants between the phases. The aqueous phase was separated from the oil by centrifugation (10 min, 210 *g*). The collected aqueous phase was centrifuged once more before analysis of antioxidant content. This experiment was performed in triplicate for each antioxidant.

Emulsifier / Water (buffer). 40 g of a mixture containing antioxidant (100 µM), 10 mM acetate-imidazol buffer (99%) and citrem (1%) was mixed. Separation of the two phases was done the next day to allow time for equilibration of antioxidants between the phases. The buffer was separated from citrem by centrifugation (3 mL, 10 min, 210 *g*) in Amicon® Ultra centrifugal filter devices (regenerated cellulose 3,000 MW cut off, Millipore, Carrigtwohill, Ireland). The buffer in the bottom of the tube was collected and the rest of the buffer-citrem solution in top was discarded every time, and the aqueous phase in the bottom was discarded the first 3 times to

saturate the filter with antioxidant. This procedure was repeated 5 times, and the last 2 fractions were collected for measuring the concentration of antioxidant in the buffer phase. This experiment was performed in triplicate for each antioxidant.

Emulsions (o/w). Antioxidant (100 μM), 10 mM acetate-imidazol buffer (94%), fish oil (5%) and citrem (1%) to a total amount of 200 g were mixed for 3 min with an Ultra-Turrax (Janke & Kunkel IKA-Labortechnik, Staufen, Germany). Separation of the two phases was done the next day to allow time for equilibration of antioxidants between the phases. The procedure described for the emulsifier / buffer system was also used to separate the buffer from the emulsion. Two emulsions were prepared for each antioxidant and duplicate separations of the aqueous phase from each emulsion were carried out.

2.9 Concentration of antioxidants.

Caffeic acid. This compound was analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) with a C18 Thermo Hypersil ODS (250, 4.6 mm) column and using a gradient elution at a flow rate of 0.7 mL/min. Solvent A was water : acetic acid (94 : 6, v/v) and solvent B was water : acetic acid : acetonitrile, 65 : 5 : 30 (v/v). Gradient condition: 0-5 min 30-100% B, 5-8 min 100% B and 8-10 min 100-30% B. Injection volume of aqueous phase containing caffeic acid was 10 μL. The content of caffeic acid was measured spectrophotometrically at 324 nm, and quantified by using a prepared calibration curve of this compound dissolved in methanol.

Dihydrocaffeic acid, octyl dihydrocaffeate and oleyl dihydrocaffeate. These compounds were analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) with a C18 Thermo Hypersil ODS (250, 4.6 mm) column and a flow rate of 1 mL/min using a methanol : water solvent (95 : 5, v/v) and an injection volume of 10 μL. Dihydrocaffeic acid, octyl dihydrocaffeate and oleyl dihydrocaffeate were measured spectrophotometrically at 205 and 280 nm,

respectively, and were quantified by using a prepared calibration curve of the compounds dissolved in methanol.

2.10 Data analysis.

Statistics. The obtained results were analyzed by two way analysis of variance (GraphPad Prism, Version 4.03, GraphPad Software, Inc). Bonferroni multiple comparison posttest was used to test differences between samples or storage time (significance level $p < 0.05$).

Multivariate data analysis – Principal Component Analysis (PCA). Results from PV, volatiles and tocopherol analysis were subjected to PCA using Unscrambler version 9.0 (Camo, Oslo, Norway). The PCA model was built on the average of the measured data and full cross validation was used to validate the model. A PCA allows detection of similarities and dissimilarities between the different samples in a score plot, whereas correlations between the measured variables are visualized in a loadings plot. Connecting these plots in this case shows the degree of oxidation between the different samples.

3 Results and Discussion

3.1 *In vitro* antioxidant assays. Radical scavenging, iron chelating and reducing power activities were measured by different *in vitro* antioxidant assays for caffeic acid, dihydrocaffeic acid and the two esters hereof, octyl dihydrocaffeate and oleyl dihydrocaffeate, and the obtained results are shown in Figure 2. It is clear that for all three assays the antioxidant activity increased with increasing antioxidant concentration.

At all concentrations (25 – 200 μM), caffeic acid had the highest radical scavenging activity and the ranking of the radical scavenging activity at 100 and 200 μM antioxidant was as follows: caffeic acid[a] > dihydrocaffeic acid[b] > octyl dihydrocaffeate[c] > oleyl dihydrocaffeate[d] > BHT[e] (positive control). Hence, lipophilization of dihydrocaffeic acid with octyl or oleyl alcohol had a negative impact on the radical scavenging activity of the antioxidant at concentrations of 50 – 200 μM. These findings are in accordance with earlier results obtained by Sabally et al. *(26)*. At all concentrations, caffeic acid had a significantly higher radical scavenging activity than dihydrocaffeic acid, although at 200 μM the difference was smaller than at concentrations between 25 – 100 μM. This conflicts with the literature, where either a higher radical scavenging activity for dihydrocaffeic acid *(6)* or similar activities of these two phenolics were reported *(27)*. Furthermore, Moon and Terao *(27)* concluded that in the concentration range from 3 – 350 μM, the double bond in the alkyl chain attached to the phenolic ring had no influence on the radical scavenging activity.

Figure 2B compares antioxidants based on their ability to chelate iron at four different concentrations. It is very clear that the positive control, EDTA, had a much stronger chelating

activity than the evaluated phenolics and phenolic esters. Moreover, among the phenolic compounds only dihydrocaffeic acid had a significant metal-binding activity. It has been reported that two neighbouring hydroxyl groups (catechol moiety) are required in the molecular structure for the compound to be able to bind metal ions *(3)*. As illustrated in Figure 1, the evaluated compounds all contain a phenolic ring with a catechol moiety. The assay for evaluating iron chelating activity of the antioxidants is based on the ability of the antioxidants to compete with the indicator ferrozine to form a complex with ferrous (Fe^{2+}) ions in solution. Using different methods earlier experiments with caffeic acid showed that caffeic acid formed a complex with ferrous ions *(3, 28)*. Taken together these findings might indicate that ferrozine had stronger capacity for binding iron than caffeic acid. Thus, it cannot be ruled out that caffeic acid may also have metal-binding capacity in a real food system, despite the finding that this compound did not show any activity in the metal chelation assay. Lipophilization of dihydrocaffeic acid resulted in markedly reduced chelation activity, which is in accordance with results obtained by Lue et al. *(29)* with lipophilized rutin. In spite of the fact that the catechol moiety has been suggested to be the site of metal chelation *(3, 4)*, the findings indicate that the alkyl chain may influence the ability of the compound to chelate metal.

The ability of the antioxidants to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion through donation of an electron is illustrated in Figure 2C. Antioxidants, which are capable of donating electrons to reactive free radicals, are promoting the termination of free radical chain reactions in lipid oxidation. However, the ability of the antioxidants to reduce ferric ion to the more active form of iron, ferrous ion, might also be indicative of their ability to act as prooxidants. Overall, the level of reducing power of the evaluated antioxidants was less different at low concentrations (25 μM) than at higher antioxidant concentrations. Moreover, at 25 and 50 μM, there was no significant

difference between the reducing power of caffeic and dihydrocaffeic acids. However, at higher concentrations, 100 and 200 μM, dihydrocaffeic acid was capable of donating electrons at a significantly higher level compared to caffeic acid. The ability of the investigated antioxidants to reduce iron at concentrations of 100 and 200 μM was as follows: dihydrocaffeic acid[a] > caffeic acid[b] > octyl dihydrocaffeate[c] > oleyl dihydrocaffeate[d]. However, ascorbic acid, applied as a positive control in this assay, had the highest reducing power. As observed for the other *in vitro* assays, lipophilization of dihydrocaffeic acid had a negative impact on its activity, which indicates that the alkyl chain has an impact on the compound's ability to donate electrons. The findings are in accordance with those of Lue et al. *(29)* with lipophilized rutin.

In all three antioxidant assays, the activity was higher for octyl dihydrocaffeate than oleyl dihydrocaffeate. Lue et al. *(29, 30)* has reported the same tendency for the reducing power assay for rutin esterified with two acyl chains of different length; 12-carbon and 16-carbon. They suggested that the formation of micellar structures in the aqueous environment of the assay could explain the different effects of the two rutin esters. Thus, rutin palmitate was anticipated to form micelles at lower concentrations due to the longer acyl chain compared to rutin laurate, resulting in less free ester and consequently lower reducing power *(29, 30)*. Furthermore, Yuji et al. *(14)* has reported that CMC decreases with increasing alkyl chain length. Thus, the reduced antioxidant activity observed in this study for oleyl dihydrocaffeate with a long alkyl chain compared to octyl dihydrocaffeate with a short alkyl chain may be due to different degrees of micelle formation.

3.2 Partitioning. The partitioning study is a "pseudo-partitioning" determination since the concentration of antioxidants was only determined in the aqueous phase. Of the four different

antioxidants evaluated, only caffeic and dihydrocaffeic acids were detected in the aqueous phase, which was separated by centrifugation from the three different systems: oil/buffer, emulsifier/buffer and emulsions (Figure 3). Phenolic acids are known as hydrophilic compounds. Hence, it is not surprising that caffeic acid was detected in the aqueous phase in all 3 systems. Slightly less caffeic acid was detected in the system containing emulsifier/buffer compared with the two other systems. This finding may indicate weak interactions between citrem and caffeic acid in spite of both compounds being negatively charged at pH 7. However, 100% caffeic acid was recovered in the aqueous phase in the emulsion system, indicating that caffeic acid was located in the aqueous phase in the emulsions without interaction with citrem (Figure 3). This may be explained by different orientation of the emulsifier in the buffer/emulsifier mixture compared with the emulsion. Surprisingly, dihydrocaffeic acid was primarily located in the oil or emulsifier phase. In the oil/buffer and emulsifier/buffer, 14 and 13 % dihydrocaffeic acid was found in the aqueous phase, respectively, whereas twice as much (27 %) dihydrocaffeic acid was located in the aqueous phase in the emulsion system. Results from earlier studies on the partitioning of caffeic and dihydrocaffeic acids partly conflicts with ours, since these studies showed that both phenolics mainly partitioned into the aqueous phase of either an octanol-water system (1:1) or an oil-water (1:9) system *(5-7)*. However, Pekkarinen et al. *(7)* observed that the emulsifier Tween 20 increased the solubilisation of caffeic acid in the lipid phase. This phenomenon was not observed in the present partitioning study. On the contrary, the concentration of dihydrocaffeic acid in the aqueous phase was increased in the emulsion system. The partitioning results for caffeic acid were thus in accordance with other results, whereas this was not the case for dihydrocaffeic acid. Dihydrocaffeic acid and caffeic acid only differs in their molecular structure by one double bond (Figure 1). Thus, it is surprising if such a small difference can result in significantly different partitioning properties of the two compounds.

When quantifying dihydrocaffeic acid by HPLC, another peak occurred very close to the wavelength we used for determining dihydrocaffeic acid concentrations. Thus, these results either indicate that small changes in the molecular structure changed the partitioning drastically, or that the obtained concentration for dihydrocaffeic acid was misleading due to interactions in the quantification methods of this compound.

As expected, lipophilization of dihydrocaffeic acid with either octyl or oleyl alcohol changed their location. As shown in Figure 3, neither of these lipophilized dihydrocaffeic acids was detected in the aqueous phase irrespective of the system analyzed. This is probably a result of interactions between octyl dihydrocaffeate or oleyl dihydrocaffeate and citrem or / and location of these lipophilized phenolics in the oil phase.

3.3 Storage experiments with emulsions.

Droplet size and content of EPA and DHA. Oil droplets in the different emulsions were measured to 110 nm ± 4 nm and no changes were observed during the entire storage period. The content of EPA and DHA at day 0 were measured to be between 7.4 – 8.1% and 10.0 – 10.3% and at day 15 between 7.4 – 7.9% and 9.3 – 9.8%, respectively. Thus, a tendency to a slightly decreased DHA content after 15 days of storage was observed. The DHA content was lowest in the emulsion with no antioxidant added indicating a protective effect of all the antioxidants.

Lipid oxidation (PV, Volatiles and Tocopherols). A PCA was made to analyze the effect of the different antioxidants on PV, volatiles and tocopherol results obtained in this experiment. Figure 4A shows the score plot, which indicate similarities between the antioxidants applied. Figure 4B shows the loading plot, which indicate correlations between the measured variables. By

connecting these two plots, the effect of the applied antioxidants on the measured PV, volatiles and tocopherol can be interpreted.

The score plot (Figure 4A) shows that the first principal component (PC 1) explains the variation between emulsions with lipophilized phenolics and the parent phenolic and PC 2 explains the variation between emulsions with and without antioxidant applied. Moreover, the locations of the control emulsion in the top of the plot and the emulsion with octyl dihydrocaffeate in the bottom of the plot indicate that these emulsions are completely opposite each other regarding lipid oxidation (PV, volatiles and tocopherols). According to their location oleyl alcohol and oleyl dihydrocaffeate are in between the control and octyl dihydrocaffeate regarding their antioxidant efficacy in o/w emulsions. Moreover, octyl dihydrocaffeate is located slightly more to the right than oleyl dihydrocaffeate, thus being more different from the phenolic acids (Figure 4A).

By comparing Figure 4A and Figure 4B, it is observed that PV for day 0, 2, 4 and 6 are located in the same area as the emulsion with no antioxidant (CON: control) and PV for day 9, 12 and 15 in an area in between the control and the emulsion with oleyl alcohol (OLAL) added. These observations indicate that the concentration of lipid hydroperoxides was highest in the emulsion with no antioxidant added in the beginning of the storage period (day 0-6). In contrast, the lowest concentration of lipid hydroperoxides was in the emulsion with caffeic acid and octyl dihydrocaffeate, as interpreted from their location opposite to the control emulsion in the scores plot (Figure 4A). Later in the storage period (day 9-15), the highest PV was found either in the control emulsion or in the emulsion with oleyl alcohol (OLAL) added, whereas the lowest concentration of lipid hydroperoxides still was observed for the emulsions with dihydrocaffeic acid and caffeic acid.

Table 1 shows the concentration of lipid hydroperoxides measured during storage. The raw data supported the observations from the multivariate analysis that the highest PV level was measured in the emulsion without antioxidant or with oleyl alcohol added and that the lowest levels was found in the emulsions with dihydrocaffeic acid or caffeic acid. The antioxidative effect of caffeic acid on PV was also observed in an earlier study in citrem stabilized emulsions with or without iron *(28)*. Furthermore, the results shown in Table 1 indicate that lipid oxidation has occurred during the processing of the emulsions due to the high PV level measured in the emulsion without antioxidant. Interestingly, the PV measured in the different emulsions shows that all the antioxidants have protected the emulsion from lipid oxidation under the production, which is observed by the lower PV level in emulsions with antioxidants than in the emulsion without antioxidant (CON).

The lower levels of PV in the beginning and end of the study, in some emulsions, indicate either inhibition of the formation of lipid hydroperoxides by the antioxidant applied or fast decomposition of lipid hydroperoxides to secondary volatile oxidation products.

Figure 4B shows that the different volatiles primarily are located in the 2^{nd} and 3^{rd} quadrant of the loadings plot. The saturated aldehydes (Δ) had generally negative PC 2 coordinates. These findings coupled to the scores plot (Figure 4A), showed that the concentration of saturated aldehydes was higher in the emulsion with caffeic acid followed by the emulsion with dihydrocaffeic acid. The other volatiles measured (2-pentenal (●), 1-penten-3-ol (■), 4-heptenal (►), 1-octen-3-ol (◄) and 2,4-heptadienal (▼)) were located with negative PC 1 values and from slightly negative to positive PC 2 values, which also indicated highest concentrations in the

emulsions with caffeic and dihydrocaffeic acids followed by the emulsion without antioxidant added. However, the volatile compound 4-heptenal was detected in the highest concentration in the control emulsion at the end of storage (day 12-15), which also is observed in Figure 5A. As evaluated from their location primarily in the opposite direction of all the evaluated volatile compounds (Figure 4), the lowest concentrations of all volatiles were observed in the emulsion with the lipophilized dihydrocaffeic acid. Moreover, the location of octyl dihydrocaffeate further to the right than oleyl dihydrocaffeate suggested that the concentration of volatiles were lower in the octyl dihydrocaffeate emulsion than in the oleyl dihydrocaffeate emulsion. The raw data obtained from volatile analysis of the different emulsions supported the conclusions drawn from the multivariate analysis. In Figure 5, the concentrations of two of the measured volatiles in the different emulsions are shown: 4-heptenal and 1-penten-3-ol, respectively. For some of the volatiles a lag phase was observed before the concentration started increasing in the different emulsions. This is clearly illustrated in Figure 5 for the development of 4-heptenal. The lag phase was longest in the emulsion with octyl dihydrocaffeate added. For the development of 1-penten-3-ol in the emulsions, the concentration was also increasing with different speed, but with no clear lag phase. Similar to the concentration of 4-heptenal, the lowest concentration of 1-penten-3-ol was observed in the emulsion with octyl dihydrocaffeate added. Contrary to the findings for 4-heptanal for which the control had the highest concentration after approx. 5 days, the emulsion with caffeic acid had the highest concentration of 1-penten-3-ol during most of the storage period.

At almost all the measured time points, the different tocopherol homologues are located in the 4^{th} quadrant in the correlation loadings plot (Figure 4B), indicating that the highest concentrations of tocopherols were determined in emulsion with octyl dihydrocaffeate followed by emulsions with

oleyl alcohol and oleyl dihydrocaffeate (Figure 4A). Again, the observation from the multivariate analysis was confirmed by the raw data and statistical analysis. The decrease of tocopherol in the different emulsions during storage is shown for α-tocopherol in Figure 6. The observed increase in the concentration of α-tocopherol in the dihydrocaffeic acid and octyl dihydrocaffeate emulsions from day 2 to 4 is difficult to explain, since it is not possible to form more tocopherol than what is present at day 0. At day 12 and 15, the ranking of α-tocopherol concentrations was as follows in the different emulsions: octyl dihydrocaffeate^{a} > oleyl alcohol^{ab} > dihydrocaffeic acid^{bc} > oleyl dihydrocaffeate^{bc} > control^{bc} > caffeic acid^{c} (as average on the two days; different superscripts indicating significant differences between the concentration in these emulsions).

Caffeic acid had the highest antioxidant activity measured by the DPPH and reducing power in the *in vitro* assays followed by dihydrocaffeic acid. These two compounds also had the lowest concentration of lipid hydroperoxides, which could indicate decreased formation of lipid hydroperoxides in emulsions with either caffeic acid or dihydrocaffeic acid. However, in this case it is more likely due to increased decomposition of lipid hydroperoxides, since the highest concentrations of different volatiles were generally observed in emulsions with these two compounds in spite of highest antioxidant activity measured in the different *in vitro* assays. Additionally, only these two compounds were detected in the aqueous phase in the emulsion system.

In contrast, the two lipophilized dihydrocaffeate had lower *in vitro* antioxidant activity and emulsions with these lipophilized compounds resulted in higher concentration of PV in the end of storage compared to the more hydrophilic compounds, but a decreased concentration of volatiles.

This indicates that decomposition of lipid hydroperoxides was slower than the formation in emulsions with lipophilized dihydrocaffeic acid.

Thus, the results partly support the polar paradox hypothesis, since the emulsion with lipophilized dihydrocaffeic acid had an increased oxidative stability compared to the emulsion with the more hydrophilic parent compound, dihydrocaffeic acid. Of the two lipophilized compounds, octyl dihydrocaffeate worked as a better antioxidant than oleyl dihydrocaffeate and neither compound were detected in the aqueous phase in either of the systems used for measuring partitioning. Additionally, oleyl alcohol was observed to be inactive or had an antioxidative effect; hence the poorer effect of oleyl dihydrocaffeate compared with octyl dihydrocaffeate was not due to impurities of oleyl alcohol.

Influence of phenolic lipophilization and the alkyl chain length. To the best of our knowledge no earlier experiments have been performed on the evaluation of the antioxidative effect of lipophilized dihydrocaffeic acid in emulsion systems. However, lately the effect of chlorogenic acids lipophilized with alkyl chain length from 1 to 20 carbon has been evaluated in emulsion *(13)*. The highest oxidative stability was obtained with a 12 carbon atoms alkyl chain length for lipophilized cholorogenic acid. A longer alkyl chain esterified to chlorogenic acid decreased the oxidative stability of the emulsions, and this was suggested to be due to micellization in the aqueous phase. This was explained by the fact that the CMC is reduced when the compounds are more lipophilic. Thereby, these compounds may not be available at the interface.

Experiments with rutin and lipophilized rutin, rutin laurate and rutin palmitate, as antioxidants in o/w emulsion reported by Lue et al. *(30)* does not support the cut-off effect, since the esters were

consistently less effective compared with rutin. However, these studies were only carried out with alkyl chain lengths of 12 or 16 carbon atoms. The shortest alkyl chain length in lipophilized rutin was lauryl, which was the optimal alkyl chain length for lipophilized chlorogenic acid in order to obtain optimal antioxidative effect in emulsions. Rutin is a more hydrophobic compound than chlorogenic and dihydrocaffeic acids due to the sugar moiety in its molecular structure, and it may therefore be suggested that the cut-off effect for an ester produced from rutin occurs before an alkyl chain length of 12 carbon atoms. Thus, it is possible that the rutin lipophilized with laurate also participated in micellization in the aqueous phase and that this could explain why lipophilization of rutin did not increase its efficacy.

In the present study, an alkyl chain length of 8 carbon atoms esterified to dihydrocaffeic acid resulted in better antioxidative protection than a chain length of 18 carbon atoms. Hence, although, the present study only evaluated two alkyl chain lengths, it supported the new cut-off effect hypothesis suggested by Laguerre et al. *(13)*. However, the obtained partitioning results indicated no presence of lipophilized dihydrocaffeic acid in the aqueous phase independent of alkyl chain lengths, and this seems to contradict the suggested cut-off effect due to assumptions of micelle formation for the lipophilized phenolic with longer chain length, which in this case is oleyl dihydrocaffeate. Nevertheless, as the lipid oxidation results support the cut off hypothesis, it may be suggested that possible formed oleyl dihydrocaffeate micelles was retained in the filter of the centrifuge tubes and that this could explain why no oleyl dihydrocaffeate was detected in the aqueous phase in the partitioning experiment.

4 Conclusion

Lipophilization of dihydrocaffeic acid reduced its antioxidant activity as measured by the *in vitro* assays suggesting that the attached alkyl chain had an impact on its activity. Moreover, antioxidant activity decreased with increasing alkyl chain length on dihydrocaffeate, which might be due to micelle formation of oleyl dihydrocaffeate in the aqueous phase.

Lipid oxidation in o/w emulsions was significantly affected by the antioxidants applied. Lipophilized dihydrocaffeic acid worked better than caffeic and dihydrocaffeic acids that had prooxidative effects on some of the oxidation parameters evaluated. Octyl dihydrocaffeate had a significantly better antioxidative effect than oleyl dihydrocaffeate in emulsions. Caffeic and dihydrocaffeic acids, but not the lipophilized compounds were detected in the aqueous phase of the emulsion system. Thus, the results partly supported the polar paradox hypothesis, since lipophilized compounds resulted in an increased oxidative stability. However, the decreased antioxidative efficacy with increasing alkyl chain length esterified to dihydrocaffeic acid was not entirely in agreement with the polar paradox oxidation hypothesis, but supported a newly suggested “cut-off effect” hypothesis. Thus, when a certain level of hydrophobicity is obtained for lipophilized dihydrocaffeic acid, the ester may form micelles in the aqueous phase rather than being located at the interface or oil phase. This explains the reduced antioxidant activity of oleyl dihydrocaffeate compared with octyl dihydrocaffeate. The effect of lipophilization was only evaluated in a simple model system rather than in a real food system. It may be interesting to evaluate if the same antioxidative effect of lipophilized dihydrocaffeic acid could be observed in real food systems.

5 Acknowledgements

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Figure legends

Figure 1 Molecular structure of the applied antioxidants: caffeic acid, dihydrocaffeic acid, octyl dihydrocaffeate and oleyl dihydrocaffeate.

Figure 2 Antioxidant activity measured in different *in vitro* assays: A) Radical scavenging activity, B) Fe^{2+} chelating activity and C) Reducing power. In all three assays a positive control was included: BHT, EDTA and ascorbic acid, respectively. The error bars indicate standard deviation of the measurements (n = 3).

Figure 3 Concentration of the different antioxidants measured in the buffer separated from 3 systems: oil (5%)-buffer (95%), emulsifier (1%)-buffer (99%) and emulsion (5% oil, 1% emulsifier and 94% buffer). The error bars indicate standard deviation of the measurements (n = 3 for the oil-buffer and emulsifier-buffer systems and n = 4 for the emulsion system).

Figure 4 Scores (A) and Correlation loadings (B) plots obtained by principal component analysis (PC 1 vs. PC 2) on PV, volatile and tocopherol results. The analysis was done with full cross validation and 77% of the variance was explained by the two first PCs, 3 PCs were validated. A) Scores, showing the samples; CON: Control (no antioxidant added); DCA: Dihydrocaffeic acid; DC:C8: Octyl dihydrocaffeate; DC:C18: Oleyl dihydrocaffeate; OLAL: Oleyl alcohol and CAF: Caffeic acid. B) Correlation loadings, showing the measured variables; PV: Lipid hydroperoxides; α-, β-, γ- and δ-: The 4 different tocopherol homologues; Δ: Saturated aldehydes (pentanal, hexanal and nonanal); ●: 2-pentenal; ■: 1-penten-3-ol; ►: 4-heptenal; ◄: 1-octen-3-ol; ▼: 2,4-heptadienal. Numbers indicate storage time [days].

Figure 5 Concentration of 4-heptenal (A) and 1-penten-3-ol (B) [ng/g emulsion] in the different emulsions during storage. The error bars indicate SD of the measurements (n = 3). Symbols: X Control; Δ Dihydrocaffeic acid; ■ Octyl dihydrocaffeate; ● Oleyl dihydrocaffeate; * Oleyl alcohol and □ Caffeic acid.

Figure 6 Concentration of α-tocopherol in the different emulsions during storage. The error bars indicate SD of the measurements (n = 4).

Table 1 PV [meq. Peroxide / kg oil] measured in the different emulsions during storage. Average ± standard deviation and different superscription indicate significant (95% - significant level) differences between samples at that specific time point (differences within a column).

Sample	Day 0	Day 2	Day 4	Day 6	Day 9	Day 12	Day 15
Control	4.0 ± 0.04^{a}	21.1 ± 0.53^{b}	43.2 ± 10.1^{c}	30.4 ± 0.17^{b}	34.0 ± 1.19^{ab}	42.3 ± 0.28^{c}	50.1 ± 8.80^{c}
Dihydrocaffeic acid	1.1 ± 0.01^{a}	11.3 ± 0.25^{a}	27.7 ± 9.18^{ab}	24.3 ± 3.23^{ab}	27.0 ± 1.54^{a}	29.5 ± 0.01^{a}	34.3 ± 0.17^{a}
Octyl dihydrocaffeate	1.2 ± 0.25^{a}	8.3 ± 1.66^{a}	21.4 ± 5.15^{a}	20.3 ± 2.59^{a}	29.7 ± 1.30^{ab}	35.2 ± 2.11^{abc}	47.0 ± 0.43^{bc}
Oleyl dihydrocaffeate	1.3 ± 0.02^{a}	11.3 ± 0.11^{a}	22.4 ± 0.30^{a}	24.7 ± 1.53^{ab}	32.2 ± 0.45^{ab}	38.6 ± 0.05^{bc}	44.3 ± 8.41^{bc}
Oleyl alcohol	1.7 ± 0.04^{a}	17.3 ± 0.41^{b}	31.4 ± 1.89^{b}	26.4 ± 2.94^{ab}	37.2 ± 1.49^{b}	42.3 ± 0.03^{c}	47.4 ± 0.79^{c}
Caffeic acid	0.9 ± 0.04^{a}	13.4 ± 0.48^{ab}	20.0 ± 0.40^{a}	20.0 ± 0.68^{a}	28.4 ± 0.41^{ab}	31.7 ± 0.14^{ab}	38.3 ± 0.35^{ab}

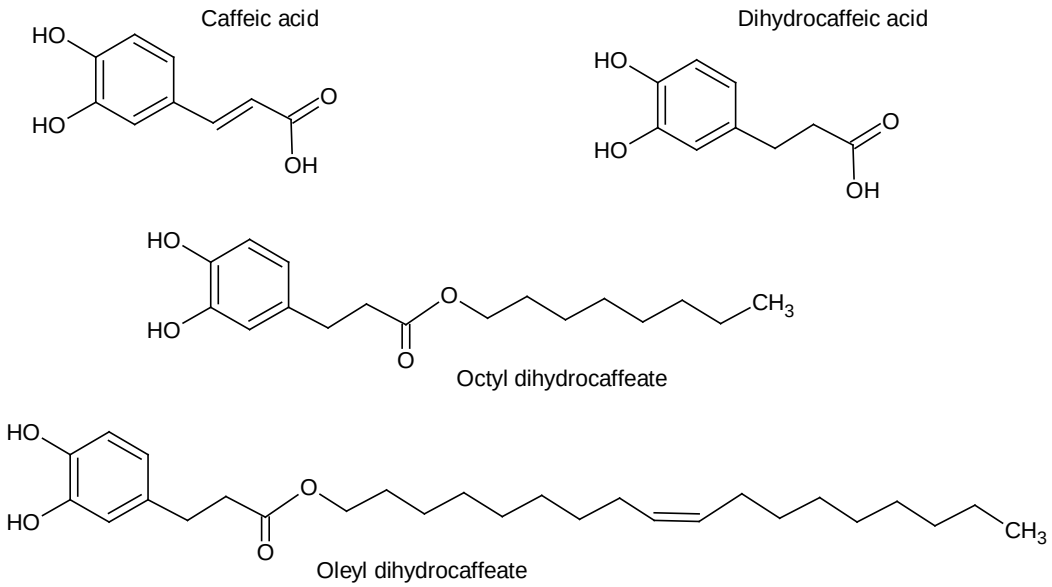
Caffeic acid
HO
HO
O
OH
Dihydrocaffeic acid
HO
HO
O
OH
HO
HO
O
O
CH3
Octyl dihydrocaffeate
HO
HO
O
O
CH3
Oleyl dihydrocaffeate

Figure 1

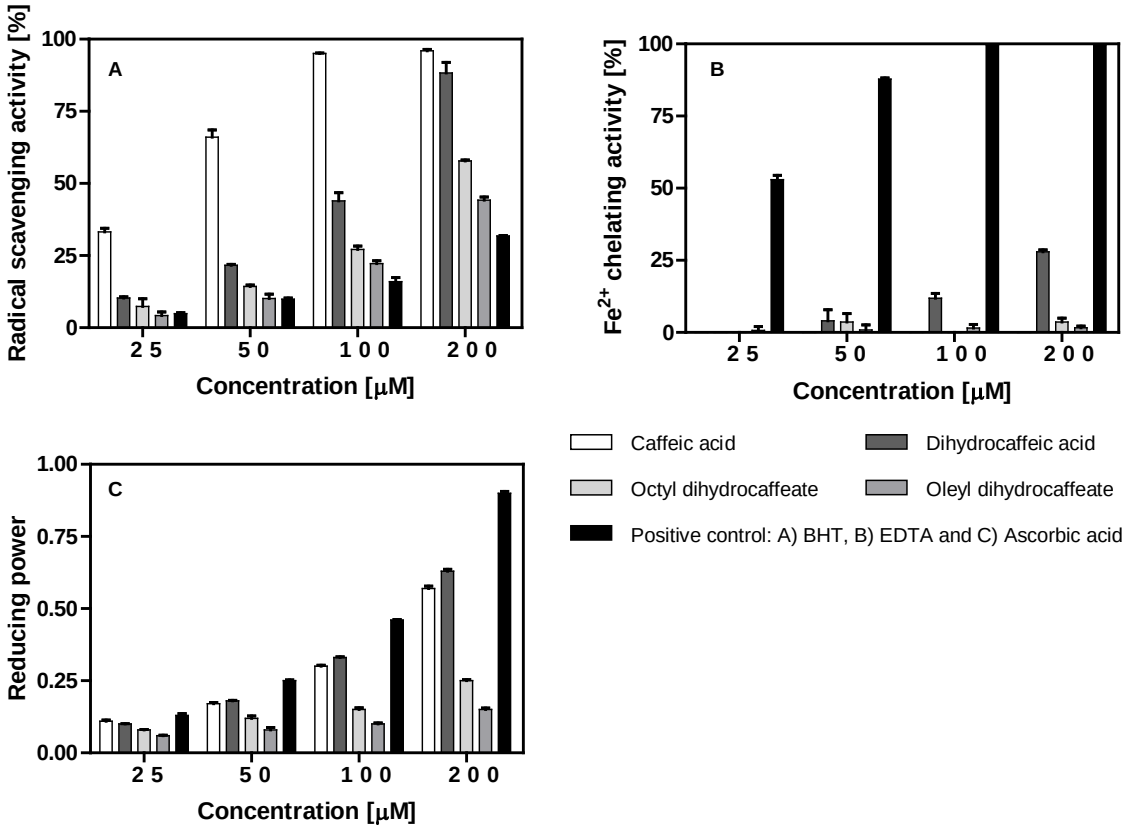
A
Radical scavenging activity [%]
100
75
50
25
0
25
50
100
200
Concentration [μM]
B
Fe2+ chelating activity [%]
100
75
50
25
0
25
50
100
200
Concentration [μM]
C
Reducing power
1.00
0.75
0.50
0.25
0.00
25
50
100
200
Concentration [μM]
Caffeic acid
Dihydrocaffeic acid
Octyl dihydrocaffeate
Oleyl dihydrocaffeate
Positive control: A) BHT, B) EDTA and C) Ascorbic acid

Figure 2

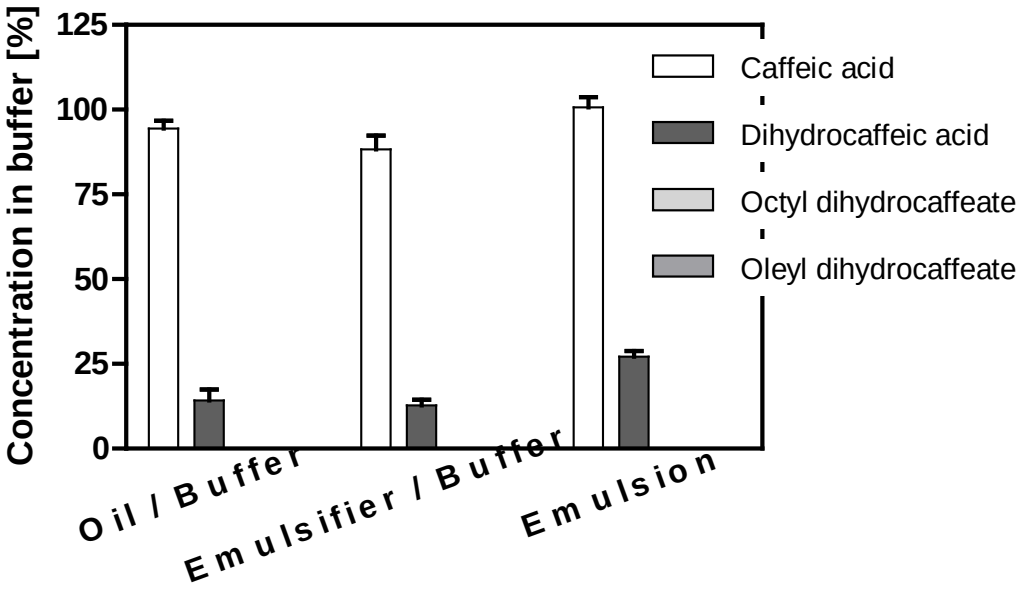
Concentration in buffer [%]
125
100
75
50
25
0
Caffeic acid
Dihydrocaffeic acid
Octyl dihydrocaffeate
Oleyl dihydrocaffeate
Oil / Buffer
Emulsifier / Buffer
Emulsion

Figure 3

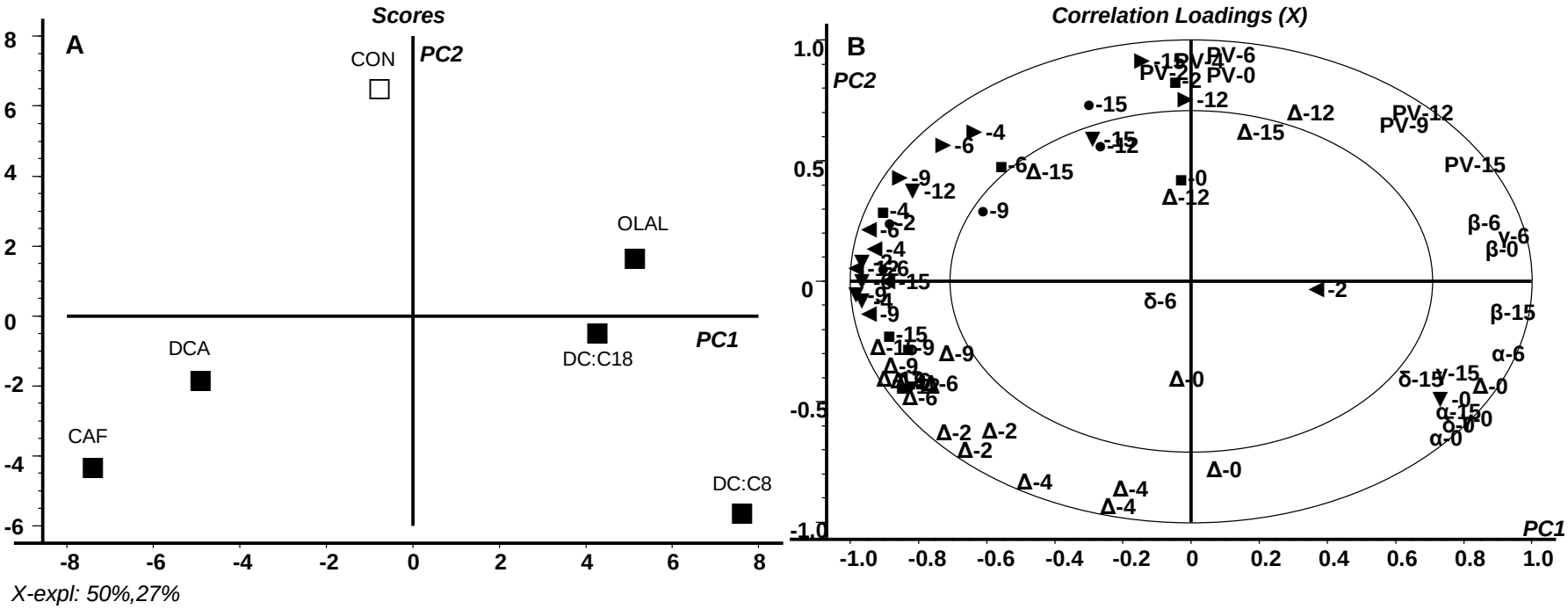
Scores
A
CON
PC2
OLAL
DCA
DC:C18
PC1
CAF
DC:C8
X-expl: 50%,27%
Correlation Loadings (X)
B
PC2
PC1

Figure 4

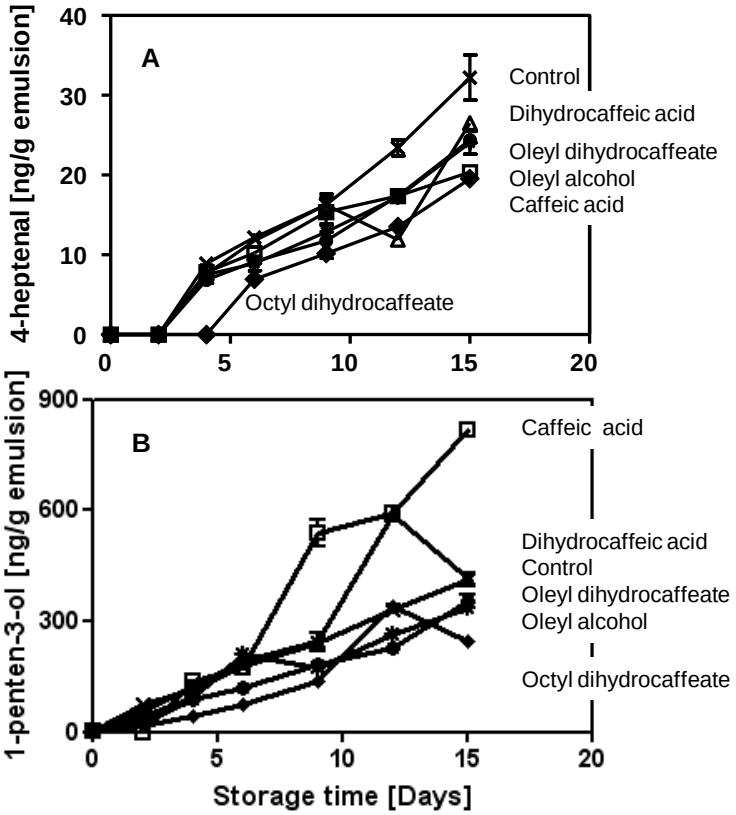
A
4-heptenal [ng/g emulsion]
40
30
20
10
0
0
5
10
15
20
Control
Dihydrocaffeic acid
Oleyl dihydrocaffeate
Oleyl alcohol
Caffeic acid
Octyl dihydrocaffeate
B
1-penten-3-ol [ng/g emulsion]
900
600
300
0
Caffeic acid
Dihydrocaffeic acid
Control
Oleyl dihydrocaffeate
Oleyl alcohol
Octyl dihydrocaffeate
Storage time [Days]

Figure 5

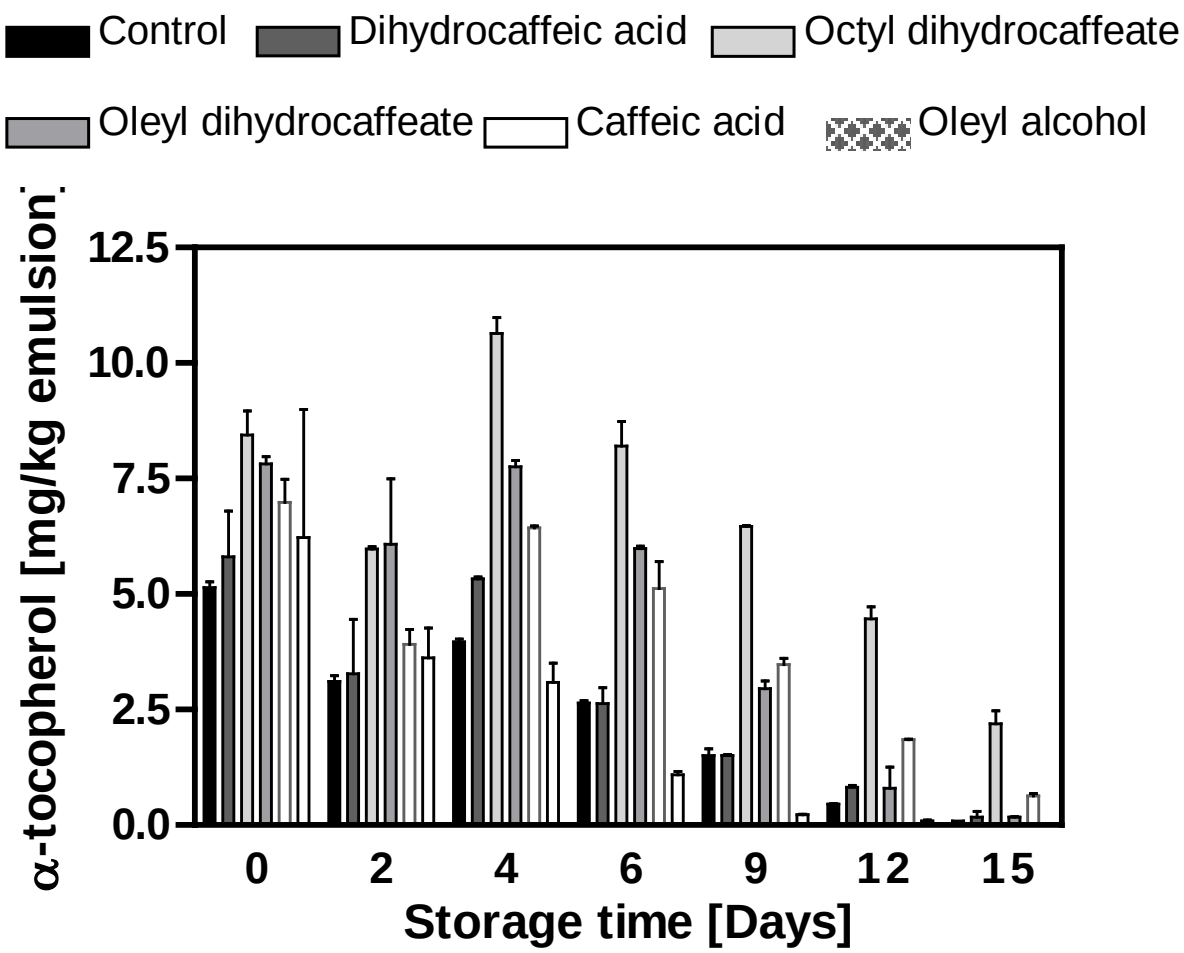
Control
Dihydrocaffeic acid
Octyl dihydrocaffeate
Oleyl dihydrocaffeate
Caffeic acid
Oleyl alcohol
α-tocopherol [mg/kg emulsion]
12.5
10.0
7.5
5.0
2.5
0.0
0
2
4
6
9
12
15
Storage time [Days]

Figure 6

PAPER V

Sørensen, A.-D.M., de Diego, S., Petersen, L.K., Nielsen, N.S., Lue, B.-M., Yang, Z., Xu, X. & Jacobsen, C.

The antioxidative effect of lipophilized dihydrocaffeic acid and rutin in fish oil enriched milk.

***In preparation*.**

The antioxidative effect of lipophilized phenolics in fish oil enriched milk

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Keywords: o/w emulsion, lipid oxidation, Rutin, Caffeic acid, Dihydrocaffeic acid

Running title: Antioxidant efficacy of lipophilized phenolics

Aim

The overall aim was based on the polar paradox and was to test the hypothesis that lipophilization of phenolics can increase their antioxidant efficacy in a complex food matrix, fish oil enriched milk was used as a test system.

Material and methods

Materials.

Fresh milk (0.5 % and 1.5 % fat content) was purchased in a local supermarket. Fish oil without antioxidant added was supplied by Maritex Norway (subsidiary of TINE BA, Norway). This oil had an initial PV at 0.1 meq peroxides / kg oil, tocopherol content of 204 mg α-tocopherol, 102 mg γ-tocopherol and 42 mg δ-tocopherol / kg oil, and the fatty acid composition was as follows: 14:0, 3.0%; 16:0, 8.7%; 16:1, 8.2%; 18:0, 1.9%; 18:1, 20.9%; 18:2, 1.8%; 18:4, 2.6%; 20:1, 12.5%; 20:5 (EPA), 9.4%; 22:1, 5.9%; 22:5, 1.1% and 22:6 (DHA), 11.6%. The total percentages of n-3 and n-6 in the oil were 24.7% and 2.7%, respectively.

Rutin (purity≥ 98%), caffeic acid (purity≥ 98%), dihydrocaffeic acid (purity ≥ 98%) and oleyl alcohol (purity 85%) were from Sigma Aldrich (Steinheim, Germany). Lipophilized rutin with lauric (C12) or palmitic (C16) acids, both with a purity of 98%, were synthesized at the National Food Institute, Division of Seafood Research (Technical University of Denmark). For further details about the lipophilization process is refer to Lue et al. *(1)*. Lipophilized dihydrocaffeic acid with octyl (C8) with a purity of 80% or oleyl alcohol (C18:1) with a purity of 60% were synthesized at the Department of Molecular Biology, Faculty of Science (Aarhus University). Chemicals were from Merck (Darmstadt, Germany) and external standards for identification and quantification of secondary volatile oxidation products were all from sigma Aldrich (Steinheim, Germany). All solvents were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland).

Production of fish oil enriched milk.

Milk with 1.5 % fat and milk with 0.5 % fat were mixed (1:1, v/v) to obtain a total fat content of 1 %. Subsequently, the milk was pasteurized at 72 °C for 15 s and the fish oil (0.5 % v/v) and antioxidant were added (for specification on antioxidant addition see Experimental design).These milk emulsions were prepared in two steps: pre-emulsification and homogenization. During pre-emulsification, the heated milk with fish oil and antioxidant added was stirred with an Ultra-Turrax (Step 7, 1 min, Janke & Kunkel IKA-Labortechnik, Staufen, Germany). The pre-emulsion was then homogenized at a pressure of 25 and 250 bar with 4 circulations of the emulsion at room temperature using a table homogenizer from GEA Niro Soavi Spa (Parma, Italy). The emulsions (100 g) were stored in 100 mL blue cap bottles at 5°C. Samples, one flask pr. code, were taken at day 0, 3, 6, 9 and 12 and divided in brown glass bottles and stored at -40°C until analyses of peroxides, volatiles, tocopherols and fatty acids were performed. The droplet size was measured at day 1, 6 and 12 without prefreezing.

Experimental design.

The experimental design for experiment 1 and 2 with sample codes is described in details below and summarized in Table 1.

Experiment 1. Five different antioxidants were evaluated, rutin, rutin laurate, rutin palmetate, Dihydrocaffeic acid and oleyl dihydrocaffeate in a concentration of 100 μM in fish oil enriched milk. Dihydrocaffeic acid and oleyl dihydrocaffeate were added directly to the milk, whereas rutin, rutin laurate and rutin palmitate were first dissolved or suspended in 1.5 mL acetone due to dissolving problems in the milk emulsion. The acetone with antioxidant was then added to the heated milk. To obtain the same condition for all emulsions, 1.5 mL acetone was added to the

milk emulsions with dihydrocaffeic acid, oleyl dihydrocaffeate and the control (no antioxidant added).

Experiment 2. Four different antioxidants were evaluated, caffeic acid, dihydrocaffeic acid, octyl dihydrocaffeate and oleyl dihydrocaffeate. Antioxidant concentration tested in the equal to experiment 1 (100 μM). The synthesized oleyl dihydrocaffeate was only 60 % pure, and contained ≤ 40% free oleyl alcohol. Therefore, an emulsion with oleyl alcohol with the same amount of oleyl alcohol (29 mg/kg) as in the emulsion with oleyl dihydrocaffeate was included to evaluate the effect of oleyl alcohol on lipid oxidation in the milk emulsion. The antioxidants were dissolved directly in the heated milk before homogenisation. Since some of the antioxidants (dihydrocaffeic acid and oleyl dihydrocaffeate) and control were repeated from experiment 1, the effect of acetone in the milk due to the antioxidants efficacy can be interpreted.

Droplet size determination

The size of the fat droplets in milk emulsions was determined by laser diffraction with a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). A few droplets of the milk emulsion were suspended directly in re-circulating water (2800 rpm, obscuration 14-16 %). The set-up used was the Fraunhofer method, which assumes that all sizes of particles scatter with equal efficiencies and that the particles is opaque and transmits no light *(2)*. The results were reported as surface mean diameter, $D_{3,2} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}$, where d is the diameter of individual droplets.

Measuring lipid oxidation

Extraction of lipids. Lipids were extracted from fish oil enriched milk (15 g) according to the method described by Bligh and Dyer *(3)* with reduced amount of solvent applied *(4)*. The analysis was done in duplicate and further used for determination of peroxide value, fatty acid composition and tocopherol concentration.

Primary oxidation products, peroxide value (PV). PV in the lipid extracts were determined by a colorimetric method based on formation of an iron-thiocyanate complex measured according to the method described by Shanta and Decker *(5)*. One determination was made on each of the two extracts prepared from the same milk sample.

Secondary volatile oxidation products – Dynamic headspace. Volatiles were collected on Tenax™ tubes (Perkin Elmer, Norwalk, CT, USA) by purging the fish oil enriched milk (8 g) with nitrogen (150 mL / min, 30 min) at 45°C. An ATD-400 automatic thermal desorber was used for thermally desorbing the collected volatiles. The transfer line of the ATD was connected to a 5890 IIA gas chromatograph (Hewlett-Packard, CA, USA) equipped with a DB wax column (length 30 m x I.D. 0.25 mm x 0.5 μm film thickness, J&W Scientific, CA, USA) coupled to a HP 5972A mass selective detector. Temperature program was as follows: 5 min at 45°C, 1.5°C/min from 45-55°C, 2.5°C/min from 55-90°C, 12°C/min from 90-220°C and hold for 4 min at 200°C. For quantification of the different volatiles, solutions with external standards at different concentrations were prepared and analyzed from milk with no fish oil added. The analysis was performed in triplicate and results given in ng / g milk.

Tocopherol concentration. Lipid extract, the same as used for PV analysis, was evaporated under nitrogen, re-dissolved in heptane and analyzed by HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) according to the AOCS method *(6)* to determine tocopherol concentration in the different milk samples. The reported tocopherol concentrations are averages of duplicate lipid extractions and duplicate analysis on the HPLC.

Fatty acid composition. Lipid extract was evaporated under nitrogen. Firstly, the glycerol bound fatty acids were trans esterified with methanolic NaOH (0.5 M). Then, hydrolytic released and free fatty acids were methylated by a boron trifluoride reagent (20%) catalyzed process. Methyl esters were extracted with heptane followed by separation on GC (HP 5890A, Hewlett Packard, CA, USA). The procedure was according to the AOCS methods *(7, 8)*. One determination was made on each of the two extracts prepared from the same mayonnaise sample.

Data analyses.

Statistic. The obtained results were analyzed by two way analysis of variance (GraphPad Prism, Version 4.03, GraphPad Software, Inc). The Bonferroni multiple comparison was used to test differences between samples or storage time (significance level $p < 0.05$).

Inhibition percentages. To compare the efficacy of the antioxidants in the two different emulsion systems, inhibition percentages ($Inhibition[\%] = \left(1 - \frac{Sample_{Antioxidant}}{Sample_{Control}}\right) \times 100$) were calculated.

Results and discussion (In preparation)

Characteristic of the fish oil enriched milk emulsions. The characteristic i.e. droplet size and content of EPA and DHA of the different fish oil enriched milk emulsions are summarized in Table 2. Average sizes of the lipid droplet in the milk were between 0.45 and 0.74 μm during the storage period. The droplet sizes measured in the different milk emulsion were stable during storage. However, the sizes differed between milk emulsions. Content of EPA and DHA at day 0 indicate similar level in the different milk samples from the beginning at 3.26-3.47 and 4.03-4.44 % EPA and DHA of total lipids, respectively. At day 12, oleyl dihydrocaffeate (experiment 2) was the only milk emulsion with a decrease in EPA and DHA > 0.1 %. However, the EPA and DHA increased in the same level in dihydrocaffeic acid (experiment 1) milk emulsion. Thus, the decreases in EPA and DHA in 2DC:C18 may be due to day to day variation for the measurements rather than an actual decrease.

Lipid oxidation in fish oil enriched milk emulsions. The lipid oxidation in the different milk emulsion were followed by measuring concentrations of primary oxidation products (PV) and secondary oxidation products (volatiles) at day 0, 3, 6, 9 and 12. The concentrations of tocophorols in the milk emulsions were measured at day 0 and 12, since they also can act as antioxidants in the emulsions.

PV's obtained in experiment 1 and 2 during storage is shown in Figure 1. For experiment 1 a lag phase was observed in Figure 1A until day 3, where no significant differences for PV level in the milk emulsions existed. At day 6 fish oil enriched milk had significantly higher PV than fish oil enriched milk with antioxidant added except for milk emulsion with dihydrocaffic acid added.

The ranging of the different milk emulsions due to the PV level at day 12 was as follows for experiment 1: control^{a} ≥ rutin palmitate^{ab} ≥ rutin^{b} = oleyl dihydrocaffeate^{b} = dihydrocaffeic acid^{b} > rutin laurate^{c}. For experiment 2 (Figure 1B) the PV level in some milk emulsions was higher than for experiment 1 e.g. for fish oil enriched milk without antioxidant added. However, the development of PVs was triggered in different rates depending on the antioxidant applied. At day 12 the ranging of milk emulsions due to PV level was as follows: oleyl alcohol^{a} > control^{b} > dihydrocaffeic acid^{c} = caffeic acid^{c} > oleyl dihydrocaffeate^{d} = octyl dihydrocaffeate^{d}. Furthermore, the chain length octyl or olyel esterified to dihydrocaffeic acid did not influence the development of peroxides differently.

Additionally, the inhibition percentages were calculated in order to be able to compare the PV level during storage between the experiments, and thereby conclude on the effect of phenolics and lipophilized phenolics on the development of PV. Inhibition percentages (Table 3) of those milk emulsions with antioxidant applied in both experiments; dihydrocaffeic acid, oleyl dihydrocaffeate, indicate better inhibition of PV development in experiment 2 without acetone. In spite of the higher PVs in experiment 2, the efficacy of antioxidants in fish oil enriched milk seems to be better for octyl and oleyl dihydrocaffeate without acetone than oleyl dihydrocaffeate, rutin laurate and rutin palmitate with acetone added. However, the most important is the decomposition rate of the PV, which is indicated by the development of volatiles.

The development of two different volatiles (1-penten-3-one and 2,6-nonadienal) in the milk emulsions is shown in Figure 2. The volatile results indicated that both lipophilized dihydrocaffeic acid and rutin had a better antioxidative effect in fish oil enriched milk compared with their more hydrophilic parent compound: dihydrocaffeic acid and rutin, respectively.

Both octyl dihydrocaffeate and oleyl dihydrocaffeate exerted stronger antioxidative effects than dihydrocaffeic acid in fish oil enriched milk. The efficacy of octyl dihydrocaffeate was slightly better than oleyl dihydrocaffeate. These findings are similar to the findings obtained for these compounds in a simple o/w emulsion. Thus, the cut-off effect with the optimal alkyl chain length below C18 long seemed to be confirmed for dihydrocaffeate in fish oil enriched milk. However, oleyl dihydrocaffeate contained impurities such as oleyl alcohol. Since oleyl alcohol was inactive or acted as a prooxidant in fish oil enriched milk, it might have reduced the antioxidative effect of oleyl dihydrocaffeate. Thus, the antioxidative effect may have been better for oleyl dihydrocaffeate if a more purified compound was used, but this needs to be further studied.

Interestingly, findings regarding the efficacy of rutin esters as antioxidants were different in fish oil enriched milk compared with the simple o/w emulsion. In fish oil enriched milk, both rutin esters had an antioxidative effect, but rutin laurate was a more efficient antioxidant than rutin palmitate. In contrast, these rutin esters were less effective antioxidants when compared with rutin in o/w emulsion *(9)*. Moreover, rutin laurate also exerted stronger antioxidative activity than rutin and rutin palmitate in a LDL assay which is a more complex system than an o/w emulsion *(10)*. Thus, these findings indicate that the cut-off effect might be influenced by the system i.e. simple o/w emulsion or more complex emulsion systems such as LDL and milk and that complexity of the system affect the cut-off effect.

Table 1 Experimental design.

Antioxidant applied	Sample code	Concentration of antioxidant	
		[µM]	**[mg/kg]**
Experiment 1: Antioxidant + 1.5 mL acetone or 1.5 mL acetone with antioxidant			
Control	1Con	-	-
Rutin	1Rut	100	61.1
Rutin laurate (C12)	1Rut:C12	100	79.3
Rutin palmitate (C16)	1Rut:C16	100	84.9
Dihydrocaffeic acid	1DCA	100	18.2
Oleyl dihydrocaffeate (C18:1)	1DC:C18	100	72.1
Experiment 2: Antioxidant added without acetone			
Control	2Con	-	-
Dihydrocaffeic acid	2DCA	100	18.2
Octyl dihydrocaffeate (C8)	2DC:C8	100	36.8
Oleyl dihydrocaffeate (C18:1)	2DC:C18	100	72.1
Oleyl alcohol	2OLAL	-	28.8
Caffeic acid	2Caf	100	18.0

Table 2 Droplet size $D_{3,2}$ [μm] for lipid droplets in fish oil enriched milk given as an average during storage (average ± SD) and content of EPA and DHA [% wt of total lipids] at day 0 and 12 in the different milk emulsions. Sample codes refer to Experimental design

Sample code		Droplet size [μm]	EPA content [%wt of total lipids]		DHA content [%wt of total lipids]	
			Day 0	Day 12	Day 0	Day 12
Exp. 1	**1Con**	0.45 ± 0.01	3.47 ± 0.08	3.51 ± 0.09	4.35 ± 0.24	4.47 ± 0.21
	1Rut	0.70 ± 0.03	3.26 ± <0.01	3.51 ± 0.15	4.03 ± 0.07	4.43 ± 0.28
	1Rut:C12	0.74 ± 0.01	3.42 ± 0.04	3.48 ± <0.01	4.26 ± 0.20	4.30 ± 0.06
	1Rut:C16	0.59 ± 0.01	3.43 ± 0.08	3.44 ± 0.01	4.26 ± 0.21	4.27 ± 0.01
	1DCA	0.70 ± 0.03	3.47 ± 0.05	3.58 ± 0.01	4.35 ± 0.15	4.57 ± <0.01
	1DC:C18	0.72 ± 0.02	3.51 ± 0.08	3.48 ± 0.01	4.44 ± 0.16	4.36 ± 0.01
Exp. 2	**2Con**	0.73 ± 0.02	3.41 ± 0.01	3.75 ± 0.07	4.15 ± 0.03	4.53 ± 0.02
	2DCA	0.65 ± 0.07	3.37 ± 0.02	3.31 ± 0.04	4.10 ± 0.04	4.04 ± 0.08
	2DC:C8	0.70 ± 0.03	3.31 ± 0.01	3.31 ± 0.07	4.05 ± 0.04	4.02 ± 0.16
	2DC:C18	0.74 ± 0.04	3.43 ± 0.08	3.26 ± 0.06	4.09 ± 0.01	3.99 ± 0.02
	2OLAL	0.73 ± 0.02	3.46 ± 0.01	3.58 ± 0.01	4.22 ± 0.04	4.45 ± 0.03
	2Caf	0.61 ± 0.09	3.37 ± 0.07	3.37 ± 0.02	4.13 ± 0.09	4.07 ± 0.04

Table 3 Calculated inhibition percentages of PV level in the different milk emulsions in both storage experiments. The values are calculated according to the control emulsions from the respective experiment.

Samples	Inhibition of PV during storage [%]				
	Day 0	**Day 3**	**Day 6**	**Day 9**	**Day 12**
Experiment 1					
Rutin	-6	18	42	34	21
Rutin laurate (C12)	27	32	59	77	78
Rutin palmitate (C16)	37	-18	57	32	11
Dihydrocaffeic acid	18	-15	31	52	29
Oleyl dihydrocaffeate (C18:1)	34	21	58	31	26
Experiment 2					
Dihydrocaffeic acid	35	29	11	15	23
Octyl dihydrocaffeate (C8)	45	63	83	57	57
Oleyl dihydrocaffeate (C18:1)	47	59	70	59	49
Oleyl alcohol	63	3	-95	26	-15
Caffeic acid	57	51	33	34	31

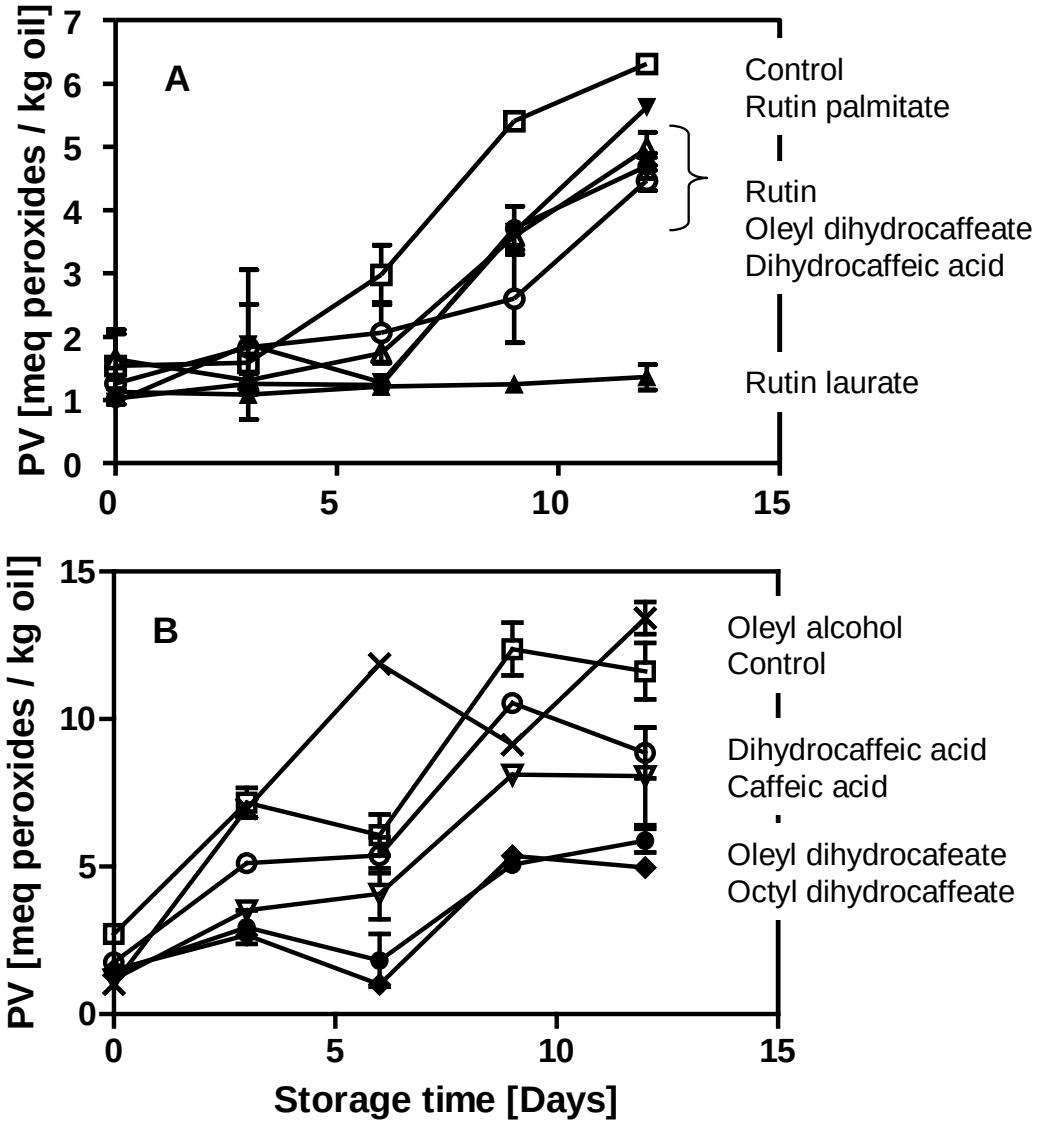

Figure 1 Concentration of peroxides measured as PV [meq. peroxides / kg oil] in the different fish oil enriched samples during storage. Error bars indicate SD of the measurements (n = 2). A) Experiment 1: control (□), rutin (Δ), rutin laurate (▲), dihydrocaffeic acid (○) and oleyl dihydrocaffeate (●). B) Experiment 2: control (□),dihydrocaffeic acid (○), octyl dihydrocaffeate (◊ solid), oleyl dihydrocaffeate (●), oleyl alcohol (X) and caffeic acid (▼unsolid).

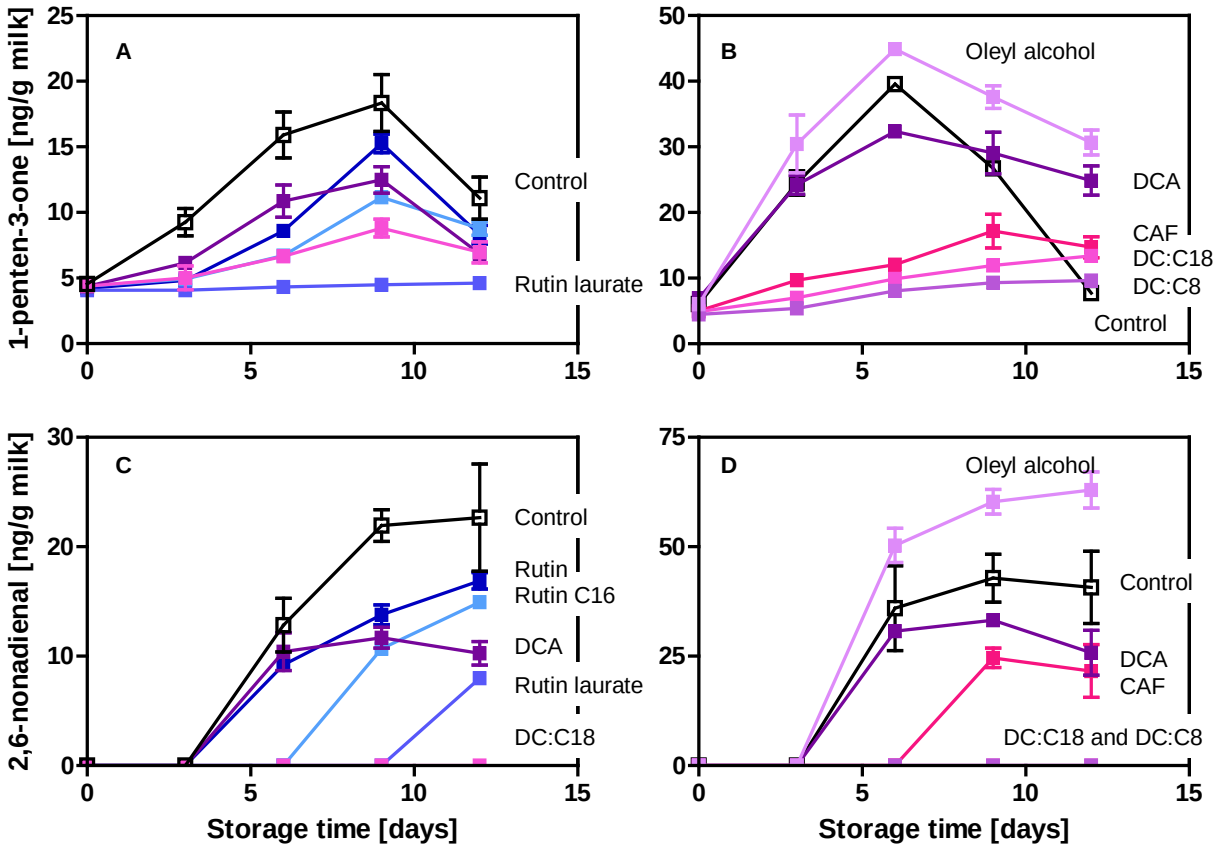

Figure 2 Concentrations [ng/g milk] of A) 1-penten-3-one (acetone added), B) 1-penten-3-one (no acetone), C) 2,6-nonadienal (acetone added) and D) 2,6-nonadienal (no acetone). Sample codes: □ Control (no antioxidant); ■ Rutin; ■ Rutin laurate (C12); ■ Rutin palmitate (C16); ■ DCA Dihydrocaffeic acid; ■ DC:C8 Octyl dihydrocaffeate; ■ DC:C18 Oleyl dihydrocaffeate; ■ CAF Caffeic acid and ■ OLAL Oleyl alcohol. Bars indicate SD of 3 measurements.

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APPENDIX I

Sørensen, A.-D.M., Nielsen, N.S. & Jacobsen, C.

Oxidative stability of fish oil enriched tuna salads with origanox

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OXIDATIVE STABILITY OF FISH OIL ENRICHED TUNA SALADS WITH ORIGANOX

Introduction

Earlier experiments with fish oil enriched tuna salad and 1% dry spices such as oregano, rosemary and thyme showed an increased oxidative stability of fish oil enriched tuna salads. All spices added additional not wanted flavour. Of the dry spices evaluated, oregano resulted in less oxidation in the fish oil enriched tuna salad (Paper I).

Aim

The aim of the experiment was to evaluate the oxidative stability of fish oil enriched tuna salad different concentration of a spice extract based on oregano: Oreganox. Moreover, the effect of Origanox was compared with that of EDTA.

Design and Analyses

Sample code	Concentration [mg/kg mayonnaise]		Concentration
	EDTA	Origanox	[mg/kg product]
TunaFO	-	-	-
TunaFO_75EDTA	75	-	25.5
TunaFO_75Origanox	-	75	25.5
TunaFO_750Origanox	-	750	255
TunaFO_1500Origanox	-	1500	510

Produced tuna salad was stored for 6 weeks at 5°C, and lipid oxidation was evaluated based on formation of lipid hydroperoxides and volatiles during storage (Week: 0, 2, 4, 5 and 6). Furthermore the tuna salads were evaluated by a sensory expert panel.

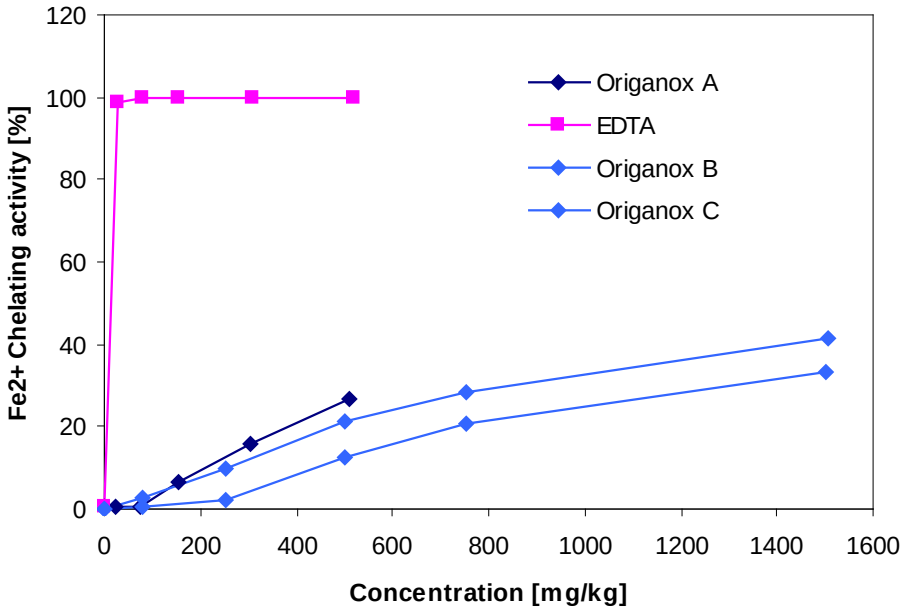
Additional, the employed antioxidants were evaluated for their antioxidant properties in *in vitro* assays. For more details refer to paper III and IV.

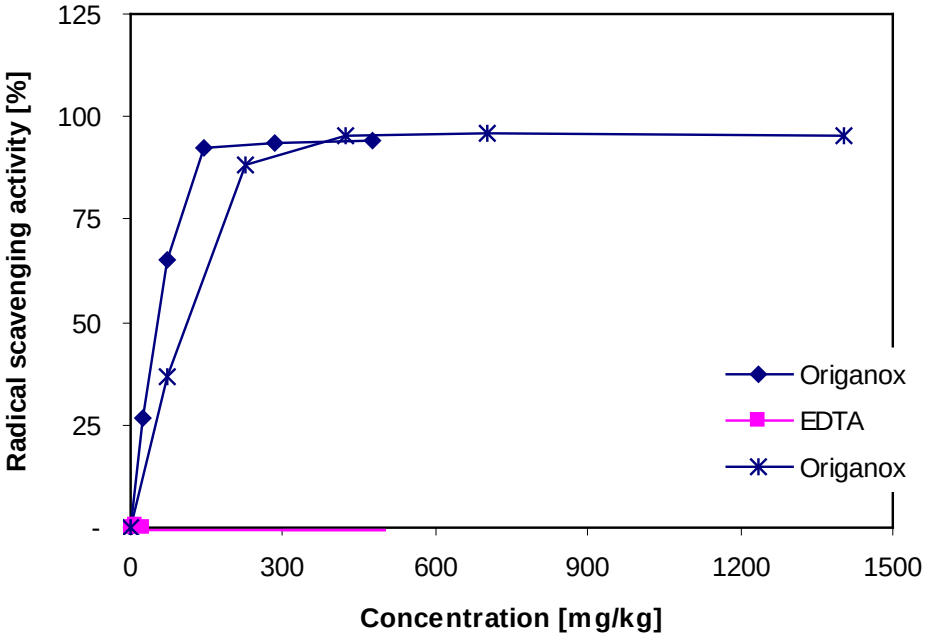
Production

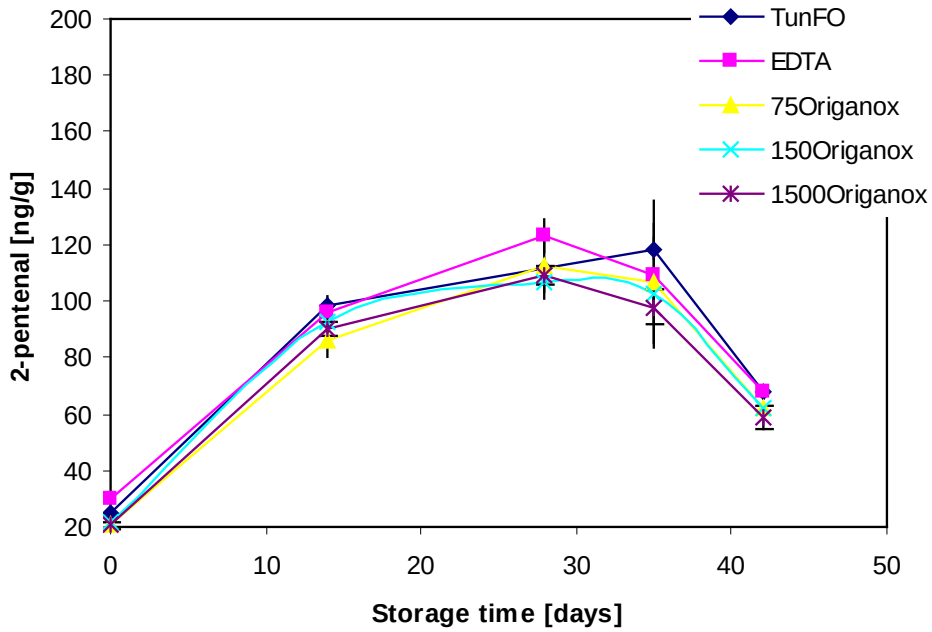
Mayonnaise with or fish oil added. Ingredients for the mayonnaise preparation were (all amounts are stated in % w/w): water (30%), oil (rapeseed oil 56.7% and cod liver oil 6.3%), pasteurized egg yolk (5%), tarragon vinegar, salt and thickeners (modified starch, guar gum, xanthan gum). Sourness was adjusted to pH 3.5-4.1 with lactic acid, sodium acetate and malic acid. Potassium sorbate and sodium benzoate were used for preservation. The ingredients were mixed and the mayonnaise was used for preparation of tuna salads. The respective concentration of Origanox or EDTA was added together with the other ingredients before mixed.

Tuna salad with or without fish oil added. The full recipe for the tuna salads was: Mayonnaise (with or without fish oil) 34%, tuna 32%, peas 10%, maize 10%, bell pepper (red) 10% and onion 0.6%. To adjust sourness to pH 5.1-5.5, ascorbic acid and citric acid were added. All the ingredients were carefully mixed and weighted into plastic trays (175 g each) with lid as previously described.

Results




125
100
75
50
25
-
Radical scavenging activity [%]
0
300
600
900
1200
1500
Concentration [mg/kg]
Origanox
EDTA
Origanox


200
180
160
140
120
100
80
60
40
20
2-pentenal [ng/g]
0
10
20
30
40
50
Storage time [days]
TunFO
EDTA
75Origanox
150Origanox
1500Origanox

APPENDIX II

Sørensen, A.-D.M., Nguyen, L., Nielsen, N.S. & Jacobsen, C.

Effect of rosemary and green tea extracts on the oxidative stability of fish oil enriched milk

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OXIDATIVE STABILITY OF FISH OIL ENRICHED MILK WITH ROSEMARY OR GREEN TEA EKSTRACTS

Aim

The aim of the experiment was to evaluate the antioxidative effect of selected plant extracts in a complex food emulsion: fish oil enriched milk.

Design

Milk samples	Concentration in the fish oil enriched milk [mg/kg]		
Reference	-	-	-
Green tea	100	300	500
Rosemary 11	100	300	500
Rosemary 201	100	300	500

Produced milk samples were stored for up to 10 days at 2°C. Lipid oxidation was evaluated based on formation of lipid hydroperoxides and volatiles during storage (Day: 0, 3, 6 and 10). Furthermore the tuna salads were evaluated by a sensory expert panel.

Production

Milk with 1.5 % fat and milk with 0.5 % fat were mixed (1:1, v/v) to obtain a total fat content of 1 %. Subsequently, the milk was pasteurized at 72°C for 15 s and the fish oil (0.5 % v/v) was added. Thereafter, milk samples were homogenized with a two-valve Rannie homogenizer (Albertslund, Denmark) at different pressures and temperatures (Table 2). Obtained milk emulsions were stored in 250 mL sterilized pyrex bottles at 2 °C in darkness.

Analyses

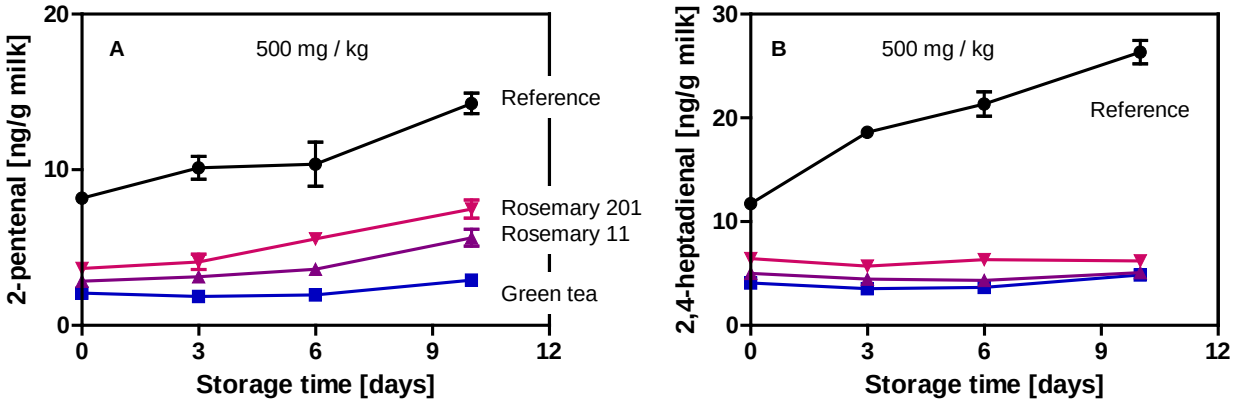
Measurement of peroxide values (PV)

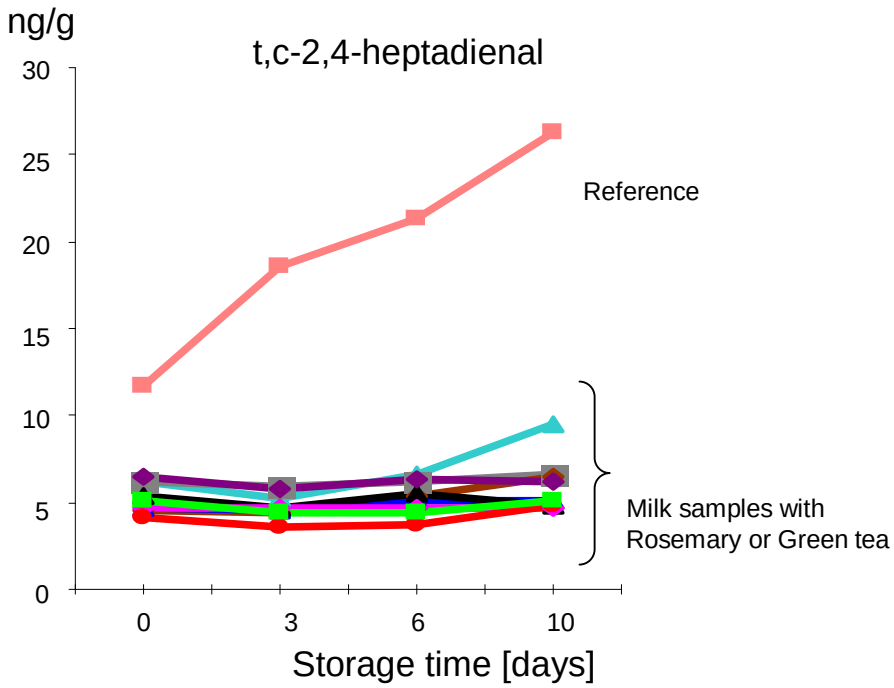
PV was determined as a measure of lipid oxidation. Lipids were extracted from the milk emulsions according to the methods described by Bligh and Dyer *(1)* using a reduced amount of solvent *(2)*. The peroxide values were determined in the lipid extract by the colorimetric ferric-thiocyanate method according to the International IDF Standards *(3)*.

Volatiles

The volatiles were analyzed by dynamic headspace, purge and trap method. The volatile components were thermally desorbed from the trap by Automated Thermal Desorption (ATD-400, Perkin Elmer, Norwalk, CN, USA) and seperated by gas chromatography (HP 5890 IIA, Hewlett Packard, Palo Alto, CA, USA). Specifications for the ATD and GC can be seen in Appendix 3. The volatile compounds were analysed by mass spectrometry (HP 5972 mass selective detector, Hewlett Packard, Palo Alto, CA, USA).

Results





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