Studies on the antioxidant activity of milk proteins in model oil-in-water emulsions

A thesis presented in partial fulfilment of the requirements
for the degree of

Doctor of Philosophy
in
Food Technology

by

Daniel Ries

Riddet Institute

Massey University, Palmerston North, New Zealand

2009

Abstract

The present study was aimed at extending our knowledge of the antioxidative properties of the milk protein products, whey protein isolate (WPI) and sodium caseinate (NaCas), in oil-in-water (O/W) emulsions rich in polyunsaturated fatty acids (PUFAs). In particular, the objective was to contribute to our understanding of the compositional and processing factors that influence the oxidative stability of protein-stabilised O/W emulsions. Linoleic acid (approximately 60 %) was used as the lipid for the oil phase (10.6 %). The emulsion samples were usually incubated at 50 ˚C to accelerate lipid oxidation. Lipid oxidation indicators were lipid hydroperoxides and headspace hexanal, determined by solid phase microextraction (SPME) combined with gas chromatography (GC).

WPI- or NaCas-stabilised emulsions were prepared using a wide range of protein concentrations (0.5, 1.0, 2.0, 3.0, 4.0, 7.0 or 10.0 %) at two droplet sizes (d_{32} = 0.31 and 0.65 µm). In general, higher lipid oxidation levels were found for the larger droplet size. Increasing protein concentration led to a decrease in the lipid oxidation rate. The greatest decrease in lipid hydroperoxide levels (values after 4 h) occurred at up to 4.0 % protein concentration. The greatest decrease in hexanal levels (values after 24 h) occurred at up to 4.0 % protein concentration in WPI emulsions (0.31 µm). The hexanal levels were more independent of the protein concentration in the other emulsion types. The hexanal level decreased at protein concentrations > 4.0 % in NaCas emulsions (0.31 and 0.65 µm) and at protein concentrations > 7.0 % in WPI emulsions (0.65 µm). The difference between lipid hydroperoxide generation in emulsions with small and large droplet sizes decreased with increasing protein concentration. This effect was more pronounced in NaCas emulsions. In general, NaCas was a better inhibitor of lipid oxidation than WPI, but WPI appeared to be the better antioxidant at some droplet size/protein concentration combinations.

The protein in the continuous phase, i.e. the unadsorbed protein, played an important role in lipid oxidation. In principal, the lipid hydroperoxide and hexanal levels showed the same development over the continuous phase protein concentration as over the

protein concentration in WPI and NaCas emulsions (d_{32} = 0.31 µm). A low NaCas level in the continuous phase already led to a relatively low hexanal level, whereas a higher WPI level was required. When NaCas solution was added to a WPI emulsion or WPI solution was added to a NaCas emulsion, a synergistic antioxidative effect was observed.

The high molecular weight fractions (molecular weight ≥ 12000–14000) of WPI and NaCas contained pro-oxidative metal ions that contributed to lipid oxidation in the emulsions. An enrichment of NaCas emulsions with the low molecular weight fraction of NaCas (with a molecular weight ≤ 12000–14000) notably inhibited lipid oxidation. An enrichment of WPI emulsions with the low molecular weight fraction of WPI (with a molecular weight ≤ 12000–14000) also seemed to inhibit lipid oxidation, but the effect was not significant. The protein solutions were enriched with these fractions before emulsion preparation.

Pure WPI solution or mixed WPI/NaCas (1:1, weight/weight) solution with 1.12 or 2.24 % protein concentration was heated at 84 ˚C for up to 40 min, cooled and then used to prepare emulsions. Lipid oxidation was generally not affected by the heat treatment or the degree of whey protein denaturation. However, at the lower WPI concentration, more hexanal was produced for the longer heating times (20, 30 and 40 min) and this appeared to be connected with the physical instability of the emulsions. Greater oxidative stability was found at the higher protein concentration and when the proteins were mixed, pointing to a possible synergistic antioxidative effect of WPI and NaCas.

The addition of the free radical source 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) greatly increased the oxygen uptake and the generation of lipid hydroperoxides in the emulsions. The oxidative stability increased with increasing protein concentration (1.0, 4.0 and 7.0 %). NaCas had a greater antioxidative effect than WPI. The inhibition of oxygen uptake appeared to be largely influenced by the free-radical-scavenging activity of the system, determined by the protein type and the protein concentration, as the radicals were produced linearly over time and oxygen was consumed linearly over time. It can therefore be concluded that free-radical-scavenging activity represents a major antioxidative mechanism of the milk proteins.

Oxygen was consumed much faster in emulsions than in protein solutions when the same level of AAPH was incorporated. In a WPI (1.0 % protein) emulsion, much lower levels of protein hydroperoxides than of lipid hydroperoxides developed. This pointed to a much greater reactivity of linoleic acid than of the milk proteins with oxygen. In contrast, the exposure of WPI to oxidising linoleic acid in an emulsion (1.0 % protein) or to AAPH in aqueous solution led to oxidative damage of the whey proteins, indicated by the loss of amino acids. The loss of specific amino acids was different for proteins in the continuous phase or cream phase of an emulsion or in WPI solution.

The present study confirms the antioxidative potential of WPI and NaCas and gives new insights into their functionality as oxidative stabilisers in O/W emulsions.

Acknowledgements

I am sincerely grateful to my chief supervisor, Professor Harjinder Singh, for his guidance, encouragement and generous help throughout the entire project. I highly appreciate his inputs and expertise. I especially thank him for preventing me from following the wrong tracks during the practical work, and for his support and guidance in the preparation of this thesis.

I am sincerely grateful to my co-supervisor, Dr Derek R Haisman. I would like to thank him for believing in my suitability for this project and bringing me to the Riddet Institute and Massey University. Also, I would like to thank him for his constant guidance with expertise, his support and encouragement throughout the practical work and during the preparation of the thesis.

I would like to thank Dr Aiqian Ye for his valuable inputs and expertise in questions about oil-in-water emulsions and his help in preparing publication material based on the findings of this project.

I wish to thank the Riddet Institute for the scholarship that enabled me to do this project.

I would like to thank Ms Leiza Turnbull, Ms Michelle Tamehana, Ms Fliss Jackson, Mr Garry Radford, Mr Steve Glasgow, Dr Kelvin Goh, Dr Lara Matia-Merino and Mr Warwick Johnson (Institute of Food, Nutrition and Human Health, Massey University), Ms Fliss Stibbards, Dr Peter Zhu, Ms Janiene Gilliland, Ms Maggie Zhou, Mr Shane Rutherfurd and Mr Chris Hall (Riddet Institute, Massey University) for their cooperation, help or assistance in experimental work or their advice. Thank you to Mr Matt Levin and Mr Peter Jeffery for their services in information technology (Institute of Food, Nutrition and Human Health, Massey University).

Thank you to Professor Eric Ainscough for his advice and the demonstrations in ESR spectroscopy, to Mr Grant Platt for his customised technical equipment used in one of the experiments (both Institute of Fundamental Sciences, Massey University), to Mr

Rob Hunter for his assistance in gel electrophoresis (Fonterra Co-operative Group Limited), to Dr Caryl Lockhart for her advice in statistical analyses (EpiCentre, Massey University) and to Dr Yacine Hemar for his inspiring contribution to the project (The MacDiarmid Institute for Advanced Materials and Nanotechnology).

A special thank-you I direct to my fellow post-graduates from the Seed Tech Hut and colleagues, to my friends and flatmates and other people I shared time with in the past years, for their company, their support, a word that cheered me up when it was tough, their prayers, for extending my knowledge in life science ☺ or insights into another culture of this world: Dr Noorzaid Muhamad, Dr Mary Eastham, Claire Hastings, Kathleen and Rick Field, Tim Coogan, Alex Kordichev, Nandu Goswami, Aniket Abhyankar, Dr Amelie Deglaire, Anwesha Sarkar, Catootjie Nalle, Dr Mischa Walton, Oni Yuliarti, Dr Supat Chaiyakul, Dr Ahmed Mohammed Rida Amerah**,** Thanuja Herath, Dr Janina Kühn, Prabhu Balan, Emmanuelle Riou, Hilary McKinnon, Visaka Anantawat, Jiahong Su, Sylvia Chung, Carolina Saavedra, Natalie Keller, Xuemei Tang, Cheryl Nkhasi, Dr Simon Loveday, Amit and Nimi Taneja, Dr Reza Mozafari, Dr Inge Merts, Dr Ajay Awati, Bonny Zhan, Liwei (Daisy) Bai, Anne Göppert, Taka Fihaki, Wiremu and Trieste Te Awe Awe, Ron Sanders, Daniel Chua, Francis Keete, Reuben Wallis, Hone Davis, Dr Caryl Lockhart, Benjamin Trembarth, Steven Evans, Birgit Schauer, Dr Simone Titus, Manfred Sutter, James and Alice Curtis, Ariunaa and Amra Mendtsoo, Dr Clyton Moyo, Bernard Phiri, Carlos Hug de Belmont, Dione Joseph, Dominic Tehan, Stephen Smith, Rachel Anderson, Emma Dodson, Penelope Byrnes, Christina Rock, Agatha Chiu, Aberry, James Valtorta, Lucy Ellis, Lynda Bottrill, Sonya House, Trudy Leslie, Albertha Lavi, Jane Dravitski, Averil Wills, Alex Ogidi, Deogratius Mohanga, Fuminori Watanabe, Kunsik Park, Kieran Udy, Ellen Schoner, Fr Marcus Francis, Fr Brian Walsh, Jonny Boon and Mary-Grace Williams, Professor Janina Mazierska, David Mullin, Kui and Helen Chen, Giovanna, Tatiana Shirokova, Sonja Koch, Karin Kohler, Stefanie Bachmann, Dominik Durner, Monique Laurie, Philipp Schenkel, Morgane Blanc, Florence, Christoph Rau, Michaela Eckstein, Kaustubh Savant, Andre Bruns, Helga Kocurek, Jozsef-Sebastian and Katrin Pap, Paul and Dianne Stock, Matthias and Victoria Herzog, Joanne Oliver, Lucia Kwa and all members of the Magnificat Community, Cross Creek, and Freedom Church, Te Rangimarie Marae, Rangiotu.

I would like to thank my family, especially my Mum, my Dad and my brothers, who were far away but still there for me when I needed them.

Finally, I want to thank God for holding me in his hands, although he does not always give me a hand. I am grateful to come to the end of this PhD journey.

Table of contents

Chapter 1

Introduction

Polyunsaturated fatty acids (PUFAs) play an important role in human nutrition. Research in recent decades has shown greatly beneficial effects for human health of diets that are high in the omega-3 (or n-3) fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). They appear to be particularly suitable for the prevention of coronary heart disease, hypertension and type 2 diabetes and are beneficial for brain development and mental health (Ruxton et al. 2004; Simopoulos 1999). Other PUFAs such as arachidonic acid, linoleic acid, γ-linolenic acid (n-6 fatty acids) and α-linolenic acid (n-3 fatty acid) are also physiologically important. In general, unsaturated fatty acids serve as building blocks for biological membranes and signal compounds (Kritchevsky 2002; Watkins and German 2002). A balanced ratio of n-6:n-3 intake is an important factor in the prevention of heart diseases. In Western countries, such as the USA and the UK, this ratio was found to be unbalanced because of a low n-3 intake and a high n-6 intake (Simopoulos 1999; Simopoulos 2001; Wijendran and Hayes 2004).

The fortification of food products with lipids high in omega-3 fatty acids could be a means of dealing with the problem of omega-3 deficiency in Western societies. However, the stability and stabilisation of PUFAs in foods is a challenge because these fatty acids are highly susceptible to lipid oxidation. Lipid oxidation leads to damage to PUFAs and thereby a decrease in their nutritional value. Volatile off-flavour compounds (e.g. aldehydes) are produced during lipid oxidation. Thus, deterioration in the quality of foods can occur and their shelf lives may be reduced. Some metals that are widely present in food, the oxidative state of the raw materials and the conditions during the production process and storage of foods can initiate and promote lipid oxidation:

• iron and copper ions;
• pre-contamination with hydroperoxides;
• presence of oxygen;
• heating during the production process, e.g. pasteurisation;

• high storage temperatures;
• light.

Therefore, effective strategies for protecting PUFAs in foods from oxidative deterioration are required. The food delivery systems for omega-3 fatty acids need to have the correct format to minimise negative influences that can promote lipid oxidation. For their incorporation at high levels into a food product, PUFAs usually need to be emulsified because of their hydrophobic nature. Examples of sources that are rich in DHA and EPA are oils of marine fish and algae (Ackman 2004; Berge and Barnathan 2005; Lagarde 2008).

The milk protein products whey protein isolate (WPI) and sodium caseinate (NaCas) are used as emulsifiers for oil-in-water (O/W) emulsions because of their remarkable emulsifying properties (see also Chapter 2, Sections 2.4 and 2.5). Furthermore, WPI and NaCas, as well as hydrolysates and peptides thereof, have shown good antioxidative potential by inhibiting the oxidative deterioration of unsaturated fatty acids, either as part of triacylglycerols or in free form (see also Chapter 2, Section 2.7). WPI and NaCas therefore appear to be useful for the design of O/W emulsions that serve as delivery systems for omega-3 fatty acids because of their dual functionality as emulsifiers and antioxidants. Such emulsions may be incorporated into real food emulsion systems, e.g. milk, yoghurt, mayonnaise or ice cream.

Although WPI and NaCas have shown antioxidative potential in O/W emulsions, a more detailed understanding of their effectiveness under certain conditions, their antioxidative mechanisms and their interaction with emulsion lipids is required. In addition, new possibilities for improving their antioxidative functionality need to be explored. These were the purposes of the present study. As more fundamental information about the characteristics and the behaviour of whey proteins and caseins in O/W emulsions is gathered, it will become easier to handle and use them for real food systems.

For the present study, an O/W emulsion test model system, in which the influence of certain parameters on oxidation could be examined, needed to be chosen. A simple,

relatively well-defined and standardised system was proposed, in order to limit the number of unknown variables and to be able to attribute changes in the oxidative stability more unequivocally to the factors that were altered. In that respect, the selection of a suitable lipid as the oil phase was important. One criterion for the lipid was a high content of PUFAs because it had to exhibit similarity to omega-3 fatty acids. Linoleic acid was selected, as it was available in purified and standardised form without antioxidants and in large quantities from a chemical supplier.

Chapter 2

Literature review

2.1 Lipids

Food lipids, fats (if they are solid) and oils (if they are liquid), are derived from both animal sources and plant sources. Triacylglycerols, which consist of three fatty acids esterified with glycerol, form the major component of food lipids. Depending on the origin of the oil or the fat, the overall fatty acid composition varies and determines the characteristics of the triacylglycerol mixture, e.g. melting point, hydrophobicity and susceptibility towards oxidation. Free fatty acids, diacylglycerols and monoacylglycerols are present in smaller quantities in food oils. Di- and monoacylglycerols are also industrially produced from triacylglycerols and are used as food emulsifiers. Another group of lipids that are present in food comprises phospholipids and glycolipids. Together with proteins, they are major components of biological membranes and are therefore present in many food products. Amongst them, lecithin is the most relevant group in foods. Lecithin is a complex mixture of glycerol esterified with two fatty acids of various kinds and orthophosphate, which is again esterified, mostly with three molecules, choline, ethanolamine or inositol. In addition, isoprenoid compounds (steroids, carotenoids and monoterpenes), tocopherols and waxes are classified as lipids (Belitz et al. 2004).

2.1.1 Fatty acids in food material

Fatty acids are the main constituent of the most common food lipids. The major saturated fatty acids are palmitic acid (11 %), stearic acid (4 %) and myristic acid (2 %) and the major unsaturated fatty acids are oleic acid (34 %), linoleic acid (34 %) and α-linolenic acid plus γ-linolenic acid (5 %), according to estimated quantities of the world production of food oils. Certain highly unsaturated fatty acids are not found in a great variety of sources but are contained in higher quantities in specific lipid-containing

biological material. Typical sources of arachidonic acid, with 20 carbon atoms and four double bonds, are liver, lard, lipids of chicken eggs and meat. Docosahexaenoic acid (DHA) consists of 22 carbon atoms and six double bonds, and eicosapentaenoic acid (EPA) consists of 20 carbon atoms and five double bonds (Belitz et al. 2004). They are contained in larger quantities in marine algae and fish oils, which represent the major source of DHA and EPA (Ackman 2004; Berge and Barnathan 2005; Lagarde 2008).

2.1.2 Fatty acids and human health

Fatty acids have important functions in the human body. Adipose tissue, consisting mainly of triacylglycerols, serves as energy storage, an insulator and a shock absorber. However, an excessive fat and oil intake is detrimental, leading to obesity with all its negative aspects. The lipophilic vitamins A, E, D and K are transported in the body in association with fat. In particular, unsaturated fatty acids are important building blocks for biological membranes and signal compounds with hormone-like character, e.g. eicosanoids (Kritchevsky 2002; Watkins and German 2002).

It is known that palmitic, stearic and oleic acids can be synthesised by humans, but the overall fatty acid metabolism is not yet fully understood. Linoleic acid and α-linolenic acid have been seen as essential fatty acids, but this has also been controversially discussed. DHA and arachidonic acid can be synthesised from α-linolenic acid and linoleic acid respectively, but at low levels that are particularly insufficient in conditions such as infancy (Cunnane 2003).

A balanced ratio of n-6 fatty acids (linoleic acid, γ-linolenic acid, arachidonic acid) to n-3 fatty acids (α-linolenic acid, DHA, EPA) was found to be an important aspect of a healthy diet to prevent heart diseases. Socio-cultural differences for this ratio were detected. The n-6:n-3 ratio was 4:1 for the average Japanese diet, whereas it was 7:1 for the UK diet and 17:1 for the USA diet (Simopoulos 2001). Ratios from 1:1 to 6:1 have been suggested for healthy adults (Simopoulos 1999; Wijendran and Hayes 2004).

The ingestion of DHA and EPA has generally shown greatly beneficial influences, as indicated by numerous studies. Anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidaemic and vasodilatory properties have been found, resulting in beneficial health effects for patients with predispositions for coronary heart disease, hypertension and type 2 diabetes and in some cases for patients with renal disease, rheumatoid arthritis, ulcerative colitis, Crohn's disease and chronic obstructive pulmonary disease (Ruxton et al. 2004; Simopoulos 1999). Furthermore, there is strong evidence that they play an important role in brain development and function. Also, mental health seems to be influenced beneficially by DHA and EPA intake. Lower plasma levels of both fatty acids were found in children with hyperactivity, pointing to a possible correlation with the symptoms. Anti-depressive effects were found and evidence for beneficial influences in the prevention or treatment of dementia, cognitive decline and Alzheimer's disease was shown (the results of these studies were partly linked to a high fish consumption) (Ruxton et al. 2004). DHA is also important for eye health, playing a particular role in the retina, with functional, structural and protective properties (Delcourt 2007).

Intake levels for DHA and EPA have been recommended by health organisations and government agencies. For health improvements, 400–600 mg/day (Harris 2007) and 450–900 mg/day (Ruxton et al. 2004) have been suggested. Although positive health effects are predominant with respect to diets that are rich in long chain polyunsaturated fatty acids (PUFAs), some studies point out that such diets are very susceptible towards oxidation and can partly increase the oxidative stress on the body. Consequences may be lower antioxidant levels (e.g. of vitamin E), which could affect their protective functions within the body. Some studies also found higher oxidation levels in low density lipoproteins (LDLs), previously linked to atherosclerosis and inflammatory processes. An adequate supplementation with antioxidants has therefore been suggested to account for possible negative side effects (Turini et al. 2001).

2.2 Oxidation of unsaturated fatty acids

Unsaturated fatty acids, in acyl or free form, are susceptible to oxidation. The major oxidation mechanism is called autoxidation and takes place when the oxidising fatty acid is not exposed to the activity of pro-oxidative enzymes (lipoxygenases, peroxidases and catalases) or light in the presence of type II photosensitisers, which would trigger photooxidation with singlet oxygen activity (Belitz et al. 2004). The basic condition for the initiation of autoxidation is the presence of small amounts of free radicals (a "free radical" or just "radical" is defined as "an atom or group of atoms with at least one unpaired electron;…" (*http://wordnetweb.princeton.edu/perl/webwn?s=free%20radical* 2009)) or a light-activated photosensitiser of type I. Both can abstract a hydrogen atom from a methylene group situated between two double bonds or as part of an allyl group and a free radical is created on the fatty acid molecule. The greater the number of double bonds present on the whole molecule, the lower is the energy required for the abstraction, as the free radical is stabilised by electron delocalisation over a larger area. Therefore, the susceptibility towards oxidation increases with an increasing degree of unsaturation (Belitz et al. 2004).

The free radical further reacts with oxygen to create a peroxyl radical (reaction a, Figure 2.1), which can again abstract a hydrogen atom from a methylene group, leading to a hydroperoxide (reaction b, Figure 2.1). This sort of hydroperoxide will generally be called lipid hydroperoxide in the present study. The greater the number of double bonds, the greater is the variety of lipid hydroperoxides that can result from one hydrogen atom abstraction. The lipid hydroperoxide can resolve into an alkoxyl radical and a hydroxyl radical in a monomolecular reaction (reaction c, Figure 2.1) or, in the presence of a metal ion (+II), an alkoxyl radical and a metal ion (+III) can be generated (reaction d, Figure 2.1). The lipid hydroperoxide can also react with a metal ion (+III), creating a peroxyl radical and a metal ion (+II) (reaction e, Figure 2.1). The most relevant pro-oxidative metal ions derive from iron, copper and cobalt. In further decomposition reactions, many volatile secondary oxidation products (mostly aldehydes, ketones, alcohols and hydrocarbons (Frankel 1998b)), which are already perceivable at low concentrations, are formed. This can cause quality deterioration in food products with even low oil or fat content. The homolytic β-scission of the alkoxyl radicals is one of

the major reactions responsible for off-flavour production. In Figure 2.2, this reaction is illustrated with the example of linoleic acid autoxidation, resulting in the aldehyde hexanal. An intermediate product is the hydroperoxide at carbon atom C_{13} (in the autoxidation of linoleic acid, hydroperoxides can also form at carbon atom C_9). Belitz et al. (2004) indicated that hexanal is the major volatile oxidation product of linoleic acid. Out of 19 secondary oxidation products, the highest concentration was found for hexanal (5100 µg/g linoleic acid after specified oxygen uptake at 20 ˚C). The second highest concentration was below 1000 µg/g and the concentration was below 500 µg/g for all other compounds.

$R_1\cdot + O_2 \rightarrow R_1OO\cdot$ (reaction a)

$R_1OO\cdot + R_2H \rightarrow R_1OOH + R_2\cdot$ (reaction b)

$R_1OOH \rightarrow R_1O\cdot + \cdot OH$ (reaction c)

$R_1OOH + Me^{n+} \rightarrow R_1O\cdot + OH^- + Me^{(n+1)+}$ (reaction d)

$R_1OOH + Me^{(n+1)+} \rightarrow R_1OO\cdot + H^+ + Me^{n+}$ (reaction e)

$R_1O\cdot + R_2H \rightarrow R_1OH + R_2\cdot$ (reaction f)

Figure 2.1: Principal reactions in the autoxidation of unsaturated fatty acids (Belitz et al. 2004)

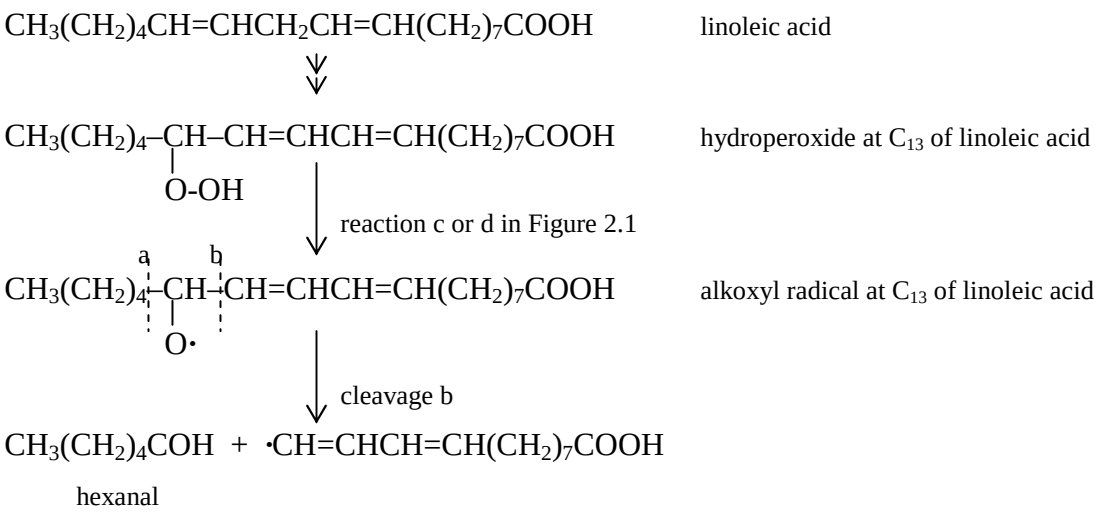


Figure 2.2: Hexanal formation in the autoxidation of linoleic acid by homolytic β-scission (Frankel 1998b; Gordon 2001)

Iron appears to be the most relevant pro-oxidative metal in foods as it is reactive and is present in relatively high concentrations. Ferrous iron is more pro-oxidative than ferric iron as it is ten times more reactive (Mei et al. 1998a) and fuels the production of alkoxyl radicals, further leading to off-flavour products (reaction d, Figure 2.1; Figure 2.2). In Decker et al. (2002), ferrous iron is stated to react 10^5 times faster than ferric iron with hydrogen peroxide. Although Cu^+ reacts 50 times faster than ferrous iron with hydrogen peroxide, it is not seen as the major pro-oxidative metal in food because of low concentration levels.

2.3 Measuring lipid oxidation

2.3.1 Oxygen consumption

In the lipid oxidation process, oxygen reacts with lipids. Therefore, the uptake of oxygen can be measured as a parameter that indicates lipid oxidation. The technique to measure dissolved oxygen comprises an oxygen electrode with a Clark-type membrane, where a current that is proportional to the concentration of dissolved oxygen is created (Clark et al. 1953). The electrode is connected to an oxygen meter, where the current is converted into oxygen concentration, shown on a display. To measure the oxygen consumption, the sample is located in a closed container. The technique has also been used for emulsion systems (Chen et al. 1998; Rival et al. 2001a; Taylor and Richardson 1980a).

2.3.2 Lipid hydroperoxides

The peroxide value (PV) method is widely used to measure the hydroperoxide concentration in bulk oils. The principle is that iodide acts as a reducing agent towards the oxidised sample. In turn, iodide is oxidised to iodine and is determined titrimetrically with sodium thiosulfate. Thiosulfate reduces iodine (yellow in solution) again to iodide (colourless). Starch can be used as an indicator towards the endpoint of

the iodine reduction. The PV is expressed as milliequivalents (meq) of iodine per kilogram of lipid or mmoles of hydroperoxide per kilogram of lipid (PV in meq/kg = 2 PV in mmol/kg). For dairy products, another more sensitive method without the time-consuming titration step is often used to determine lipid hydroperoxides. Its basic principle is the oxidation of ferrous iron to ferric iron when in contact with lipid hydroperoxides. Ferric iron reacts with thiocyanate (reactant) to form a coloured complex that can be determined spectrophotometrically. In the assay, the lipids are first extracted from the sample with organic solvents before this reaction takes place (Frankel 1998c; Nuchi et al. 2001; Shantha and Decker 1994).

2.3.3 Conjugated diene hydroperoxides

With this method, conjugated diene hydroperoxides, generated during the course of fatty acid oxidation, can be measured. The samples (oils, fats or emulsions) are diluted in organic solvents. The conjugated diene hydroperoxides have a strong absorption maximum at 234 nm and are determined directly at this wavelength. Thus, the method is straightforward, but the complication is that it can deliver reliable results only in the early stage of lipid oxidation. Secondary oxidation products and polymers generated from the decomposition of lipid hydroperoxides at later stages of oxidation also absorb at 234 nm. Therefore, it can become unclear to what extent the measured concentration is due to the presence of conjugated diene hydroperoxides or secondary oxidation products. The method has also been described as being unsuitable for samples containing free PUFAs (Frankel 1998c).

2.3.4 2-Thiobarbituric acid (TBA) value

TBA reacts with many secondary oxidation products of unsaturated lipids (called TBA-reactive substances or TBARS), forming compounds that absorb at 532–535 nm. The reaction is not specific to a certain oxidation product, although the colour development (pink) was originally thought to be due to the reaction of only malonaldehyde with TBA (in quantitative analyses, 1,1,3,3-tetraethoxypropane is used as a standard compound as

it decomposes to malonaldehyde in the assay). Various factors influence the generation of the coloured compounds and they vary depending partly on the version of the assay: pH (low pH is a reaction condition), temperature (the assay involves heating to 100 ˚C), the presence of metal ions (ferric iron promotes the reaction) and antioxidants (butylhydroxytoluol and EDTA suppress the reaction). Therefore, a negative aspect is also that the reaction conditions are suitable for altering the original lipid oxidation status of the sample. Furthermore, other materials present in complex food systems, such as amino acids and carbohydrates, may affect the result as they can contribute to the colour reaction (Frankel 1998c; Hegenauer et al. 1979a; McDonald and Hultin 1987).

2.3.5 Carbonyl compounds

Carbonyls (ketones and aldehydes) can be determined by their reaction with 2,4-dinitrophenylhydrazine as they form coloured hydrazones that absorb at 430–460 nm. In the classical method, trichloroacetic acid is used as a catalyst but this is disadvantageous in lipid oxidation studies as it promotes the decomposition of hydroperoxides. To eliminate the problem, the hydroperoxides can be reduced by stannous chloride or the hydrazones can be derivatised and analysed after their removal by vacuum distillation (Frankel 1998c). In food products with a naturally high content of carbonyls, high values might be obtained for the control sample, independent of the status of lipid oxidation, reducing the sensitivity of the method. The method has not been widely used for measuring lipid oxidation.

Another method, the p-anisidine test, can be used to measure the content of saturated and unsaturated carbonyls in triacylglycerols. An aliquot of the fat or oil is dissolved in isooctane and the carbonyls react with the reagent p-anisidine in the acetic acidic milieu, forming a coloured adduct. The absorbance at 350 nm, multiplied by a factor and divided by the weight (grams) of the sample, is defined as the p-anisidine value (Frankel 1998c; IUPAC 1987).

2.3.6 Gas chromatographic (GC) analysis

Volatile compounds of a sample can be analysed using gas chromatography, which is therefore useful for determining lipid oxidation products such as aldehydes and hydrocarbons. The sample is added into a container with a headspace and the container is sealed. Volatile compounds present in the sample are transferred into the headspace by heating and/or agitation for a set time. In the static headspace technique, an aliquot of the headspace is taken (with or without previous purging of the sample with an inert gas) and is injected on to the GC column. In the dynamic headspace (or "purge and trap") technique, the sample is first purged with an inert gas. Volatiles evaporate with the gas stream and are then adsorbed or "trapped" and thus concentrated on a short column. From there, they are transferred on to the GC column with the carrier gas. In the solid phase microextraction (SPME) technique, the simplicity of the static headspace method is combined with the greater sensitivity of the dynamic headspace technique. A fibre that is capable of adsorbing volatile compounds (e.g. polydimethylsiloxane) is exposed to the gas in the headspace of the measurement container. Under set conditions, the volatiles adsorb to the fibre material and concentrate. The fibre is then inserted directly into the injection port of the GC column. The volatiles pass through the GC column (most commonly capillary columns) with the inert carrier gas at various velocities, as they are differentially retained by the column material, and thus are separated. At the end of the GC column, they are qualified (e.g. by using a standard of the targeted compound) and quantified with a detector (e.g. a flame ionisation detector (FID) or a mass spectrometric (MS) detector) (Frankel 1998c). Examples where the SPME technique has been used to determine secondary lipid oxidation products are the studies of Beltran et al. (2005) (Tween-20-stabilised sunflower O/W emulsions), Lee et al. (2003) (DHA-enriched fish oil) and Jonsdottir et al. (2005) (microencapsulated fish oil).

2.4 Milk proteins

2.4.1 Caseins

Pasteurised skimmed bovine milk is acidified with hydrochloric acid, lactic acid (particularly in New Zealand by fermentation with lactic acid bacteria (Fox and McSweeney 1998a)) or sulphuric acid. The acid is added gradually while stirring at 30 or 45 ˚C. Caseins precipitate at pH 4.6 (concomitantly, the casein micelles are disrupted and calcium phosphate is set free as a solute (Dalgleish 2004)). The casein precipitate is then separated by centrifugation or vibrating sieve, washed with potable water to remove lactose and salts, dehumidified by pressing and dried with belt or drum driers to give the so-called acid casein. The casein fraction represents about 80 % of the total milk protein (about 3.5 %). Sodium caseinate (NaCas) is produced by dissolving acid casein in sodium hydroxide solution and subsequent spray drying (by analogy, calcium and potassium caseinates are obtained by the use of calcium and potassium hydroxides). NaCas forms colloidal solutions in water and has a bland flavour if the pH value is maintained at around pH 7 during production (Tamime et al. 2007).

Bovine casein consists of four proteins, with a molecular weight between 20 and 24 kDa (Patel 2007a), that are separately generated by gene expression: α_{s1}-casein (37 %), α_{s2}-casein (10 %), β-casein (35 %) and κ-casein (12 %). β-Casein is the major substrate of plasmin, a native proteinase in milk. When β-casein is hydrolysed by plasmin, γ-caseins and proteose-peptones are formed (Fox and McSweeney 1998a).

Caseins have a low level of cysteine/cystine compared with whey proteins and no free sulfhydryl groups. Only α_{s2}-casein and κ-casein contain cysteine (two per molecule), which is present in the disulfide form of cystine under normal non-reducing conditions. In α_{s2}-casein, two cysteine molecules are linked by disulfide bridges and κ-casein forms oligomers from two to ten molecules interlinked by disulfide bridges. Caseins have a high phosphorus content because of the esterification of monophosphate with the hydroxyl group of serine and, to a minor degree, threonine. The possible numbers of such phosphorylated residues decrease as follows: α_{s2}-casein (10–13) > α_{s1}-casein (8–9)

> β-casein (4–5) > κ-casein (1–3). The phosphate groups contribute to the hydrophilicity of the caseins and can interact with cations because of their negative charge. κ-Casein is glycosylated. Some κ-casein molecules are linked to tri- or tetrasaccharides of N-acetylneuraminic acid, galactose and N-acetylgalactosamine via a threonine residue. The oligosaccharide residues contribute to the hydrophilicity of κ-casein (Fox and McSweeney 1998a). β-Casein is also glycosylated (with galactose, galactosamine and N-acetylneuraminic acid (Fox 2003)).

Caseins are widely used in food processing. They exhibit excellent emulsifying and foam-stabilising properties and can be used to modify the texture of foods (Chandan 1997). A good overview of the wide variety of food applications is given by Fox and McSweeney (1998a). The good emulsifying characteristics of caseins are due to hydrophobic and hydrophilic moieties, as opposed to an even distribution of amino acid residues with various polarities. Caseins are very stable to thermal denaturation (sodium caseinate is stable at 140 ˚C, pH 6.5–7.0, for more than 1 h before visible physical effects occur (Fox and McSweeney 1998b)). Little secondary and tertiary protein structure, as a result of a high proline content, is likely to account for the thermal stability (Fox and McSweeney 1998a).

2.4.2 Whey proteins

In the production of acid casein, acid whey is obtained after the removal of the precipitated caseins. Lactose and salts are removed from the whey to a large degree by ion-exchange chromatography and ultrafiltration. Thereafter, the solution is spray-dried to yield whey protein isolate (WPI) with a protein content of about 95 %. Whey protein represents about 20 % of the total milk protein (Fox and McSweeney 1998a).

Whey protein consists of many proteins. The major proteins are β-lactoglobulin (50 %), α-lactalbumin (20 %), bovine serum albumin (10 %) and immunoglobulins (IgG_1, IgG_2, IgA, IgM) (10 %). The remaining 10 % consists mainly of non-protein nitrogen compounds and small amounts of a large variety of proteins (lactoferrin, serotransferrin and about 60 enzymes) (Fox 2003; Fox and McSweeney 1998a).

β-Lactoglobulin (molecular weight 18.3 kDa) contains two disulfide bonds and one free sulfhydryl group, which is buried in the interior of the protein. It can form polymers, depending on the environment. α-Lactalbumin (molecular weight 14.2 kDa) contains four disulfide bonds and no free sulfhydryl group and exists as a monomer. One disulfide bond is less stable than the others and might be disrupted more easily, depending on the conditions. A possible disruption leads to a conformational change, with greater exposure of hydrophobic regions. This can increase the surface activity and might influence the antioxidative properties of the protein. The α-lactalbumin in milk was found to be glycosylated to some degree. Bovine serum albumin (molecular weight 66.4 kDa) contains 17 disulfide bonds and one free sulfhydryl group and exists as a monomer. Immunoglobulins (molecular weight 150–900 kDa) exist as monomers or as polymers, in which four molecules are linked by four disulfide bonds (Fox 2003; Fox and McSweeney 1998a).

In comparison with caseins, whey proteins are very susceptible to heat denaturation, which begins at temperatures ≥ 70 ˚C (Smith and Campbell 2007). The whey proteins in milk are completely denatured when heated at 90 ˚C for 10 min (Fox and McSweeney 1998a). This sensitivity can be explained by a greater presence of secondary and tertiary structure in comparison with caseins, partially caused by a lower proline content. The structures are largely determined by globular conformation (Patel 2007a).

In foods, WPI can be used as a stabiliser and fat mimetic (yoghurt, bakery mixes, dietetic foods, infant foods, confections), as a substitute for egg white to reduce costs, and as an ingredient or in pure form for high protein dietary formulations. It exhibits water-binding and gel-forming properties and has a bland flavour. As native whey proteins do not precipitate and give stable colloidal solutions over a wide pH range, they can therefore also be used to protein-fortify acidic fruit or soft drinks (Chandan 1997). They also have good emulsifying properties (Ye and Singh 2000). A comprehensive overview of their applications in food is given by Fox and McSweeney (1998a).

Amongst the whey proteins, β-lactoglobulin and α-lactalbumin exhibit the best emulsifying properties. They form stable oil-in-water emulsions that are only slightly less stable than casein-stabilised emulsions (Hunt and Dalgleish 1994; Ye and Singh

2000). It has also been shown that the emulsifying properties of whey proteins are improved by partial hydrolysis. This may be partly due to the formation of larger surface-active peptides (Agboola et al. 1998; Dalgleish 2004; Huang et al. 1996).

2.5 Oil-in-water (O/W) emulsions

O/W emulsions are common on the food product market, for example as milk, mayonnaise, creamers, ice cream mixes, gravies, whippable toppings, salad dressings or cream liqueurs. Ideal O/W emulsions consist of a hydrophilic continuous phase surrounding a hydrophobic oil phase, which exists as dispersed and discrete spherical droplets of various sizes. The droplets are kinetically stabilised and prevented from coalescing by the presence of a surfactant/emulsifier, which reduces the interfacial tension between the two phases by forming an intermediate layer, the droplet interface. The lower the interfacial tension, the lower is the tendency of the two phases to form the smallest possible contact surface. Surfactants are molecules of an amphiphilic nature, with a non-polar part and a polar part. The non-polar part reaches into the hydrophobic oil phase whereas the polar part moves into the hydrophilic continuous phase (Dalgleish 2004). The ability of a surfactant to reduce the interfacial tension is given by the Gibbs' equation ((Equation 1) (Fox and McSweeney 1998c)). In protein-stabilised emulsions, Γ represents the protein load (mg/m^2) on the interface (Dalgleish et al. 1995).

$$d\gamma = -R*T*\Gamma*d\ln a \qquad \text{(Equation 1)}$$

where γ is the interfacial tension, Γ is the excess concentration of the surfactant at the interface over the concentration in the continuous phase, a is the activity of the surfactant in the continuous phase, R is the universal gas constant and T is the absolute temperature. Higher values of Γ and a as well as higher temperatures result in a decrease in the interfacial tension γ.

In food O/W emulsions, the oil phase generally consists mainly of triacylglycerols and the continuous phase consists of water, surfactants and other solutes. To give stable

O/W emulsions, surfactants are added in excess and are therefore also present in the continuous phase. Often solids and gas bubbles are also contained in the system. Proteins, in particular milk proteins, are widely used as emulsifiers in food emulsions. Further details about applications and properties of specific milk proteins are given in Section 2.4.

Colloid mills or high pressure homogenisers are used for the production of food O/W emulsions. Colloid mills consist of a rotor and a stator, with a narrow gap between the two. When a mixture of oil, aqueous solution and emulsifier passes through that gap during the rotation process, shear forces tear the oil phase into small droplets and surfactant molecules instantly adsorb to the newly created surfaces. Droplets of about 2 µm can be created with colloid mills. Smaller droplets are obtained with high pressure homogenisers, in which the shearing forces are generated by pressure differences. The components are usually pre-emulsified, e.g. with a high speed blender of the rotor–stator type, before they are passed through the homogeniser. The main factors that influence the droplet size are homogenisation pressure, design of the homogeniser, number of passes, type and amount of surfactants and viscosity. At low emulsifier concentrations, the formation of small droplet sizes is limited. The droplets coalesce and form larger droplets, if the total droplet surface is too large to be covered (Dalgleish 2004). Monomodal, bimodal or multimodal droplet size distributions are obtained by emulsification; they are assumed to be single (monomodal) or overlaying (bimodal and multimodal) log–normal distributions (van Duynhoven et al. 2002). In a monomodal droplet size distribution, the mean represents the average droplet size. In general, monomodal distributions are targeted as such emulsions are more controllable.

The techniques that are widely used to measure the droplet size distribution and the average droplet size are based on light scattering, such as integrated light scattering (ILS) and dynamic light scattering (DLS). In the measurement process, the emulsion is highly diluted with water. Light passes through the sample from a light source located at one side of the sample and is scattered in a characteristic way that depends on the droplet size distribution. In ILS, the intensity of the scattered light is measured from various angles. In DLS, the light scattered to a fixed angle of 90 ˚ within a short period of time is measured. In each case, the data obtained are processed by software that

calculates the most likely size distribution and average droplet size by generating a regression function. Droplet size measurement is useful for controlling the effectiveness and reproducibility of the emulsification process and allows the emulsion stability to be monitored over time (Dalgleish 2004). The droplet sizes in food emulsions generally range between 0.5 and 2.5 µm (Dalgleish 1997) or between 0.1 and 100 µm (Dickinson 1992; Dickinson and Stainsby 1982; McClements and Decker 2000).

When proteins adsorb at the oil–water interface, their conformation changes (e.g. as found for β-lactoglobulin and α-lactalbumin). Such structural changes cannot be investigated for caseins because of a naturally simpler conformation and limitations in the measurement techniques. The denaturation can be reversible to some degree, as found for α-lactalbumin, or irreversible, as observed for β-lactoglobulin. In the adsorption process, the protein molecules unfold and spread out to maximise the contact of lipophilic moieties with the oil phase, but structural elements such as the polypeptide backbone also restrain close adsorption. However, even a few contact points can be sufficient to stabilise the droplets towards coalescence, as for example in the case of the egg protein phosvitin, which is a good emulsifier although it is relatively hydrophilic. Other proteins, such as lysozyme and also gelatin, are poor emulsifiers because their structure constrains effective adsorption (Dalgleish 2004).

The emulsifying properties of proteins can also change depending on the conditions or the treatment. For example, pH reduction or chelator treatment can cause removal of naturally bound Ca^{2+} from α-lactalbumin. This improves its emulsifying properties because of greater flexibility. Also, the surface activity of β-lactoglobulin has been reported to be pH dependent. Protein interfaces are less closely packed than interfaces formed by surfactants of smaller size, such as mono- and diglycerides or phospholipids. They can reduce the interfacial tension more effectively, but proteins often better stabilise the droplets towards disruptive forces by forming more cohesive and viscous interfaces. It has been reported that the interfaces formed by caseins are about 10 nm thick. Whey proteins form thinner layers of about 2 nm. Small molecule surfactants are able to replace proteins from the interface after emulsification (e.g. sodium dodecyl sulfate (SDS)). Some studies have looked at the proportion in which different milk proteins adsorb to droplet interfaces when mixed protein solutions are used for

emulsification. In most cases, and when a mix of whey protein and NaCas was used, a balanced adsorption of all proteins according to their concentration was found (Dalgleish 2004; Dalgleish et al. 1995).

In protein-based food emulsions, droplets are stabilised by charge repulsion and steric repulsion. The droplet surface is often negatively charged as the pH may be above the isoelectric point (pI) of the proteins under weakly acidic or neutral pH conditions (pH 5–7). The droplets are thereby stabilised by charge repulsion. In steric repulsion, structural features of the proteins prevent droplets from close approach and aggregation, e.g. hydrophilic residues in β-casein with a high lysine and arginine content, which protrude into the continuous phase (Dalgleish 2004; Dalgleish et al. 1995).

Proteins, adsorbed at the interface, can polymerise, as for example observed for β-lactoglobulin and α-lactalbumin, by disulfide formation. In whey-protein-based emulsions, droplets can take part in gel formation by binding to proteins in the continuous phase. Interactions between interfacial and continuous phase proteins can also result in mono- or multi-molecular protein layers. Multi-layers are reported to form more easily at whey protein interfaces than at casein interfaces as the molecules do not reach as far into the continuous phase and therefore the steric repulsion against approaching protein molecules is weaker. However, multi-layer formation also seems to occur in NaCas-based emulsions. The stability and the properties of multi-layers, in comparison with mono-layers, and the conditions for their formation have not yet been fully elucidated. By the interaction of interfacial proteins present on different droplets, e.g. disulfide linkage between whey proteins, the emulsion can physically destabilise as droplets aggregate. In casein-based emulsions, the addition of cations such as Ca^{2+} can reduce the charge repulsion between droplets, and organic solvents such as ethanol can affect the steric stabilisation of the droplets. Both can result in aggregate formation (Dalgleish 2004). Calcium has also caused aggregation in whey protein emulsions (Chanamai and McClements 2002). In contrast, when calcium is present in the form of calcium caseinate, casein clusters are formed in aqueous solution and, during emulsification, these clusters can adsorb at the interface, leading to a higher protein load and emulsion stability (Srinivasan et al. 1999). In general, the temperature (e.g. flocculation occurred in whey-protein-stabilised emulsions at neutral pH at 70 ˚C), the

pH value and the ionic strength also influence the stability of protein-based emulsions (Chanamai and McClements 2002; Dalgleish 2004).

2.6 Lipid oxidation in O/W emulsions

In the O/W emulsion system, unsaturated fatty acids are present as part of tri-, di- and monoacylglycerols, and as free fatty acids. They are mostly located within the oil droplets and a few molecules can also be present in the interface (McClements and Decker 2000). Lipid hydroperoxides are a vital element in the free-radical-mediated propagation of lipid oxidation. They are more polar than the unoxidised lipid molecules and therefore a higher concentration can be found at the interface than in the more hydrophobic core of the oil droplet (McClements and Decker 2000; Nuchi et al. 2002). The interface has also been suggested to be the most relevant region for lipid oxidation to take place and surface-active antioxidants would be particularly effective oxidation inhibitors (McClements and Decker 2000). In general, the autoxidation mechanisms in O/W emulsions are thought to be the same as in bulk oils (Genot et al. 2003). As described earlier, metal ions and free radicals play an important role in the autoxidation process of lipids. The limitation of these factors is also most relevant for the oxidative stability of O/W emulsions (Decker et al. 2002).

2.6.1 Role of pro-oxidative metal ions, chelators, the pH and the surface charge

A basic requirement for low oxidative sensitivity is that the materials used for emulsification, in particular the oil phase and the emulsifier, contain the lowest possible levels of hydroperoxides (as they are a source of free radicals upon decomposition) and iron and copper (as they lead to decomposition of hydroperoxides) (McClements and Decker 2000). For example, Mancuso et al. (1999a) found 4–35 µmol peroxide/g surfactant in lecithin, Brijs and Tweens.

The presence of effective chelators in the system makes metal ions unavailable for the lipid oxidation process. The addition of artificial chelators such as EDTA has a marked

antioxidative effect. EDTA can also be added legally as an antioxidant in food O/W emulsions (e.g. in the USA with a permitted dose of 75–100 ppm (FDA 2007)). Chelating properties of proteins have also been reported, as discussed later. Not only the continuous phase but also the oil phase can be a source of metal ions (water, oil, surfactants, buffer substances (Mei et al. 1998a)). Proteins naturally contain minerals and water may also be a source of pro-oxidative metal ions. Food oil can be contaminated with low levels of metals from the production process (Benjelloun et al. 1991). The location of chelators is therefore a relevant factor for an effective chelation process.

The solubility of iron in water increases with decreasing pH (Donnelly et al. 1998; Graf et al. 1984; Mancuso et al. 1999b). Thus, more lipid oxidation occurred in a Tween-20-based emulsion at pH 3 than at pH 7, when oxidation was promoted by Fe^{3+} and ascorbate, creating Fe^{2+}. Metal repulsion would have been similar at both pH conditions because of a non-ionic droplet surface (Donnelly et al. 1998). In another study, in which Tween 20 was also used as the emulsifier and no iron was added, lipid oxidation was greater at pH 7 than at pH 3. An increased iron concentration on the droplet surface was found at pH 7, which indicated that more iron had precipitated on the droplet surface at the higher pH (Mancuso et al. 1999b). An explanation for these seemingly contradictory results is offered by Mei et al. (1998a); the solubility of ferric iron increases from pH 7 to pH 3, whereas the solubility of ferrous iron increases from pH 3 to pH 7. In contrast, ferrous iron is apparently still 10^{17} (pH 7) and 10^{13} (pH 3) times more water soluble than ferric iron (Decker et al. 2002). The concentration of the respective redox form therefore appears to be a relevant factor for how much iron can precipitate on the droplets at a certain pH, affecting the oxidative stability of the emulsion.

Iron on the droplet interface can also be removed by continuous phase chelators. This was shown in a study of Mei et al. (1998a), as the addition of EDTA or phytate to an SDS-stabilised hexadecane O/W emulsion with ferrous iron lowered the zeta-potential on the droplets. In the same study, the lipid oxidation rate in SDS-stabilised salmon O/W emulsions with ferrous iron decreased when EDTA or phytate was added. An example of a real food product is mayonnaise, stabilised with the protein phosvitin from egg yolk. Phosvitin has a high iron content and lipid oxidation may be accelerated by

the release of iron at the droplet interface under acidic conditions. EDTA can remove iron from phosvitin at the interface and thus inhibit lipid oxidation (Jacobsen et al. 2001; Thomsen et al. 2000). In a study of Tong et al. (2000b), the addition of ferrous iron to a hexadecane O/W emulsion (pH 7) stabilised with bovine serum albumin (0.1 %) led to an increase in the zeta-potential at the droplet surface, indicating iron association. When increasing levels of a high molecular weight fraction of whey (molecular weight ≥ 3500) were incorporated, the zeta-potential decreased. At the highest whey protein level (14.0 µg/ml), the zeta-potential had decreased more than when 20 µmol EDTA/l was added and the level was below the initial level before iron addition. It was concluded that both components were able to remove iron from the interface by chelation. In the same study, the lipid oxidation rate in a Tween-20-stabilised salmon O/W emulsion (pH 7) to which the same whey fraction had been added also decreased. This pointed to the possibility that the removal of iron from the interface into the continuous phase may have contributed to the improved oxidative stability. Other milk proteins also exhibit metal-binding abilities and can thus enhance the oxidative stability of emulsions. This is discussed in more detail in a later section.

The surface charge of the droplets also plays an important role in the lipid oxidation process. Pro-oxidative cations can be electrostatically repelled into the continuous phase by a positive surface charge, away from the interface where lipid hydroperoxides are prone to metal-induced decomposition. In contrast, a negatively charged droplet surface can promote lipid oxidation by attracting pro-oxidative cations (Genot et al. 2003; McClements and Decker 2000). These effects were shown when emulsions stabilised with an anionic surfactant (SDS) oxidised more than emulsions stabilised with non-ionic (Tween 20 or Brij 35) or cationic (dodecyltrimethylammonium bromide) surfactants, when iron (Mei et al. 1998b) or no iron (Mancuso et al. 1999b) was added. In a study of Mei et al. (1998b), sodium chloride (1.0 %) reduced iron-promoted lipid oxidation in an SDS-stabilised emulsion with a net negative surface charge by 20 %, but had no effect in emulsions with non-ionic or cationic surfactants. The authors pointed to the possibility that other cations, which are not pro-oxidative, such as sodium ions in this particular case, may compete with iron ions for negatively charged binding sites on the droplet surface. This can lead to lower iron adsorption on the droplets and thus decreased lipid oxidation.

It was also found that the access of antioxidants from the continuous phase to the interface can be limited, and their effectiveness reduced, if their electric charge has the same sign as the droplet surface charge. For example, partially negatively charged galloyl derivatives did not associate with SDS-stabilised droplets with a negative surface charge at pH 7.0 (Mei et al. 1999).

In general, the repelling or attracting force on the droplet surface depends on the surface charge density (McClements and Decker 2000). The pH determines the surface charge of protein molecules. In protein-based emulsions, the pH is therefore an influential factor on the oxidative stability. Proteins are more likely to repel iron and copper ions when they are positively charged at pH conditions below their isoelectric point. At the interface, metal ions from the continuous phase would be repelled, but continuous phase proteins are also less capable of binding metal ions. When a fraction of acid whey (molecular weight $\leq$ 5000) was added to a phosphatidylcholine liposome system, the inhibition of TBARS formation increased with increasing pH from pH 5 to pH 7. This might have been due to greater metal-binding capacity, keeping metal ions away from the liposomes (Colbert and Decker 1991). In a study of Donnelly et al. (1998), WPI-stabilised menhaden O/W emulsions with iron had more oxidative stability at pH 3.0 than at pH 7.0. This observation was attributed to the greater repulsion of iron from the net positively charged protein interface at low pH. Similar results were obtained by Faraji et al. (2004); less lipid hydroperoxides and propanal were produced in menhaden O/W emulsions stabilised with 0.5 % WPI at pH 3 than at pH 7. In a study by Hu et al. (2003a), various whey proteins (WPI, sweet whey, α-lactalbumin, β-lactoglobulin) were used to produce salmon O/W emulsions. Increasing pH led to decreasing droplet surface charge (indicated by a decrease in the zeta-potential). At the same time, the lipid oxidation rates increased. However, amongst the emulsions prepared with different proteins (pH 3), the order of oxidative stability and zeta-potential did not totally correspond, showing that the droplet surface charge was not the only factor that influenced the oxidative stability.

2.6.2 Primary antioxidants

Primary antioxidants (also called chain-breaking antioxidants) are able to inhibit the lipid oxidation process by scavenging free radicals. The antioxidant donates a hydrogen atom to the free-radical-bearing molecule (e.g. lipid radical, hydroxyl radical, alkoxyl radical or peroxyl radical). The free radical is thereby transferred to the antioxidant molecule, on which it is stabilised and less reactive (Genot et al. 2003; Yanishlieva-Maslarova 2001). Primary antioxidants are reducing agents.

In studies on the effectiveness of primary antioxidants in O/W emulsions and how it depended on their polarity, a tendency for antioxidants to achieve greater oxidative stability the more lipophilic they were was found. Lipophilic antioxidants can be more effective as they accumulate in the oil phase and at the droplet interface, where higher levels of lipid hydroperoxides and free radicals may be present. In contrast, hydrophilic antioxidants can be more effective in bulk oils where they are located at the oil−air interface (McClements and Decker 2000). This phenomenon is sometimes called “polar paradox”. Partly conflicting results about the relationship between the lipophilicity of antioxidants and their effectiveness in O/W emulsions have been obtained (Genot et al. 2003). To some degree, free radicals can also be found in the continuous phase, e.g. because they were generated during emulsification (Coupland et al. 1996). A study of Mei et al. (1999) pointed to the relevance of free-radical-scavenging activity in the continuous phase, as galloyl derivatives with the ability to scavenge free radicals were able to inhibit lipid oxidation in an O/W emulsion when they were not associated with the droplet interface. Thus, the overall effectiveness of free radical scavenging depends on the polarity, location and oxidisability of the primary antioxidants and the type and concentration of free radicals in the different phases. Examples of primary antioxidants are α-tocopherol, ascorbyl palmitate and carnosol (lipophilic) and Trolox, ascorbic acid, carnosic acid and rosmarinic acid (hydrophilic) (McClements and Decker 2000).

In the presence of ferric iron, some reducing agents may have a pro-oxidative effect (e.g. reducing sugars, glutathione, ascorbate or tocopherol) as they reduce ferric iron to more pro-oxidative ferrous iron (McClements and Decker 2000; Mei et al. 1998a). In a study of Mei et al. (1999), galloyl derivatives, which are also used as antioxidants,

showed strong reducing activity for ferric iron at pH 3.0. Also sulfhydryl groups, e.g. present in cysteine residues in proteins, were suspected to reduce ferric iron to ferrous iron (Pazos et al. 2006). Cysteine also promoted the free-radical-scavenging activity in herring O/W emulsions (Marcuse 1960) and linoleic acid O/W emulsions (Marcuse 1962).

Proteins, in particular milk proteins, have shown a great potential for free-radical-scavenging activity. This is discussed in more detail in a later section. In some studies, proteins also influenced the effectiveness of other antioxidants by interacting with them (Genot et al. 2003).

2.6.3 Oxygen

In the pre-blending and emulsification process, the newly formed emulsion is enriched with oxygen. Oxygen is an important reactant in lipid oxidation as it contributes to lipid hydroperoxide formation. The solubility of oxygen is higher in food oil than in water (about three times (Ke and Ackman 1973) or 4.4 times (Genot et al. 2003)). With an increase in the oil volume fraction in an O/W emulsion, the total amount of contained oxygen increased (conditions: 25 ˚C, saturation with air). In contrast, the oxygen concentration in the oil (µg O_2/g oil) decreased. The greatest decrease was found with an increase in the oil content up to 10 % (Genot et al. 2003). With increasing temperature, the solubility of oxygen decreases and may become a factor that limits the oxidation rate. At the same time, autoxidation reactions are accelerated with increasing temperature. The oxygen solubility in the continuous phase also decreases with increasing concentration of dissolved compounds. Oxygen exclusion, for example by vacuum storage or the use of a protective inert gas such as nitrogen, can inhibit lipid oxidation in real food emulsions until the product container is opened. In studies in which the ability of an emulsion to resist oxidation is investigated, the oxygen partial pressure above the emulsion should not be a limiting factor. It has also been found that the production of secondary oxidation products is influenced by the oxygen concentration. In the autoxidation of linoleic acid, 2,4-decadienal becomes a more prominent product over hexanal at lower oxygen concentrations (Genot et al. 2003).

As oxygen is consumed in an emulsion, oxygen diffuses from the air into the continuous phase and further via the droplet interface into the oil phase. Agitation accelerates this process. The physical characteristics of an emulsion, e.g. viscosity, cream layer at the air−water interface and structure of the droplet interface, may also influence oxygen diffusion and thus lipid oxidation (Genot et al. 2003).

2.6.4 The droplet interface as a physical barrier

The oxidative stability of a Brij-stabilised O/W emulsion was greater when the size of the hydrophilic part of the surfactant was larger. This suggested that the interface would play a role as a physical barrier towards pro-oxidants from the continuous phase (Silvestre et al. 2000). As proteins form relatively thick interfaces, they may be an effective physical barrier against not only oxygen but also metal ions from the continuous phase (Genot et al. 2003; McClements and Decker 2000). In a study of Donnelly et al. (1998), WPI was partially removed from the droplet interface of a WPI-based emulsion by the addition of Tween 20. Lipid oxidation increased in the iron-fortified system and it was suggested that it could be attributed to the thinner Tween 20 interface exhibiting a less protective physical barrier towards pro-oxidants from the continuous phase. In a study of Kellerby et al. (2006), NaCas was used as a stabiliser of menhaden O/W emulsions (pH 7). The interfacial casein was cross-linked by transglutaminase and could not be replaced by Tween 20. In spite of the more cohesive interface, lipid hydroperoxide and TBARS generation was not more inhibited than in the untreated emulsion. The increased rigidity of the interface did not seem to influence the migration of pro-oxidants. It was suggested that pro-oxidative metals could still have transferred across the protein layer. Kiokias et al. (2007) found greater oxidative stability of whey-protein-stabilised sunflower O/W emulsions when the protein solutions had been heated before emulsification. The authors suggested that the heated whey proteins could have formed a more effective physical barrier towards pro-oxidants. They referred to a previous study (Kiokias and Bot 2006), in which the same emulsion system was used and twice as much whey protein associated with the oil phase at elevated emulsification temperatures between 65 and 85 ˚C. Kiokias et al. (2006) found greater oxidative stability and smaller droplet sizes for NaCas-stabilised

cottonseed O/W emulsions at higher protein concentrations. They mentioned the possibility that thicker interfaces and thus greater protection from pro-oxidants could have played a role for the improved oxidative stability at higher protein concentrations. They referred to the findings of Kiokias and Bot (2006) who observed thicker protein layers at the droplet interfaces at higher protein concentrations.

2.6.5 Viscosity

The influence of viscosity on the oxidative stability of O/W emulsions has been discussed in association with the presence of polysaccharides, added to achieve textural modification. It was found that the molecular movement of small molecules was not hindered in polysaccharide networks. In contrast, xanthan gum was found to chelate metal ions and tragacanth exhibited free-radical-scavenging activity in an O/W emulsion; both activities led to improved oxidative stability (McClements and Decker 2000; Shimada et al. 1992; Shimada et al. 1994). Therefore, the oxidative stability in O/W emulsions with higher viscosity may not be primarily related to viscosity, but to the ingredients' ability to chelate metal ions, scavenge free radicals and build a protective droplet interface (McClements and Decker 2000). In a study of Jacobsen et al. (2001), the incorporation of antioxidants into a fish-oil-enriched mayonnaise changed the gel strength and the viscosity. The oxidative stability was also examined, but it was not clear if the viscosity influenced lipid oxidation. In another study, gum arabic, xanthan gum and propylene glycol alginate solutions made with lemon juice were used to produce olive O/W emulsions. These were of various viscosities. Higher viscosity was caused by a higher incorporation level of polysaccharides and resulted in greater oxidative stability (Paraskevopoulou et al. 2007). The increase in viscosity was again not disconnected from the possible antioxidative influence of the polysaccharides.

2.6.6 Oil phase concentration

The oil phase content of O/W emulsions and its influence on lipid oxidation is a factor that has not yet been extensively studied. In a study of Kiokias et al. (2006), decreasing

oil content (40, 30, 20 and 10 %) resulted in greater oxidative stability (conjugated diene hydroperoxides were determined) for sunflower O/W emulsions (pH 7) stabilised with 2 % NaCas. Sun and Gunasekaran (2009) observed less oxidative stability (lipid hydroperoxides and anisidine values were determined) at the lowest oil content in WPI-stabilised menhaden O/W emulsions (pH 6.7–7.0; oil contents: 40, 20 and 5 %; WPI contents: 2.0, 1.0 and 0.2 %), but there was no consistent link between oil content and oxidative stability. Other factors such as droplet size, surface charge, creaming stability and viscosity were also influenced by changes in the oil content. Sun and Gunasekaran (2009) referred to other studies in which safflower O/W emulsions (Sims et al. 1979) and canola O/W emulsions (Osborn and Akoh 2004) had less oxidative stability at lower oil contents. Some possible explanations for the influence of the oil content were given. The number of radicals generated per droplet may be higher at low oil contents as the concentration of oil droplets is lower, leading to more lipid oxidation (McClements and Decker 2000; Osborn and Akoh 2004). The droplet surface may be less covered with surfactant at high oil contents and thus pro-oxidants may interact more easily with the oil phase, leading to more lipid oxidation (Sun and Gunasekaran 2009).

2.6.7 Droplet size

The droplet size is another physical characteristic of O/W emulsions that influences their oxidative stability. Controversial results for the effect of the droplet size have been obtained.

Lethuaut et al. (2002) reported that bovine-serum-albumin-stabilised sunflower O/W emulsions with different droplet sizes showed the trend of more oxidative stability at larger droplet sizes (0.5, 1.9, 27 µm), by measuring the oxygen uptake and the conjugated diene formation in emulsions stored in closed containers. Hexanal was also measured but no differences with varying droplet size were found. The differences in oxidative stability were observed for the first stage of storage when oxygen was not limiting. It was assumed that the hydroperoxide decomposition and the transfer of decomposition products into the continuous phase were independent of the droplet size. Lipid hydroperoxide decomposition possibly depended on the lipid hydroperoxide

concentration. More lipid oxidation in smaller droplets was attributed to a larger droplet surface area and thus better access for pro-oxidants to the interface and the oil phase. As bovine serum albumin could probably also better interact with the droplet surface, this may have partly offset the pro-oxidative effect.

Gohtani et al. (1999) found greater oxidative stability for larger droplet sized decaglycerin-monostearate-stabilised DHA emulsions (droplet sizes: 6.4, 3.4 µm), when the peroxide development was followed. It was speculated that the effect of a larger droplet surface area in emulsions with smaller droplet size led to more oxidation.

In a study of Jacobsen et al. (2000), the oxidative stability of fish-oil-enriched mayonnaises with a larger mean droplet size was higher in the early stage of storage (20 °C). Fewer free radicals, lipid hydroperoxides and volatiles were generated. After 3–4 weeks, no difference was found and the authors concluded that, in the state of propagation, the oxidation was independent of the droplet size. Mayonnaise with small droplets showed greater gel strength. In the initiation stage of lipid oxidation, the contact of phosvitin (major protein of egg yolk serving as the emulsifier) with a larger interfacial area might have been a pro-oxidative factor. Phosvitin has a high content of iron, which is mostly bound to phosphoserine groups. Iron might have been partly released and enhanced lipid oxidation. At a later stage, similar or even faster oxidation occurred in large droplets, which was attributed to a greater oil volume in which free radical chain reactions could take place more unhindered. As more creaming occurred with the larger droplet size after advanced storage time, it was also speculated that a better exposure of the cream phase to oxygen could have led to more lipid oxidation.

In a study of Rampon et al. (2001), bovine-serum-albumin-stabilised sunflower O/W emulsions of two mean droplet sizes (d_{32}: 1.20, 0.36 µm) were produced. They were incubated at 37 and 47 ˚C. After 15 days, a stronger fluorescence (emission at 425 nm), indicating reactions between the protein and lipid oxidation products, was observed in the small droplet sized emulsion for both incubation temperatures. For lipid hydroperoxide and pentane production, no difference was found for the two droplet sizes. The stronger fluorescence was attributed to a larger interfacial area, enabling

more interaction between oxidising lipids and the amino groups (e.g. in lysyl residues) of bovine serum albumin.

Nakaya et al. (2005) reported higher lipid hydroperoxide and hexanal levels as well as lower residual oxygen concentrations in cod liver or soybean O/W emulsions stabilised with sucrose lauryl ester or decaglycerol lauryl ester at larger droplet sizes. They offered two hypotheses for the findings. Firstly, at a smaller droplet size, the concentration of oxidisable lipid per droplet was lower and the ratio of surfactant to oxidisable lipid per droplet was higher. This affected the oxidation process. Secondly, the hydrophobic tails of the surfactants would have reached further into the interior of smaller droplets because of a smaller droplet diameter and may have affected the free movement of the oxidisable triacylglycerols, thereby limiting the free radical chain reactions. This was called a "wedge effect". The decreased mobility of oxidisable lipid was shown by ^{1}H-NMR (nuclear magnetic resonance) with motional spin–spin relaxation times of protons in acyl residues. The motional frequency was shorter in small droplet sized emulsions. In larger droplets, in contrast, the autoxidation in the droplet core was not hindered by the hydrophobic tails of surfactants as they were located only near the interface.

In O/W emulsions based on methyl linoleate and decaglycerol monolaurate, small droplets also gave better oxidative stability. The authors explained the finding by the above-described "wedge effect" (Imai et al. 2008).

In a study of Let et al. (2007b), fish oil was homogenised with milk at 50 and 72 ˚C. Smaller droplets were created by homogenising at 72 ˚C than at 50 ˚C. Also, in emulsions with smaller droplet sizes, fewer lipid hydroperoxides and volatile oxidation products developed. However, the effect of greater oxidative stability could not be directly attributed to the droplet size as the conformation of some milk proteins, in particular β-lactoglobulin, changes at temperatures ≥ 65 ˚C. This creates different characteristics in adsorption and also exposes sulfhydryl groups with free-radical-scavenging activity, which were previously buried in the interior of the protein structure. The researchers attributed the greater oxidative stability to a better interaction of those groups with oxidisable lipid.

Kiokias et al. (2006) produced cotton seed O/W emulsions emulsified with NaCas at various concentrations. An increasing NaCas concentration led to decreasing droplet size (d_{32} = 3.04 µm (0.5 % NaCas), d_{32} = 2.32 µm (1 % NaCas), d_{32} = 1.40 µm (2% NaCas)) and also greater oxidative stability as fewer conjugated dienes were generated. Greater oxidative stability was attributed to a larger interfacial area in which NaCas could interact with the oxidisable lipid and deploy antioxidative effects. However, whether smaller droplet sizes actually contributed to less oxidative stability could not be ruled out, with the increasing protein concentration more than offsetting this influence.

In other studies, the variation of the droplet size of emulsions did not influence the oxidative stability (Kiokias et al. 2007; Kiokias et al. 2006; Osborn and Akoh 2004; Ponginebbi et al. 1999; Roozen et al. 1994; Shimada et al. 1996).

2.6.8 Retention of volatiles

Secondary lipid oxidation products affect the flavour quality of O/W emulsions. Once the compounds are formed in the interior of a droplet or at the interface, they still need to migrate to the continuous phase and from there pass into the air phase to cause the off-flavour sensation. Shen et al. (2007) gave an overview of interactions between flavour compounds and proteins. Flavour retention has been observed in aqueous solutions and emulsions as a result of the interactions between proteins and flavour compounds. Hydrophobic interactions, and also covalent binding, in particular with aldehydes, between proteins and aldehydes, ketones, esters and lactones were found. In one study, the binding of butanal and hexanal to whey protein increased markedly with an increase in the pH from 6 to 9 (Weel et al. 2003). An increase in the pH in protein-based emulsions may therefore increase the lipid oxidation rate because of an anionic droplet surface that attracts pro-oxidative metal ions, but a stronger retention of secondary oxidation products may partly reduce the negative influence on the flavour quality.

2.7 Antioxidative influence of milk proteins in O/W emulsions and similar systems

2.7.1 General findings

Taylor and Richardson (1980b) found that the addition of skim milk to a methyl linoleate emulsion improved its oxidative stability. To determine the contribution of caseins and whey proteins to the oxidative stability, solutions of single proteins of concentration equal to that in skim milk were produced and added to the linoleate emulsion (pH 6.7). The proteins showed decreasing antioxidative behaviour in the order β-casein (2.40) > κ-casein (2.20) > α_s-casein (1.81) > α-lactalbumin (1.20) > β-lactoglobulin (1.11) (the numbers indicate the relative effectiveness). Therefore, the caseins exhibited greater antioxidative potential than the whey proteins.

In a study of Diaz et al. (2003), Brij-35-stabilised corn O/W emulsions (pH 3 and pH 7) were fortified with caseinophosphopeptides (CPPs) from a tryptic digest of casein, which resulted in reduced lipid hydroperoxide and hexanal generation at both pH conditions. In phosphatidylcholine liposome systems (pH 7), the addition of CPPs and casein hydrolysates reduced the production of TBARS (Diaz and Decker 2004).

Various whey proteins or protein fractions showed antioxidative activity (inhibition of lipid hydroperoxide and propanal production) when they were used to stabilise O/W emulsions (pH 3) (Hu et al. 2003a). The oxidative stability decreased in the order β-lactoglobulin ≥ sweet whey > α-lactalbumin ≥ WPI.

In corn O/W emulsions (pH 3), stabilised by NaCas, WPI or soy protein isolate, the oxidative stability (measured by lipid hydroperoxides and hexanal) decreased in the following order: NaCas > WPI > soy protein isolate. Emulsions of two protein concentrations (0.5 and 1.5 %) were prepared. A similar lipid oxidation rate was found at both protein levels, with partly even higher values at the higher protein concentration. In NaCas emulsions, the hexanal lag time was twice as long at the 1.5 % protein level as at the 0.5 % protein level (Hu et al. 2003b).

In a study of Djordjevic et al. (2004), NaCas gave better protection than WPI towards lipid hydroperoxide generation, when the proteins were used to stabilise algal O/W emulsions (pH 3). The generation of propanal was not significantly different in either emulsion type.

Allen and Wrieden (1982) produced triolein or sunflower O/W emulsions (pH 6.8) stabilised with lysophosphatidylcholine. When casein or whey protein was added to the emulsions, greater oxidative stability was achieved with casein, measured by oxygen consumption and the generation of TBARS. α-Lactalbumin also showed some antioxidative activity when added to the emulsion.

In a study of Kiokias et al. (2006), cottonseed O/W emulsions (pH 7) were prepared with 1 % whey protein concentrate (WPC), NaCas or Tween 20. Conjugated diene production decreased in the order Tween 20 (176 % increase after 10 days) > NaCas (53 % increase after 10 days) > WPC (29 % increase after 10 days). WPC gave the greatest oxidative stability. Furthermore, NaCas was used to emulsify cottonseed O/W emulsions at levels of 0.5, 1 and 2 %. Conjugated diene production decreased in the order 0.5 % (99 % increase after 10 days) > 1 % (53 % increase after 10 days) > 2 % (25 % increase after 10 days). An increase in the NaCas concentration clearly increased the oxidative stability.

Casein, β-lactoglobulin or lactoferrin was added to a system with multilamellar vesicles consisting of dipalmitoylphosphatidylcholine and arachidonic acid. Ferrous iron and ascorbate were also added to promote oxidation. Casein showed the greatest inhibitive effect towards TBARS production. The induction periods decreased as follows: casein (20 min) > lactoferrin (10 min) > β-lactoglobulin (7 min) > control without protein (5 min) (Cervato et al. 1999).

In a study of Sun and Gunasekaran (2009), menhaden O/W emulsions (pH 6.7–7.0) were prepared with WPI at protein concentrations of 0.2, 1.0 and 2.0 %. More lipid hydroperoxides and secondary lipid oxidation products developed at low WPI concentrations.

2.7.2 Influence of continuous phase protein on oxidative stability

Faraji et al. (2004) found that the lipid hydroperoxide production decreased in a WPI-stabilised menhaden O/W emulsion (pH 7) when the protein concentration was increased. The WPI levels were 0.25, 0.5, 1.0 and 1.5 %. More lipid hydroperoxides were generated in these emulsions when the continuous phase was substituted with buffer solution containing no protein. The measured continuous phase protein concentration was higher with increasing initial protein concentration, used to prepare the emulsions. The order of oxidative stability remained the same as in the original emulsions without continuous phase replacement. In contrast, a menhaden O/W emulsion stabilised with 1 % WPI at pH 3 showed slightly lower lipid hydroperoxide and propanal levels when the continuous phase was replaced with protein-free buffer solution. Therefore, the presence of WPI in the continuous phase only appeared to contribute to oxidative stability at neutral pH. Firstly, the researchers suggested that the removal of continuous phase protein was in particular pro-oxidative at neutral pH above the isoelectric point as the continuous phase proteins had a net negative surface charge and might have been able to chelate metal ions from the interface or the oil phase. Secondly, it was suggested that low molecular weight compounds of WPI, which were active only at neutral pH but not at pH 3, might have been present in the continuous phase. The removal of continuous phase protein thus led to different effects at the two pH conditions. In the same study, the continuous phase of a WPI-stabilised menhaden O/W emulsion (pH 7) was replaced with solutions of NaCas, soy protein isolate or WPI. The oxidative stability (measured by lipid hydroperoxides and propanal) decreased in the order: soy protein isolate > NaCas > WPI.

Elias et al. (2005) produced Brij-35-stabilised menhaden O/W emulsions (pH 7) and replaced the continuous phase with β-lactoglobulin solution at levels of 250 or 750 µg/ml emulsion or buffer solution. Lipid hydroperoxide and TBARS generation was effectively reduced with increasing continuous phase levels of β-lactoglobulin. In a subsequent study by Elias et al. (2007), the same emulsion system was used. The continuous phase was again substituted with β-lactoglobulin solution, with final protein levels of 0, 50 and 500 µg/g oil in the emulsion. The production of lipid hydroperoxides and TBARS decreased with increasing level of continuous phase protein.

Buffer solution or a high molecular weight fraction of acid whey (molecular weight ≥ 3500) was added to a Tween-20-stabilised salmon O/W emulsion (pH 7) at various levels (700, 1400, 4900 and 9800 µg/ml emulsion). Increasing continuous phase protein levels led to a decrease in lipid hydroperoxide and TBARS generation (Tong et al. 2000b). In a similar study of Tong et al. (2000a), when the same emulsion system was used, dilution with various acid whey fractions also lowered lipid hydroperoxide and TBARS generation. The oxidative stability decreased in the following order: addition of whole whey > addition of high molecular weight fraction (≥ 3500) of whey > addition of low molecular weight fraction (≤ 3000) of whey > addition of buffer solution.

In a study of Donnelly et al. (1998), increasing continuous phase levels of WPI (0–1.0 %) led to increasing lipid hydroperoxide and TBARS levels in a Tween-20-stabilised menhaden O/W emulsion (pH 7) when lipid oxidation was accelerated by iron addition. An amount of Tween 20 such that there was no excess surfactant in the continuous phase was used. The pro-oxidative influence of the whey proteins was explained by several possible mechanisms: removal of antioxidants from the interface by protein micelles, transfer of lipid peroxides to the continuous phase by protein micelles increasing their exposure to pro-oxidants, and residual lipids in WPI possibly exhibited a free radical source in combination with the higher iron level in the continuous phase. The addition of WPI plus additional Tween 20 led to greater oxidative stability. This was attributed to a greater exposure of free-radical-scavenging or chelating amino acid residues, as Tween 20 may have denatured the whey proteins.

2.7.3 Influence of heat treatment of milk proteins on oxidative stability

In a study of Tong et al. (2000a), a high molecular weight fraction of raw whey (molecular weight ≥ 3500) was heated at room temperature (control sample), 60, 70, 80 or 90 ˚C for 15 min. The samples were added to Tween-20-stabilised salmon O/W emulsions (pH 7) to test their influence on oxidative stability. When the control sample was added, slightly higher lipid hydroperoxide and TBARS levels were measured. When the sample heated at 60 ˚C was added, a clearly higher TBARS level and a slightly higher lipid hydroperoxide level were measured. In contrast, the heat treatments

at both 80 and 90 ˚C led to a considerable and similar decrease in lipid hydroperoxide and TBARS levels. The effect of heat treatment at 70 ˚C was not tested.

More aromatic amino acid residues (tyrosyl, phenylalanyl, tryptophan) and free sulfhydryl groups became exposed with increasing heating temperature of the solutions, indicating unfolding of the protein. The free sulfhydryl concentration decreased again at 90 ˚C, as sulfhydryl groups possibly oxidised under these conditions. The total sulfhydryl concentration decreased with increasing heating temperature. The antioxidative influence of the solutions heated at 80 and 90 ˚C in the emulsion was attributed to the greater exposure of free-radical-scavenging amino acid residues (aromatic residues and free sulfhydryl groups), whereas the pro-oxidative influence found for the sample treated at 60 ˚C and the control could not be explained (Tong et al. 2000a).

In a study of Elias et al. (2007), the continuous phase of a Brij-35-stabilised menhaden O/W emulsion (pH 7) was replaced with β-lactoglobulin solution that had been heated at various temperatures (95, 70, 50 and control 20 °C) for 15 min. During the storage period of the emulsions, the lipid hydroperoxide and TBARS levels were nearly 90 % lower in the emulsion with the 95 °C sample (after one week), whereas the addition of the other two heat-treated samples did not significantly inhibit lipid oxidation compared with the 20 °C sample. β-Lactoglobulin exhibited ferric-iron-binding activity after all heat treatments but it was similar for the native protein and when heated at 70 ˚C (500 µmol/mg protein), slightly higher for the protein heated at 50 ˚C (550 µmol/mg protein) and lowest for the protein heated at 95 ˚C (300 µmol/mg protein). A maximum concentration of free sulfhydryls was found at 70 °C and a minimum was found at 95 °C. The tryptophan fluorescence increased with increasing heating temperature, indicating that more tryptophan residues became exposed. The free-radical-scavenging ability of β-lactoglobulin towards radicals from the radical-generating azo compound 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH) clearly improved with increasing heating temperature. It was suggested that the increased oxidative stability of the 95 °C sample could have been due to a greater exposure of free-radical-scavenging amino acid residues (e.g. tryptophan, tyrosine, phenylalanine or methionine) on β-lactoglobulin and/or a greater interface contact of the protein because of increased

hydrophobicity, both leading to improved effectiveness of the free-radical-scavenging process. This positive influence on the oxidative stability appeared to have outweighed the negative influence of reduced metal chelation ability.

In a study of Taylor and Richardson (1980b), skim milk fractions of pH 6.7 (skim milk, sweet whey, acid whey and casein) were heated at various temperatures (room temperature, 70, 90, 110 and 130 ˚C) and added to a linoleate emulsion. The heat treatments increased the oxidative stability of the emulsion. The free sulfhydryl concentration increased and the total sulfhydryl concentration decreased through the influence of elevated temperatures.

Kiokias et al. (2007) prepared WPC-stabilised sunflower O/W emulsions with 3 % protein content (pH 6.78) at various temperatures, using solutions of WPC heated at 40, 60, 72, 75 and 85 °C. The solutions of the respective temperature were added to sunflower oil, pre-emulsified by sonication in an ice bath and then further emulsified by passing through a two-valve high pressure homogeniser. The researchers showed that the oxidative stability of the emulsions (measured by conjugated diene hydroperoxides) improved with increasing degree of β-lactoglobulin denaturation. An increased exposure of sulfhydryl groups and aromatic amino acid residues as a result of protein unfolding was thought to be partially responsible for the greater inhibition of lipid oxidation.

A heat-treated (90 ˚C, 30 min) fraction of acid whey with a molecular weight below 5000 had basically the same antioxidative activity as without heating (activity before heating: 100 %; activity after heating: 96–99 %). The samples were incorporated into a phosphatidylcholine liposome system (pH 7) at a 20 % level and TBARS and lipid hydroperoxides were determined as lipid oxidation indicators (Colbert and Decker 1991).

2.7.4 Influence of high and low molecular weight protein fractions, protein hydrolysates and peptides on oxidative stability

Colbert and Decker (1991) tested acid whey for its antioxidative properties in a phosphatidylcholine liposome system (pH 7), in which oxidation was promoted by ferric iron plus ascorbic acid (generating ferrous iron). The inhibition of lipid hydroperoxide and TBARS formation increased with increasing incorporation of whey and reached a maximum at the highest whey concentration (20 %): 86 % lipid hydroperoxide and 90 % TBARS inhibition. Whole whey was also fractionated according to molecular weight and incorporated into the liposome system at a level equivalent to 20 % whey. The antioxidative activity of the various fractions decreased in the following way: whole whey (90 %) > molecular weight below 5000 (80 %) > molecular weight above 6000 (18 %) > molecular weight below 500 (2 %). This indicated great antioxidative activity for the molecular weight fraction between 500 and 5000. In the liposome system, the molecular weight < 5000 fraction and whey were also compared for their antioxidative activity towards metmyoglobin plus hydrogen peroxide (45–50 % inhibition) and soybean lipoxygenase plus linoleic acid (75 % inhibition with the molecular weight < 5000 fraction and 92 % inhibition with whey). The molecular weight < 5000 fraction of whey inhibited TBARS formation better with increasing pH from pH 5 to pH 7.

In the study of Tong et al. (2000a), a Tween-20-stabilised salmon O/W emulsion (pH 7) was diluted with various fractions of acid whey. Dilution with the low molecular weight fraction of whey (molecular weight ≤ 3000) improved the oxidative stability towards lipid hydroperoxide and TBARS formation compared with the control, but the stabilising effect was greater for the high molecular weight fraction of whey (molecular weight ≥ 3500) and was greatest for whole whey. The protein contents of the added solutions were not equal. Whole whey was fractionated and therefore the protein concentration of the various whey fractions probably decreased in the following order: whole whey > high molecular weight fraction of whey > low molecular weight fraction of whey. This order also mirrored the oxidative stability. The antioxidative activity was attributed to whey proteins in the high molecular weight fraction and possibly to

peptides and amino acids in the low molecular weight fraction, as suggested by Colbert and Decker (1991).

WPI was hydrolysed with pure enzymes (pepsin, trypsin, chymotrypsin and papain) or commercial crude proteases (protease F, protease P or protease A). The hydrolysates were added to a liposome system (pH 7) with ascorbic acid and ferric iron. The hydrolysates obtained by treatment with the commercial proteases inhibited TBARS formation (in particular protease F), but not the hydrolysates produced by the pure enzymes. Protease F was noted as the only enzyme with exopeptidase activity, producing single amino acids. A positive correlation between the degree of hydrolysis and the antioxidative activity was found for only the hydrolysate produced by protease P. A higher degree of hydrolysis, with increased peptide content, gave greater oxidative stability (Pena-Ramos and Xiong 2001).

In a study of Diaz et al. (2003), casein, tryptic casein hydrolysate and a low molecular weight fraction of tryptic casein hydrolysate (molecular weight ≤ 10000) were added to a Brij-35-stabilised corn O/W emulsion (pH 7) to test their influence on oxidative stability. They were added in quantities of equal nitrogen and phosphorus content. The ability to inhibit lipid hydroperoxide and hexanal generation generally decreased in the following order: low molecular weight casein hydrolysate > casein hydrolysate > casein.

In a phosphatidylcholine liposome system (pH 7), in which oxidation was accelerated with ferric iron plus ascorbic acid, enriched CPPs, tryptic casein hydrolysate or a low molecular weight fraction of casein hydrolysate (molecular weight ≤ 10000) was added on an equal nitrogen basis. Casein hydrolysate and low molecular weight casein hydrolysate inhibited lipid oxidation (measured by TBARS) at low and high incorporation levels, but CPPs inhibited lipid oxidation only at low concentrations (all samples showed a similar inhibition). High incorporation levels of CPPs were pro-oxidative. When oxidation was promoted by the addition of the free radical source AAPH, lipid oxidation inhibition was greater at higher incorporation levels of the respective fraction and generally decreased as follows: low molecular weight casein hydrolysate > casein hydrolysate > enriched CPPs (Diaz and Decker 2004).

In a study of Let et al. (2007a), cod liver oil was incorporated into milk, yoghurt or salad dressing (emulsified with denatured whey protein, acidic because of 5 % acetic acid content). The fish oil either was incorporated directly with subsequent emulsification or was added after pre-emulsification with a solution of denatured whey protein. The pH conditions of these emulsions were pH 6.7 (milk), pH 4.4 (yoghurt) and pH 4.0 (salad dressing). Lower levels of lipid hydroperoxides and off-flavour volatiles developed in yoghurt and salad dressing than in milk. The lipid hydroperoxide production was similar in yoghurt and salad dressing, but lower levels of off-flavour compounds were produced in fish-oil-enriched yoghurt. Therefore, it was hypothesised that metal ions were less available for the lipid oxidation process in the yoghurt (less decomposition of lipid hydroperoxides into alkoxyl radicals). Furthermore, the microbial activity in yoghurt was mentioned as a possible reason for the limited metal availability. This would make sense from the point of view that, in yoghurt, lactic acid bacteria produce peptides from milk proteins by protease activity (Renye and Somkuti 2008; Tzvetkova et al. 2007). Peptides derived from milk proteins had also exhibited antioxidative activity in the previously mentioned studies (Diaz et al. 2003; Pena-Ramos and Xiong 2001) and they have shown the ability to chelate metal ions in many cases (discussed in the next section). The potential of yoghurt to give oxidative stability to incorporated susceptible lipids has also been observed in other studies (Jacobsen et al. 2006; Nielsen et al. 2007).

2.8 Antioxidative mechanisms of milk proteins, milk protein hydrolysates and peptides

Milk proteins and peptides have shown great potential to prevent lipid oxidation in O/W emulsions and similar systems, e.g. with liposomes. The amphiphilic nature of proteins prevents their solubility in the oil phase of emulsions but makes them available for antioxidative mechanisms at the interface and in the continuous phase. Some of their lipophilic moieties may reach from the interface into the oil phase. In the following, an overview of the antioxidative mechanisms of proteins and peptides is given, with the focus on milk proteins and derived materials.

2.8.1 Hydrophobicity

One characteristic that has been discussed is the hydrophobicity of proteins, peptides and amino acids. The hydrophobicity of proteinaceous material is likely to influence its contact to the lipid phase of an emulsion and would thereby influence the lipid oxidation process.

In the study of Saiga et al. (2003), a peptide/amino acid mixture that stemmed from a papain-generated protein hydrolysate contained in total more hydrophobic amino acids than the corresponding peptide/amino acid mixture generated by actinase E. The papain-generated mixture also showed a greater ability to inhibit lipid hydroperoxide formation in a linolenic acid/Triton X-100 emulsion system.

Murase et al. (1993) investigated the antioxidative activity of histidine and carnosine with attached long chain fatty acid moieties (oleate C 18:1, decanoate C 10:0). They added the compounds to a mixture of methyl linoleate in hexane/2-propanol and also incorporated them into phosphatidylcholine liposome membranes. In the solution system, the antioxidative activity towards lipid hydroperoxide production was 20 % higher for the N-$C_{18:1}$-histidine than for the N-$C_{10:0}$-histidine. The carnosine derivatives exhibited a corresponding behaviour. N-$C_{18:1}$-glycine showed no inhibition effect. In the liposome system, carnosine showed only a slight TBARS inhibition when incorporated into the membrane compared with its presence in the aqueous phase. The incorporation of the more lipophilic derivatives into the membrane resulted in more TBARS inhibition compared with carnosine and the inhibition was greater for N-$C_{18:1}$-carnosine than for N-$C_{10:0}$-carnosine. The incorporation of $C_{18:1}$/$C_{10:0}$ histidine derivatives into the membrane led to less TBARS generation than for the control without added compound, but there was no difference between the two. N-$C_{18:1}$-glycine showed the same TBARS development as the control sample.

Chen et al. (1998) analysed the hydrophobicity of 22 histidine-, leucine- and proline-containing peptides found in digests of soybean protein. The general composition but not the position of amino acids in the peptide had an effect on the retention time on a reversed-phase high performance liquid chromatography (HPLC) column. However, no

correlation between the hydrophobicity and the antioxidative behaviour of the peptides was found when they were added to a linoleic acid/Triton X-100 emulsion system. The oxygen consumption had been measured as a lipid oxidation indicator.

A specific antioxidative mechanism for the hydrophobic maize protein zein has been reported. Its antioxidative function was attributed to the ability to bind to lipids susceptible to oxidation and to bury them in the protein structure (Arcan and Yemenicioglu 2007; Chiue et al. 1997).

2.8.2 Metal binding

The presence of pro-oxidative iron and copper ions is an important factor in the lipid oxidation process. Milk proteins as well as other proteins and peptides thereof contribute to the oxidative stability of O/W emulsions because of their ability to bind metal ions. In general, the chelation ability of proteins, resulting in improved oxidative stability, was found to be related to the reduction in the chemical reactivity of the metal ions, the formation of insoluble metal complexes, the removal of metal ions from their location in proximity to lipids and/or the blocking of reaction sites at which metals and lipids could interact (Diaz et al. 2003; Elias et al. 2007).

Phosphorylated serine residues exist in caseins. They are present in hydrophilic domains of α_{s1}-, α_{s2}- and β-caseins. Certain sequences of the primary structure, such as -SerP-SerP-SerP-Glu-Glu-, exhibit particularly good chelating capacity towards iron, copper, zinc and calcium ions (Baumy and Brule 1988; Bennett et al. 2000; Kitts 2005). The -SerP-SerP-SerP-Glu-Glu- sequence is present in β-casein-4P (1–25), α_{s1}-casein-5P (59–79), α_{s2}-casein-4P (1–21) and α_{s2}-casein-4P (46–70) (Kitts 2005). In general, the numbers of phosphoseryl residues per molecule of casein are 7–9 (α_{s1}-casein), 5 (β-casein), 10–13 (α_{s2}-casein) and 1–2 (κ-casein) (Leonil et al. 1995).

Unlike caseins, whey proteins have no phosphoseryl residues but other metal-binding mechanisms exist, such as the involvement of carboxyl groups (Vegarud et al. 2000), present in aspartic and glutamic acid residues. For example, Shears et al. (1987) showed

that iron was complexed by carboxyl groups in a bovine serum albumin digest. Elias et al. (2008) attributed the metal chelation abilities of proteins to residues of histidine, glutamic acid and aspartic acid. Arcan and Yemenicioglu (2007) mentioned chelating properties for acidic (aspartate and glutamate) and basic (lysine and arginine) amino acid residues (Rajapakse et al. 2005; Saiga et al. 2003) as well as histidinyl residues (Je et al. 2005; Rajapakse et al. 2005). Because of the general presence of these amino acid residues in proteins, proteins would generally be able to chelate metal ions to a certain degree. For caseins, the chelating properties of phosphoseryl residues add to these features.

The metal-binding abilities of whey proteins were summarised in a review by Vegarud et al. (2000). Iron and/or copper binding to α-lactalbumin (Baumy and Brule 1988; Hirai et al. 1992; Svenning and Vegarud 1998), bovine serum albumin (Mossini and Feather 1998; Nagasako et al. 1993), β-lactoglobulin (Baumy and Brule 1988; Svenning and Vegarud 1998) and lactoferrin (Feng et al. 1995; Nagasako et al. 1993) was reported. Svenning and Vegarud (1998) found iron chelation activity for α-lactalbumin and β-lactoglobulin. Hydrolysis of the proteins yielded peptides with greater iron chelation activity.

Kim et al. (2007b) looked at the binding ability of WPC and WPC hydrolysates for ferrous iron. The hydrolysates were produced using several proteolytic enzymes (alcalase, esperase, flavourzyme, neutrase, pancreatin, papain, pepsin, trypsin and a protease from *Bacillus stearothermophilus*). A high iron-binding capacity between 72 % (WPC) and up to 98 % (alcalase-generated hydrolysate) was found in solutions with 5 % protein or hydrolysate and 0.1 % ferrous sulfate. Pancreatin-generated hydrolysate exhibited the lowest iron-binding activity amongst the hydrolysates. After 240 min hydrolysis time, WPC treated with pancreatin had the highest degree of hydrolysis (13.9 %) and WPC treated with alcalase had the second highest (13.6 %). The lowest degree of hydrolysis was 7.5 % (with papain). Therefore, the degree of hydrolysis (according to the concentration of primary amines (Adler-Nissen 1979)) seemed not to be the most influential factor for ferrous iron binding. In contrast, in the alcalase-generated hydrolysate, bovine serum albumin, β-lactoglobulin and α-lactalbumin were the most hydrolysed compared with the other hydrolysates (measured by tricine SDS

polyacrylamide gel electrophoresis). Furthermore, it was pointed out that a main fraction of the alcalase hydrolysate had a higher content of lysine, histidine and phenylalanine than WPC. The authors finally attributed the various iron-binding abilities to differences in the amino acid types (Doucet et al. 2003) and the net charge and length of the peptides (Chaud et al. 2002).

Lactoferrin inhibited lipid oxidation in soybean-lecithin-stabilised corn O/W emulsions, but was partially pro-oxidative in lecithin liposome systems (both pH 6.6). In the emulsion systems, lactoferrin inhibited lipid oxidation promoted with ferrous iron. Lipid oxidation was more promoted by the addition of copper (II) than ferrous iron and lactoferrin did not reduce the pro-oxidative effect of copper. The findings pointed to the ability of lactoferrin to bind iron and to inactivate it for redox reactions. Furthermore, lactoferrin either did not chelate cupric ions or copper–lactoferrin complexes might still have been pro-oxidative (Huang et al. 1999). Smith et al. (1994) had found that cupric ions bound to the same sites as ferric iron. In a study of Nielsen et al. (2004), lactoferrin showed antioxidative activity up to 8 µmol/l in mayonnaise and 12 µmol/l in milk, but was pro-oxidative at higher incorporation levels.

Phillipe et al. (2005) gave an overview of the metal-binding abilities of casein and casein fractions. Iron binding to CPP (1–25) from β-casein (Bouhallab et al. 1991; Gaucheron et al. 1995), ferric iron binding to α_{s1}-casein (Reddy and Mahoney 1991a; Reddy and Mahoney 1991b) and ferrous iron binding to NaCas (Gaucheron et al. 1996) were reported. Vegarud et al. (2000) referred to findings about iron and/or copper binding to peptides derived from β-casein and α_{s1}-casein (Baumy and Brule 1988; Bouhallab et al. 1991; Gaucheron et al. 1996).

Gallaher et al. (2005) added water or milk (skim milk, 2 % milk fat milk or whole milk) to Tween-20-stabilised algal O/W emulsions (pH 7) with ferric iron. The addition of milk greatly reduced the lipid oxidation rate. The authors attributed the effect to iron-binding activity, mostly caused by casein as a main constituent of milk that had previously exhibited strong metal-binding activity (Diaz et al. 2003; Hegenauer et al. 1979c). Gaucheron et al. (1997b) observed iron binding mainly for α_{s1}-casein, but not for α_{s2}-casein, β-casein, β-lactoglobulin or α-lactalbumin, when skim milk was

supplemented with ferrous iron and ferric iron (reversed-phase HPLC analysis combined with photospectrometry). However, when skim milk was ultracentrifuged to remove casein micelles, 7.8–11 % of supplemented iron was found in the whey protein fraction. When skim milk was ultrafiltered to separate whey proteins and caseins from low molecular weight compounds, 3.1–5.6 % of supplemented iron was found in the low molecular weight fraction (atomic absorption spectrometry). The studies of Baumy and Brule (1988) and Nagasako et al. (1993), who found iron binding to the whey proteins α-lactalbumin, β-lactoglobulin and lactoferrin, were referred to. The following compounds were suggested to possibly have bound iron in the low molecular weight fraction: citrate, organic and inorganic phosphate, lactose (Bachran and Bernhard 1980), orotic acid and sulfur-containing compounds.

Faraji et al. (2004) determined the chelating ability of NaCas, WPI and soy protein isolate for ferric iron at pH 7. The iron-binding capacity decreased in the order: NaCas > soy protein isolate > WPI.

Emery (1992) showed that casein supported the oxidation of ferrous iron to ferric iron in aqueous solution (pH 6.5) and that casein bound strongly to ferric iron. The binding of ferric iron to casein was evident as the metal could be recovered quantitatively after isoelectric precipitation. Under the same aerobic conditions without casein, the ferrous iron concentration hardly decreased. It was shown that the iron-binding activity increased with increasing levels of α-casein. Partially dephosphorylated casein exhibited a much lower iron-binding ability, which pointed to the importance of phosphorylated serine residues for the chelation activity of casein. Similar observations were made by Hegenauer et al. (1979b), who also found ferrous iron oxidation promoted by caseins and the binding of ferric iron to caseins. Manson and Cannon (1978) reported the reaction of α_{s1}-casein and β-casein with ferrous iron in the presence of oxygen.

Cervato et al. (1999) observed a correlation between the phosphoseryl content (α- > β- > κ-casein) of casein fractions and their antioxidative activity towards TBARS formation in a liposome system containing arachidonic acid (pH 7.4). It was also found that dephosphorylation only partially restricted the antioxidative activity of these fractions, pointing to the existence of other chelation or antioxidative mechanisms. Furthermore,

orthophosphate and phosphoserine were also added to the liposome system. Both inhibited TBARS formation to some degree, with phosphate showing a moderately better inhibition, but the phosphorylated caseins had a much greater antioxidative capacity. The antioxidative influence of phosphate and phosphoserine was probably due to their chelation ability. Also, Diaz et al. (2003) looked at the importance of phosphoseryl groups for the antioxidative activity of caseins. They added several casein fractions with equal phosphorus content to a Brij-35-stabilised corn O/W emulsion (pH 7) and followed the development of lipid hydroperoxides and hexanal. Hydrolysate of low molecular weight casein inhibited lipid oxidation the most of all fractions, followed by casein hydrolysate, casein and finally enriched CPPs (containing 86 % CPPs). When the fractions were added on the basis of equal nitrogen content, only the order of casein and CPPs was changed. The results suggested that the content of phosphoseryl residues was not the most important antioxidative factor and that other protection mechanisms must have existed.

In a study of Rival et al. (2001a), β-casein showed the greatest chelation activity towards ferrous iron, followed by casein. Dephosphorylation of β-casein markedly reduced its chelation activity. Other casein fractions (κ-casein and dephosphorylated α-casein) and bovine serum albumin had a similar and lower chelation activity compared with β-casein. The chelation activity considerably increased (except for β-casein) upon hydrolysis with various proteolytic enzymes (trypsin, subtilisin and clostripain) and reached a similar level for the various fractions. The compounds were tested for their antioxidative activity in a linoleic acid/Triton X-100 emulsion (pH 7) with the free radical source AAPH (oxygen uptake measured). The addition of dephosphorylated casein and β-casein caused a greater inhibition of lipid oxidation than the addition of the respective untreated fraction. Bovine serum albumin showed a similar antioxidative activity to the caseins. Protein hydrolysis generally increased the antioxidative activity (four out of five samples), but this also seemed to depend on the protease.

In a study of Kim et al. (2007a), CPPs were isolated from NaCas hydrolysate produced by alcalase. The CPPs were aggregated by calcium chloride addition under different pH conditions (from pH 3 to pH 8) and were precipitated with ethanol. The fraction produced at pH 3 chelated ferrous iron the least effectively. It also had a lower glutamic

acid, phosphorus and serine content than the other fractions. It was suggested that a lower level of phosphoseryl and glutamic acid residues might have affected the chelation ability. Furthermore, it was observed that the reducing capacity for Cu^{2+} to Cu^{+} was different for the various CPP fractions. Increasing pH yielded fractions with a lower Cu^{2+} reducing capacity.

Tong et al. (2000b) found that whey proteins in the continuous phase of a bovine-serum-albumin-stabilised hexadecane O/W emulsion were able to chelate and remove pro-oxidative ferrous iron from the interface at neutral pH. Also Faraji et al. (2004) suggested this as an antioxidative mechanism of proteins in the continuous phase. Hu et al. (2003b) pointed out that, for whey proteins, this mechanism might be impaired at pH 3 because of their net positive surface charge, whereas caseins are probably still able to chelate metal ions and remove them into the continuous phase at pH 3 as phosphoseryl groups are negatively charged at low pH (Gaucheron et al. 1996). Other findings would support this suggestion, as the binding of iron to caseins has been reported to be independent of the pH between pH 2.5 and pH 8.0 (Baumy and Brule 1988; Diaz et al. 2003; Gaucheron et al. 1996; Gaucheron et al. 1997a; Hekmat and McMahon 1998). Gaucheron et al. (1997a) studied the binding of ferric iron and Cu^{2+} ions to NaCas. The binding of iron, but not the binding of copper, was apparently independent of the pH. In general, decreasing pH leads to the release of more metal ions from proteins as H^{+} or H_3O^{+} ions compete for cation-binding sites (Diaz et al. 2003). For example, Katz et al. (1980) showed that the binding ability of bovine serum albumin for Cu^{2+} ions increased from pH 5.3 to pH 7.4.

In a study of Villiere et al. (2005), bovine serum albumin and NaCas were tested for their capacity to bind ferrous iron in aqueous solutions (pH 6.5; 1 g/l protein content). All ferrous iron was retained by NaCas up to the maximum dose of 5.2 mg/l. Bovine serum albumin retained basically all ferrous iron at the lowest dosage of 0.7 mg/l, but the uptake levelled off at a maximum of 1.4 mg/l at higher iron levels. In sunflower O/W emulsions (pH 6.8 at the beginning and pH 6.1 at the end of storage), prepared with the respective protein (1.4 % protein concentration), moderately less oxygen was consumed and conjugated dienes were generated in the bovine serum albumin emulsion, up to 30 h of storage at 50 ˚C. Thereafter, both oxidation indicators showed the same

development in either emulsion type. Zeta-potential measurements showed that droplets stabilised with caseins had a more negative surface charge (−80 mV) than droplets stabilised with bovine serum albumin (−20 mV). When EDTA was incorporated into the emulsions (100 μmol EDTA/l), which had been added to the protein solutions before emulsification, the steep rise in oxygen uptake was generally delayed (after 8 h without EDTA). A much later rise in oxygen uptake was observed in the NaCas emulsion (after 52 h) than in the bovine serum albumin emulsion (after 16 h) with EDTA. The iron concentration in the emulsions, considering only iron incorporated by the proteins, was 2.27 μmol/l (bovine serum albumin emulsion) and 1.34 μmol/l (NaCas emulsion).

These findings showed that caseins had a greater binding capacity for pro-oxidative ferrous iron than the whey protein bovine serum albumin. In this case, a greater metal-binding ability may have led to the slightly greater lipid oxidation rate in the emulsions. The droplet surface charge in the NaCas emulsion was more negative (compared with the bovine serum albumin emulsion), which could have resulted in a higher concentration of pro-oxidative metal ions at the interface, leading to more lipid oxidation. The authors suggested that the addition of NaCas solution after emulsification might have improved the oxidative stability, as caseins could then have removed detrimental metal ions away from the interface into the continuous phase. When pro-oxidative metal ions were sequestered by EDTA, NaCas was considerably more antioxidative than bovine serum albumin. According to the authors (Villiere et al. 2005), this might have been due to a greater free-radical-scavenging activity of the caseins on the interface.

Decker et al. (2001) tested carnosine (β-alanylhistidine) and its constituent amino acid histidine for their antioxidative properties in an LDL system, in which oxidation was promoted by cupric ions. Both compounds had an antioxidative influence. In the same system, when oxidation was promoted by free radicals from AAPH, they showed only a minor antioxidative activity. This indicated that copper-chelating and not free-radical-scavenging activity was their main antioxidative mechanism.

Chan and Decker (1994) showed that carnosine−metal chelates scavenged superoxide radicals. It was concluded that simply chelating and thereby keeping metal ions away

from lipids in an O/W emulsion is only one aspect of the antioxidative influence of chelation. In addition, the chelates themselves could have pro- or antioxidative character.

In most studies that looked at the antioxidative influence of milk proteins and derived peptides in O/W emulsions or similar systems (Section 0), metal chelation was referred to as an important antioxidative mechanism of the proteinaceous material. Chelation was mentioned in 13 articles and chelation abilities were examined in five articles.

2.8.3 Free-radical-scavenging ability

Free-radical-mediated reactions are a substantial element of lipid autoxidation. Extensive research has been conducted to replace synthetic primary antioxidants with free-radical-scavenging activity (e.g. butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)) with natural compounds that are in accordance with consumer preferences. Proteins and peptides from many animal and plant sources (e.g. bovine milk, porcine, eggs, fish, soy, legumes, cereals etc.) have been studied and there is great evidence for their free-radical-scavenging capacities. The ability to scavenge free radicals derives from their constituent amino acids, such as cysteine, tyrosine, tryptophan, phenylalanine and histidine (Arcan and Yemenicioglu 2007; Moure et al. 2006). However, it was found that amino acid residues alone did not account for the whole free-radical-scavenging activity of peptides (Hernandez-Ledesma et al. 2005). In the following, the free-radical-scavenging abilities of milk proteins, milk protein hydrolysates and peptides are reported.

Diaz and Decker (2004) studied the ability of enriched CPPs, tryptic casein hydrolysate or low molecular weight casein hydrolysate (molecular weight $\leq$ 10000) to inhibit TBARS formation in a phosphatidylcholine liposome system (pH 7) with the free radical source AAPH. Furthermore, they investigated the ability of the three fractions to scavenge free radicals derived from AAPH in aqueous solution (pH 7). The antioxidative activity in the liposome system corresponded with the free-radical-

scavenging activity and decreased in the following order: low molecular weight casein hydrolysate > casein hydrolysate > CPPs.

In a study of Rival et al. (2001a), caseins, casein hydrolysates (produced with the proteolytic enzymes trypsin, subtilisin and clostripain) and bovine serum albumin exhibited AAPH-radical-scavenging activity, as indicated by reduced oxygen uptake in a linoleic acid/Triton X-100 emulsion system (pH 7.0). The tested compounds were in detail: casein, dephosphorylated casein, β-casein, dephosphorylated β-casein, dephosphorylated α-casein, κ-casein and bovine serum albumin. Dephosphorylated casein and β-casein were more antioxidative than the respective native protein. In general, the antioxidative activity (for four out of five samples) increased upon hydrolysis.

In a test system containing linoleic acid and soy bean lipoxygenase in aqueous solution (pH 9.0), these protein fractions were also antioxidative and may have scavenged free radicals (the mechanism of lipoxygenase-induced oxidation was mentioned to include carbon- and oxygen-centred radicals). The effect of hydrolysis on the antioxidative activity was very variable and depended on the enzyme and the protein. Dephosphorylated fractions seemed to have a lower antioxidative activity (Rival et al. 2001a).

In another test, all protein fractions showed free-radical-scavenging activity in a system with linoleic acid hydroperoxides and haemoglobin (pH 9.0). Linoleic acid hydroperoxide and haemoglobin react with each other, generating a haemoglobin-derived peroxyl radical. Upon hydrolysis, the antioxidative activity either generally decreased or remained the same. Dephosphorylated fractions showed a greater antioxidative activity. In aqueous solution (pH 7.0) with the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), unhydrolysed caseins and bovine serum albumin were effective in DPPH radical scavenging, but not the hydrolysates. Dephosphorylated casein and β-casein had a lower DPPH-radical-scavenging activity than the respective native protein (Rival et al. 2001a).

The different test systems to investigate antioxidative activities contained different types of radicals. It was suggested that the different degrees of hydrophobicity of the incorporated radicals influenced the possibility of interaction between them and the respective protein fraction (Rival et al. 2001a). This suggestion was based on a former finding of Dean et al. (1991), that hydrophobic radicals, unlike hydrophilic radicals, hardly interacted with bovine serum albumin. In a previous study of Rival et al. (2001b), whole casein and α-casein, β-casein and κ-casein (native or dephosphorylated) had also shown lipoxygenase-inhibiting activity in a Tween 20/linoleic acid system (pH 9.0), indicated by a lower oxygen consumption rate.

In a study of Cervato et al. (1999), the deteriorative effect of hydroxyl and superoxide radicals (pH 7.4) towards deoxyribose and nitroblue tetrazolium in aqueous solution was 30–40 % in the presence of caseins (α-casein, β-casein, κ-casein and dephosphorylated α-casein) compared with control solutions without protein. Dephosphorylated β-casein showed the greatest free-radical-scavenging ability as the deteriorative effect of the radicals was only 12 %. Hydroxyl radicals had been generated by combining ascorbate, ferric iron and hydrogen peroxide. Therefore, for this study as well as for some of the following examples, the antioxidative activity might have been partially due to metal ion chelation activity and not just the ability to scavenge hydroxyl radicals.

Suetsuna et al. (2000) produced a casein hydrolysate with pepsin. Peptide fractions exhibited scavenging activity for superoxide anion radicals. A peptide was isolated from the most antioxidative fraction. The most important amino acid residues for the scavenging activity were tyrosine, phenylalanine, glutamic acid and leucine. The peptide also exhibited scavenging ability for hydroxyl radicals (produced by hydrogen peroxide plus ferrous iron) and DPPH radicals. In comparison with the antioxidative peptides gluthathione and carnosine, the free-radical-scavenging activity was as follows (smaller numbers indicate higher activity): casein peptide (251) > carnosine (654) > glutathione (661) for hydroxyl radicals and glutathione (6) > carnosine (23) > casein peptide (98) for DPPH radicals.

In a study of Kim et al. (2007a), CPPs were isolated from NaCas hydrolysate produced by alcalase. The CPPs were aggregated by calcium chloride addition under different pH conditions (from pH 3 to pH 8) and precipitated with ethanol. The fraction produced at pH 3 had the greatest scavenging activity for AAPH-derived radicals. This fraction also had a higher content of cysteine (possibly derived from κ-casein), histidine and tyrosine residues. These residues were pointed out as being effective free radical scavengers.

Chin and Kitts (2004) reported on the scavenging activity of CPPs for 2,2'-azino-bis-(3-ethylenzothiazoline-6-sulfonic acid) (ABTS) radicals in aqueous solution.

Buttermilk solids scavenged hydroxyl radicals in an aqueous solution with hydrogen peroxide and ferrous iron (pH 7.4) at concentrations ≥ 2.5 mg/ml, indicated by the inhibition of deoxyribose degradation. The maximal inhibition of free radicals was 60 % at the highest buttermilk solids concentration (10 mg/ml). However, at concentrations between 0.1 and 1.0 mg/ml, buttermilk solids promoted the deteriorative effect of free radicals. Free-radical-scavenging activity was partly ascribed to sulfhydryl groups. In contrast, the reducing capacity of sulfhydryl groups for ferric iron might have contributed to the pro-oxidative effect at lower buttermilk solids levels (Wong and Kitts 2003).

In a study of Tong et al. (2000b), a high molecular weight fraction of whey protein scavenged AAPH radicals, indicated by inhibition of β-phycoerythrin decay in aqueous solution (pH 7.0). The whey protein fraction also inhibited lipid hydroperoxide and TBARS production in a Tween-20-stabilised salmon O/W emulsion (pH 7). When sulfhydryl groups in the whey protein fraction were blocked with N-ethylmaleimide (NEM), the inhibitive effect towards TBARS production was affected to some degree and the free-radical-scavenging activity decreased by 20 %. It was therefore suggested that the whey protein fraction inhibited lipid oxidation in the emulsion partly by free radical scavenging with sulfhydryl groups, but that possibly other residues such as tyrosine were also involved. The protein fraction also exhibited chelation ability for ferrous iron.

Hernandez-Ledesma et al. (2005) identified several AAPH-radical-scavenging peptides from enzymatic (pepsin, trypsin, chymotrypsin, thermolysin and corolase PP) hydrolysates of β-lactoglobulin and α-lactalbumin in the oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay, as they inhibited the decay of fluorescein. The peptide with the greatest radical-scavenging activity had a Trolox equivalent value of 2.62, which was higher than for butylated hydroxyanisole (BHA) with a Trolox equivalent value of 2.43. An equimolar mixture of the 11 constituent amino acids of that peptide was more antioxidative than the peptide itself (Trolox equivalent value 4.3). The same comparison was done for the second best radical-scavenging peptide (containing five amino acids). In this case, the peptide was more antioxidative than the amino acid mixture (Trolox equivalent value 0.8 versus 0.4). The ORAC-FL assay was also run with pure amino acids. The molar Trolox equivalents were: 4.6 (tryptophan), 1.6 (tyrosine), 1.1 (methionine), 0.15 (cysteine), 0.07 (histidine) and 0.0025 (phenylalanine). All other amino acids tested (arginine, asparagine, glutamine, aspartic acid, proline, alanine, valine, lysine, isoleucine) showed values below 0.00001. It was concluded that the antioxidative properties of the peptides were influenced by the sequence of the amino acids and the structure of the peptide. The amino acid sequence may have a synergistic or antagonistic effect for the antioxidative activity in comparison with equimolar amino acid mixtures.

In a study of Bayram et al. (2008), whey proteins were directly separated from whey solution via size exclusion chromatography or whey solution underwent hydrolysis with pepsin or trypsin with subsequent chromatographic separation of the fractions obtained. The various fractions were tested for their scavenging activity towards superoxide radicals in aqueous solution at pH 7.8 (in the following, low numbers indicate high scavenging activity). The first fraction obtained without hydrolysis was high in bovine serum albumin and immunoglobulins and showed the greatest scavenging activity (9). The second fraction had a high content of α-lactalbumin and β-lactoglobulin and showed the lowest scavenging activity (176), but the activity greatly increased (16) upon hydrolysis with pepsin. The third fraction without hydrolysis contained small peptides (91). Other fractions obtained after hydrolysis and chromatography had the following activities: high in bovine serum albumin (21), high in β-lactoglobulin (88),

small peptides (143) (all obtained after trypsin treatment) and high in α-lactalbumin (60) (obtained after pepsin treatment).

Elias et al. (2006) compared the antioxidative properties of β-lactoglobulin and a chymotryptic hydrolysate of β-lactoglobulin in a Brij-35-stabilised menhaden O/W emulsion (pH 7.0). They also measured the free-radical-scavenging activity towards radicals from AAPH and the ability to chelate ferric iron. In the emulsion, the hydrolysate inhibited lipid hydroperoxide and TBARS production more than the unhydrolysed protein. Both fractions also exhibited free-radical-scavenging and chelation activity, with the hydrolysate being more effective than the unhydrolysed protein. The activities increased with increasing concentration.

A substantial decrease in the concentration of specific tyrosine (38 % after day 4) and methionine (55 % after day 4) residues, but not phenylalanine residues (8 % after day 8), was observed in peptides of the hydrolysate present in the continuous phase of the emulsion. The decrease in concentration was attributed to the oxidation of these residues, possibly indicating free-radical-scavenging activity. The loss of tyrosine and methionine residues set in earlier than the generation of lipid hydroperoxide and propanal, suggesting antioxidative activity towards lipids. The loss of phenylalanine was detected after lipid oxidation had set in. It was therefore suggested that phenylalanine did not exhibit notable antioxidative activity in this particular system. Elias et al. (2006) also referred to a previous study (Elias et al. 2005), using the same emulsion system. When methionine was part of native β-lactoglobulin in the continuous phase, its concentration was stable. It was therefore suggested that methionine was more exposed to the solvent as part of the peptide and was thus able to take part in oxidation reactions. In contrast, Elias et al. (2005) observed a decrease in free sulfhydryl groups and thereafter tryptophan residues in the continuous phase β-lactoglobulin before the onset of lipid oxidation.

Taylor and Richardson (1980a) found that, amongst 15 amino acids added to a linoleic acid methyl ester/Tween 80 emulsion, only cysteine had a dose-dependent protective effect in terms of lower oxygen consumption. Also protein solutions (bovine serum albumin, ribonuclease, lysozyme, trypsin, metallothionein) were added to the system.

The generation of more free sulfhydryl groups by treating the proteins with $NaBH_4$ led to lower oxygen consumption compared with the unmodified proteins. When bovine serum albumin and lysozyme were further treated with iodoacetic acid, free sulfhydryl groups were oxidised and, as a consequence, a marked increase in oxygen uptake was observed.

In a second study, when the same test system was used and only milk proteins were added, Taylor and Richardson (1980b) confirmed the antioxidative effect of free sulfhydryls, but also pointed out that this would not explain the entire antioxidative activity of the proteins. Also skim milk was added to the emulsion. It was found that whey proteins contained most of the sulfhydryl groups in skim milk but that they were only slightly responsible for its total antioxidative activity. Skim milk treated with $NaBH_4$ caused a marked increase in the sulfhydryl concentration but did not greatly lower the oxygen uptake in the emulsion.

Tong et al. (2000b) added a high molecular weight fraction of whey protein to a salmon oil/Tween 20 emulsion and followed the free sulfhydryl concentration over incubation time. After 10 days, the free sulfhydryl concentration was no longer detectable but the lipid hydroperoxide and TBARS concentrations did not increase steeply afterwards. Blocking of free sulfhydryl groups with NEM resulted in 60 % less effective TBARS inhibition. In aqueous solution, the whey fraction lost 20 % of its AAPH-radical-scavenging activity when sulfhydryl groups were blocked with NEM.

Faraji et al. (2004) replaced the continuous phase of a WPI-stabilised menhaden O/W emulsion with WPI or soy protein isolate treated with NEM. The inhibition of lipid oxidation was partly affected compared with the untreated continuous phase proteins.

Hu et al. (2003a) prepared salmon O/W emulsions with different whey proteins and found that NEM addition did not influence lipid hydroperoxide generation. The propanal production was more inhibited in the NEM-treated samples.

In another study, patatin, a potato protein, showed scavenging activity of DPPH and hydroxyl radicals in aqueous solution. The activity was reduced from 20 to 5 % and 30

to 10 % for the two radical types respectively when sulfhydryl groups were alkylated (Liu et al. 2003). They also reported that the modification of tryptophan side chains of patatin with N-bromosuccinimide led to a reduction in free-radical-scavenging activities from 20 to 5 % (DPPH radicals) and from 30 to 1 % (hydroxyl radicals) in aqueous solution.

Wong and Kitts (2003) added 0.1 % buttermilk solids and whey protein to fish oil/Tween 20 emulsions and found inhibition of lipid hydroperoxide development of 56 and 46 % respectively. Although the whey protein had two to three times more free sulfhydryl groups than the buttermilk solids fraction, lipid hydroperoxides were not more inhibited. Furthermore, the reduction of ferric iron to ferrous iron was greater for the whey proteins and it correlated with the free sulfhydryl content.

In most studies that looked at the antioxidative influence of milk proteins and derived peptides in O/W emulsions or similar systems (Section 0), free radical scavenging was referred to as an important antioxidative mechanism of the proteinaceous material (17 articles). In the following, amino acid residues that have been associated with free-radical-scavenging activity in those articles are indicated: cysteine/sulfhydryl groups (in 11 articles), tyrosine (in eight articles), tryptophan (in three articles), methionine (in two articles), histidine, phenylalanine, proline and lysine (in one article each).

Chapter 3

Materials and methods

3.1 General materials

3.1.1 Emulsion lipid

Linoleic acid, specified as approximately 60 %, was of the brand Sigma, purchased from Sigma-Aldrich Co., St. Louis, Missouri, USA (catalogue number L1626) and stored in the dark at 5 °C under oxygen-free nitrogen (gas code 152, purchased from BOC New Zealand Ltd., Auckland). According to the supplier, the original plant source was soybean oil and besides linoleic acid the product contained oleic acid and linolenic acids based on gas chromatographic analysis. Several batches were used throughout the project.

3.1.2 Proteins

Whey protein isolate (WPI; product name Alacen 895) and sodium caseinate (NaCas; product name Alanate 180) powders were obtained from Fonterra Cooperative Group New Zealand. Throughout the whole project the same batch of the respective protein type was used. Sodium azide, extra pure, purchased from Merck KGaA, Darmstadt, Germany, was used as microbiological stabiliser in the protein solutions.

3.1.3 Chemicals for lipid hydroperoxide and hexanal determination

Ethanol (95 %), n-hexane (95 %), ethyl acetate (min. 99.5 %), methanol (99.8 %) were obtained from Biolab Ltd., Scoresby, Victoria, Australia, and butan-1-ol from BDH (VWR International, West Chester, PA, USA). Ammonium thiocyanate (min. 98.0 %) and barium chloride (min. 99.0 %) were from Ajax Chemicals (Ajax Finechem Pty.

Ltd., Taren Point, Australia) ferrous sulphate heptahydrate (min. 99.5 %) from Merck and cumene hydroperoxide (88 %) and hexanal (98 %) from Aldrich (Sigma-Aldrich Co., St. Louis, Missouri, USA).

3.2 General methods

3.2.1 Determination of the iron and copper content of the protein powders

Iron and copper contents of the proteins were determined by acid digestion and subsequent ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) determination. The measurements were sub-contracted to Hill Laboratories, Hamilton, New Zealand.

3.2.2 Preparation of protein solutions

Protein solutions of various protein contents were prepared with water purified by reverse osmosis (RO). Sodium azide was added to inhibit microbial growth in the protein solutions and emulsions. The concentration of sodium azide in the protein solutions was adjusted to give 200 ppm in the emulsions. Gallaher et al. (2005) had shown that sodium azide had no effect on lipid oxidation in O/W emulsions at this concentration. The solutions were stirred with a magnetic bar for at least 8 h at 50°C, until all protein was dissolved and then stored at 5 ˚C until further use.

3.2.3 Preparation of emulsions

Emulsions containing various protein contents and 10.6 % lipid were prepared with solutions of either WPI or NaCas and linoleic acid. Each solution was added at a temperature of about 5°C to linoleic acid and a coarse emulsion was created by high speed blending at 20500 rpm with a Diax 600 blender from Heidolph Instruments GmbH & Co.KG, Schwabach, Germany. Complete emulsification was achieved with a

two-valve Rannie homogeniser, model APV-1000, from APV New Zealand Ltd., Auckland. The pressure stages generally used for the two valves were 320 bar and 30 bar (320/30 bar), giving physically stable emulsions. The emulsions were passed through the homogeniser three times to ensure complete emulsification.

3.2.4 Acceleration of the lipid oxidation rate by emulsion storage at elevated temperature

The emulsion samples were incubated at 50 ± 0.5 °C in the dark using a Contherm, model 120 MC (Contherm Scientific Ltd., Lower Hutt, New Zealand) as thermostat. These conditions were chosen to allow reasonably fast autoxidation rates of linoleic acid with presumably the same oxidation mechanisms as at room temperature. Other research has found that for incubation tests at 60 °C lipid oxidation correlated well with shelf life tests at room temperature and side reactions were limited compared to higher temperatures (Frankel 1993). Also the results of Hamm et al. (1968) pointed to the same mechanisms in the autoxidation of milk fat up to a temperature of 50 ˚C, according to the volatile compounds produced. The start of the incubation period followed as soon as possible after the emulsification. After emulsion preparation, emulsion aliquots were distributed into containers resulting in either 93 % or 85 % headspace for the lipid hydroperoxide samples or the hexanal samples respectively. The aliquots were 1 ml (lipid hydroperoxide measurements) and 3 ml (hexanal measurements). Sample containers for lipid hydroperoxide measurements were 15 ml plastic centrifuge tubes from Sarstedt (air tight sealed with plastic screw cap) and 20 ml glass vials for gas chromatographic measurements from Phenomenex (air tight sealed with rubber/teflon septum and tin cap). The headspace was regarded as a factor that did not limit lipid hydroperoxide formation as the molar ratio of oxygen initially present in the headspace (20 °C, 1013 hPa) to the highest molarity of lipid hydroperoxides created (WPI (0.5 %) emulsion, d_{32} = 0.6 µm, in Chapter 4) was 130:1. Samples were incubated in triplicate. Usually, samples were taken at 0, 2 and 4 hours for lipid hydroperoxide and at 0, 12 and 24 h for hexanal measurements. The samples were immediately put into the freezer (-18 °C) at the end of the incubation period. Each container represented an experimental unit for single measurements of either the lipid hydroperoxide or hexanal concentration.

Lipid hydroperoxides and hexanal were determined within 1-2 weeks. In a preliminary experiment samples kept at -18 °C were found to have oxidative stability for at least 3 weeks.

3.2.5 Measurement of the droplet size

Droplet size was measured as Sauter Mean Diameter (d_{32}) in micrometer with a Mastersizer/E from Malvern Instruments Ltd., Malvern, Worcestershire, UK. The presentation code for these measurements was 2NAD. In some experiments, the droplet size was measured as Z-average in micrometer with a Zetasizer Nano-ZS from Malvern Instruments Ltd. In general, the relative refractive index (N) i.e. the ratio of refractive index of fat or lipid globule (1.456) and that of the dispersion medium (1.33) was 1.095. Over the whole incubation period, the droplet size was measured at various times to monitor the physical stability of the emulsions. Independent sample containers were used for measurements at different times. Measurements were carried out in duplicate. This was done separately for all emulsions of an experiment.

3.2.6 Measurement of the pH value

The pH was measured using an Orion 520 pH meter from Thermo Fisher Scientific Inc., Waltham, MA, USA. The pH meter was calibrated daily with a two point calibration at pH 4.0 and 7.0. Emulsions prepared with WPI or sodium caseinate and linoleic acid appeared to be well buffered systems at protein concentrations from 0.5 to 10.0 %. A stable pH of 6.0 ± 0.2 was found over 24 h incubation time (Chapter 4). Therefore the pH was not measured explicitly in all experiments.

3.2.7 Lipid hydroperoxide determination

For the determination of lipid hydroperoxides, a method adapted from Shantha and Decker (1994) and Nuchi et al. (2001) was applied. To frozen 1 ml emulsion aliquots, 5 ml of an effective extraction solvent (ethanol/ethyl acetate/n-hexane, 1:1:1 (V/V/V)),

was added and left thawing at room temperature for 20 min. The solvent mixture had been formerly used successfully by Satue-Gracia et al. (2000) to extract fat from infant formulas. A vortexing procedure to extract the lipids from the samples and centrifugation step followed. Supernatant (0.5 ml) was transferred to a reaction tube with 4 ml of methanol/butanol (2:1 (V/V)). Thiocyanate (30 µl) and ferrous iron solution (30 µl) were added. After each addition, the reaction tubes were vortexed for a few seconds. Three reagent control samples were prepared in the same way, but using the extraction solvent instead of sample extract. The tubes were kept in the dark at room temperature for 20 min before measuring the absorption at 510 nm. A standard curve made with cumene hydroperoxide was used to quantify the lipid hydroperoxides. For this purpose, cumene hydroperoxide was diluted with the extraction solvent to various concentrations. These solutions were used instead of sample extracts in the assay.

Principle of the chemical reaction:

$$Fe^{2+} \xrightarrow{\text{lipid hydroperoxides}} Fe^{3+}$$

$$Fe^{3+} + \text{thiocyanate} \longrightarrow \text{coloured complex (absorption maximum at 510 nm)}$$

3.2.8 Hexanal determination

A characteristic secondary oxidation product of linoleic acid is hexanal. The samples were subjected to solid phase microextraction (SPME) that followed hexanal quantification by gas chromatography (GC) using a flame ionisation detector (FID). The principle of SPME is that a solid fibre capable of adsorbing volatiles is exposed to the headspace in the sample vials. After a specified agitation procedure that helps to release volatiles from the sample the fibre is exposed in the injector of the GC and releases the adsorbed compounds into the column gas stream. Characteristics of the SPME procedure were as follows: polydimethylsiloxane fibre (diameter 100 µm), incubation and extraction temperature 50 °C, pre-incubation before extraction at 50 °C for15 min at constant agitation speed, extraction time 20 min at same constant agitation speed. For

the gas chromatograph measurements an Rtx-1701 (30 m, 0.25 mm inner diameter, 1.0 µm film thickness) column was used. A split ratio of 1.0 was applied for the column gas stream. Column temperature program was 45 °C for 1 min, 5 °C/min up to 100 °C and 10 °C/min up to the end temperature of 230 °C. The injector and FID temperature were set at 250 °C. A hexanal external standard curve was created for the quantification of hexanal in the samples. For this purpose hexanal was added at different levels to a freshly prepared emulsion of 4.7 % NaCas and WPI respectively. The average of the two regression curves was used as standard curve.

3.2.9 Spectrophotometric measurements

All spectrophotometric measurements were carried out with an Ultrospec II photometer from Pharmacia LKB (Thermo Fisher Scientific Inc., Waltham, MA, USA). The samples were measured in 4 ml plastic cuvettes.

3.2.10 Statistical analysis

Mean values are shown with the standard error of the mean value (SEM) as error bars to indicate the variability of the single values (calculated in Microsoft Excel). Multifactorial analyses of variance (ANOVA) according to the general linear model were carried out with the lipid hydroperoxide and hexanal concentration as responses. Two sample t-tests were carried out to test if two mean values were significantly different from each other. For these purposes Minitab, version 14 or 15, was used.

3.3 Materials and methods in Chapter 4

3.3.1 Methods: Chapter 4.1

Incubation of emulsion samples

The containers for lipid hydroperoxide measurements were triplicates. The containers for hexanal measurements were duplicates for the first emulsion of each droplet size and triplicates for the second one.

Statistical analysis

The total number of samples was n = 72 for lipid hydroperoxide measurements and n = 60 for hexanal measurements. Several ANOVA were carried out. The factors in the analyses were droplet size and incubation time. In each ANOVA either lipid hydroperoxide or hexanal data of emulsions with two different droplet sizes was compared over the whole incubation time range. This was done to show statistical evidence if lipid oxidation had occurred significantly differently in samples with different droplet sizes.

3.3.2 Methods: Chapter 4.2

pH

The pH of the samples was 6.0 ± 0.2 over the entire incubation period. It was measured after 0, 4, 12 and 24 h of incubation time to monitor pH stability. Two independent sample containers were observed over 24 h for all emulsion types.

Statistical analysis

The total number of samples was n = 324 for lipid hydroperoxide measurements (n = 111 for samples incubated for 4 h) and n = 294 for hexanal measurements (n = 98 for samples incubated for 24 h). The number of replicates for one sample type ranged from three to six (lipid hydroperoxide measurements) and two to six (hexanal measurements). One ANOVA was carried out for the lipid hydroperoxide and hexanal data respectively,

including the experimental factors protein type, protein concentration, droplet size and incubation time (to obtain lipid hydroperoxide and hexanal means over the protein concentration). Another ANOVA was carried out for the lipid hydroperoxide and hexanal data respectively with the experimental factors protein concentration, droplet size and incubation time (to evaluate the influence of the droplet size in WPI and NaCas emulsions with increasing protein concentration).

3.3.3 Methods: Chapter 4.3

Determination of the protein surface concentration

The measurement of the surface protein concentration was based on the procedure described by Srinivasan et al. (1996).

Interface protein in 11 g emulsion:
0.033732 g NaCas in NaCas emulsion with 1.0 % protein content
0.025636 g WPI in WPI emulsion with 1.0 % protein content

The interface protein content was calculated by subtracting the protein content of the subnatant (obtained by centrifugation of four times 11 g of the emulsion at 45,000 g for 60 min with a Sorvall centrifuge type RC 5C) from the total protein content of 11 g of WPI and NaCas emulsions (1.0 % protein). The protein content of the subnatant was determined using the "Leco, total combustion method" according to AOAC Official Method 968.06 (Dumas Method) (AOAC 2005a)).

Content of oil phase in 11 g of emulsion:
11 g emulsion with 10.6 % linoleic acid content => oil phase content = 11 g * 0.106 = 1.166 g

Approximate specific surface area (SSA) for d_{32} = 0.31 µm (average droplet size)
= (20.5583 m^2/g (in d_{32} = 0.29 µm) + 18.1221 m^2/g (in d_{32} = 0.33 µm)) / 2
= 19.3402 m^2/g

Surface area = SSA (m^2/g) * oil phase content (g) = 19.3402 m^2/g * 1.166 g
= 22.55 m^2

Surface protein concentration in WPI emulsion with 1.0 % protein content
= 25.636 mg / 22.55 m^2 = 1.1369 mg/ m^2 = approximately 1.1 mg/m^2

Surface protein concentration in NaCas emulsion with 1.0 % protein content
= 33.732 mg / 22.55 m^2 = 1.4959 mg/ m^2 = approximately 1.5 mg/m^2

Replacement of the continuous phase of emulsions with protein solutions

Plastic centrifuge tubes (50 ml) were filled with freshly prepared emulsion. The tubes were then centrifuged with a CR 22GII centrifuge from Hitachi at 36,000 g for 30min at 5 °C. The cream phase was separated from the continuous phase of the emulsion. The subnatant was the continuous phase, which was carefully taken out with a syringe. The syringes that were used were 10 ml plastic ones with a blunt steel tip screwed on top. The continuous phase was substituted with an equivalent amount of RO water or protein solution (four replicates per sample type). The tubes were then vortexed for 5 min at moderate speed with a PV-1 from Grant-bio until the cream was totally suspended again. Samples in which the continuous phase was substituted with RO water were regarded as control samples for the effect of continuous phase protein on lipid oxidation. In the experiment where emulsions with 4.0 % protein were prepared, one part of the emulsion was put on the side for which the continuous phase was not replaced. During the replacement process for the rest of the emulsion, this part was kept at the same temperature before the samples were incubated together at 50 ˚C.

3.4 Materials and methods in Chapter 5

3.4.1 Materials: Chapter 5

Disodium EDTA and trisodium citrate of analytical grade were of the brand BDH from VWR International, West Chester, PA, USA. Dialysis tubing was a Spectra/Por®

Membrane with a molecular weight cut-off of 12000-14000 purchased from Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA.

3.4.2 Methods: Chapter 5.1

Preparation of protein solutions

WPI and NaCas solutions of 5 % were prepared without sodium azide. The protein was dissolved in half of the final amount of RO water. The other half of aqueous solution was added to it and consisted of RO water with various levels of disodium EDTA or trisodium citrate. One part of each protein solution was used for the dialysis process. The other part was adjusted to 3.36 % protein concentration with RO water.

Dialysis process

For the dialysis process, 80 ml of the respective protein solution was added into a dialysis tube (each end sealed with plastic clips) and dialysed for 42 h against 20 litres of RO water at 5 °C under stirring with a magnetic bar. Every 14 h the water was replaced with fresh RO water. After the dialysis process the volume in the dialysis tube had increased due to osmosis. The new volume varied depending on the original protein solution. They were all adjusted to the same protein concentration (3.36 %) with RO water.

Preparation of emulsions

After the usual emulsification process, 200 ppm sodium azide was added with 35 µl RO water per g of emulsion.

3.4.3 Methods: Chapter 5.2

Preparation of protein solutions and dialysis process

Protein solutions used in the dialysis process were prepared with 5.5 % WPI or NaCas and without sodium azide. Two dialysis tubes were filled with 100 ml of RO water and the ends were closed with plastic clips. One tube was placed into 2 litres of the WPI

solution, the other one into 2 litres of the NaCas solution. The protein solutions were in glass beakers with a magnetic bar which were placed on a magnetic stirrer. Dialysis took place at 5 ˚C for 42 h and the protein solution was replaced with fresh one every 14 h. After the dialysis the tubes were cut open and the protein content of the dialysate (solution in the tube) was determined using a Kjehldahl method. The dialysates were diluted with RO water or left undiluted and used instead of RO water to produce protein solutions as usual which were further used to produce emulsions. For one control emulsion with NaCas (5.0 %) and 100 ppm EDTA, the protein solution was prepared by dissolving the EDTA in RO water before the right amount of protein was added.

Protein content of the dialysates

The total nitrogen content of the dialysates was determined in triplicate and the protein content calculated with a Kjehldahl method: "AOAC Official Method 991.20", Nitrogen (Total) in Milk (AOAC 2005c).

3.5 Materials and methods in Chapter 6

3.5.1 Materials: Chapter 6

For the gel electrophoresis, a Mini-Protean® 3 Cell system from Bio-Rad Laboratories Inc., Hercules, CA, USA, was used. The power supply used for the electrophoresis was an EC 135 from E-C Apparatus Corporation St. Petersburg, Florida, USA. For the scanning procedure, a Molecular Dynamics personal densitometer, model PD-SI computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA) was used, equipped with the software ImageQuant for Windows NT, Version 5.0 (Molecular Dynamics, Sunnyvale, CA, USA) to quantify the colour intensity of the bands. All chemicals used were of analytical reagent grade. Ammonium persulfate and acrylamide/bis 37.5:1 premixed powder electrophoresis purity reagent (consisting of acrylamide plus N,N'methylene-bis-acrylamide) were products from Bio-Rad Laboratories. N,N,N`,N`-Tetramethylethylene-diamine (TEMED) for electrophoresis and glycine for electrophoresis were obtained from Sigma.

Tris(hydroxymethyl)methylamine of molecular biology grade, hydrochloric acid (min 35.4 %) and glacial acetic acid were products from BDH. Glycerol and sodium hydroxide pellets were purchased from Univar AG, Bever, Switzerland. Bromphenol blue, pH indicator (pH 3.0–4.6), amido black 10B for electrophoresis and redox indicator, and 2-propanol were products from Merck.

3.5.2 Methods: Chapter 6

***Heat treatment of protein solutions at 84* °C**

Protein solution (250 g) was transferred into a glass beaker (400 ml) plus a magnetic stirring bar. The container was covered with transparent food wrap to prevent water evaporation during the heat treatment and placed into a waterbath above a magnetic stirrer plate. A thermometer was placed into the solution. The temperature in the solution reached 83 °C after 10 min. The maximal temperature of 84 ± 0.3 °C was reached after 15 min. Before heating, the solutions had room temperature and after heating they were put on ice immediately. After 30 min on ice they were stored at 5 °C.

Native PAGE (polyacryl amide gel electrophoresis) method

The method is suitable to qualify and quantify whey proteins and was adapted from Havea et al. (1998) and Patel (2007b). For all preparations and procedures extra pure demineralised water (Milli-Q water) was used. The term %C stands for % w/w of crosslinking N,N'methylene-bis-acrylamide and %T stands for the concentration/strength of acrylamide.

Native-PAGE resolving gel was prepared as follows: 2.0 ml native resolving gel buffer, 6.0 ml water and 8 ml acrylamide/bis 37.5:1 solution (30 %) were pipetted into a vacuum flask, mixed well and degassed while stirring with a magnetic bar under vacuum for 20 min. The native resolving gel buffer consisted of 3.0 M tris(hydroxymethyl)methylamine adjusted to pH 8.8 with hydrochloric acid. Immediately after the degassing procedure, 8 µl of TEMED and 80 µl ammonium persulphate (10 %) were added to the degassed solution and mixed gently to initiate the polymerisation reaction leading to polyacrylamide. Then 3.3 ml of the solution was

quickly pipetted between two glass plates, sealed on the bottom and at the sides (Mini-Protean® 3 Cell equipment). Water (0.5 to 1 ml) was pipetted on top of the solution between the glass plates, to give the gel a smooth and level surface upon setting. After 30 min at room temperature, the resolving gel (15 % T, 2.67 % C) had formed and the water was removed from the top layer using filter paper.

Native-PAGE stacking gel was prepared by mixing 2.0 ml stacking gel buffer, 5.0 ml water and 1.0 ml acrylamide/bis 37.5:1 solution (30 %) in a vacuum flask, followed by the degassing procedure described above. The native stacking gel buffer consisted of 0.5 M tris(hydroxymethyl)methylamine adjusted to pH 6.8 with hydrochloric acid. Immediately after the degassing procedure, 40 µl ammonium persulphate (10 %) and 8 µl TEMED were added to the degassed solution and mixed gently. The mixture was then pipetted between the glass plates on top of the pre-formed resolving gel. A plastic comb with ten slots was inserted, to form sample loading wells in the stacking gel. It was taken care that no air bubbles were embedded during the insertion. After 90 min at room temperature, the glass plates were removed from the apparatus, as the gel formation was completed.

Two gels, both consisting of a stacking and resolving gel part situated between two glass plates, had been prepared at a time. They were placed into an electrophoresis chamber filled with native-PAGE electrode buffer solution, pH 8.3 (which consisted of 0.125 M tris(hydroxymethyl)methylamine and 0.95 M glycine, diluted 1:4 with water).

Prepared sub-samples (10 µl) were charged into the sample loading wells on top of the stacking gel with a Hamilton syringe (Hamilton Company, Reno, Nevada, USA). The sub-samples consisted of a mixture of 50 µl protein solution (original sample) plus 950 µl native gel sample buffer (pH 6.8). Native gel sample buffer (pH 6.8) had been prepared by combining 200 ml native stacking gel buffer (0.5 M tris(hydroxymethyl)methylamine adjusted to pH 6.8 with hydrochloric acid), 20 ml Bromphenol blue solution (0.4 %), 600 ml water and 80 ml glycerol.

Then, electrophoresis was carried out at a maximum voltage of 200 V, with a final current of 70 mA, for a run time of approximately 70 min; power was turned off when

the bromphenol blue dye band reached the bottom of the resolving gel. The gels were then removed from the glass plates and transferred to polyethylene containers for the staining and destaining procedure.

For the staining procedure, the gels where immersed in 50 ml amido black 10B dye (0.1 % (weight/volume) in 10 % acetic acid) for 60 min. Then the solution was removed and the gels drained. Afterwards, 100 ml acetic acid (10 % (volume/volume)) was added for destaining. The solution was replaced after 1 h and one more time after 10 h with 100 ml of acetic acid (10 % (volume/volume)). To support the staining and destaining processes they took place on a platform rocker. After 20 h destaining time, the gels were scanned and the colour intensity of the ß-lactoglobulin bands was quantified.

Four gels were prepared to run all together six replicates of each protein solution sample. On each gel two replicates of the protein solution sample without heat treatment (control) was run. The entire procedure was repeated once with a separate sub-sample preparation. The controls represented on each gel 0 % protein denaturation and showed the darkest stains. The colour intensity decreased, the higher the degree of denaturation was. The reduction in colour intensity of a stain in comparison to the average intensity of the two controls, represented the degree of protein denaturation.

Total sulfhydryl determination

Total sulfhydryls were determined as described in Section 3.6.4, Methods: Chapter 7.2. The same chemicals were used.

Free sulfhydryl determination

Protein solution (2000 µl) or RO water for controls, 200 µl phosphate buffer solution and 500 µl DTNB solution were added into test tubes, with vortex mixing after each addition (triplicates). The tubes then were put in the dark for 30 min at room temperature. The assay was other than that the same as for the determination of total sulfhydryls described in Section 3.6.4, Methods: Chapter 7.2. The same chemicals were used. For free sulfhydryl determination a standard curve was created using cysteine solutions (based on RO water) of different concentration levels instead of protein solution.

3.6 Materials and methods in Chapter 7

3.6.1 Materials: Chapter 7

The oxygen concentration was measured with an YSI model 57 Dissolved Oxygenmeter plus oxygen electrode from YSI Incorporated, Yellow Springs, OH, USA. For calibration the oxygen electrode was inserted into a 250 ml glass bottle, the bottom covered with 2-3 cm water. The bottle was immersed in a waterbath at 40 ˚C for approximately one hour until the temperature in the bottle had equilibrated to 37 ˚C (measured with the internal thermometer at the oxygen electrode). The needle of the oxygen meter was then adjusted to 6.73 mg/l, which is the solubility of oxygen in water exposed to water saturated air at 37 ˚C (YSI model 57 Operating Instructions).

The free radical agent 2,2`-azobis(2methylpropion-amidine) dihydrochloride (AAPH) (97 %), 5,5`-dithio-bis(2-nitrobenzoic acid) (DTNB) (99 %) and xylenol orange (no purity stated) were of the brand Aldrich from Sigma-Aldrich Co., St. Louis, Missouri, USA. Sodium dihydrogen orthophosphate 1-hydrate (99.0 – 102.0 %), tris(hydroxymethyl)methylamine (≥ 99 %), sodium dodecyl sulphate (SDS), ferrous sulphate 7-hydrate, hydrogen peroxide (30 %), sodium acetate-3-hydrate (99.5 %), phosphoric acid (~ 85 %) and trisodium phosphate-12-hydrate (99 %), all analytical reagents, were of the brand BDH from VWR International, West Chester, PA, USA. Disodium hydrogen orthophosphate 7-hydrate (≥ 99 %) and sodium hydroxide (NaOH, analytical reagent) were of the brand Ajax Chemicals from Ajax Finechem Pty. Ltd., Taren Point, Australia. Cysteine (no purity stated) was of the brand USB from Affymetrix Inc., Santa Clara, CA, USA. Oxygen free nitrogen, gas code 152, was purchased from BOC New Zealand Ltd., Auckland.

3.6.2 Methods: Chapter 7

Preparation of phosphate buffer (0.1 M), pH 6.0

Phosphate buffer solution (0.1 M) of pH 6.0 was prepared by combining 0.1 M solutions of sodium dihydrogen orthophosphate 1-hydrate and disodium hydrogen orthophosphate 7-hydrate in RO water.

3.6.3 Methods: Chapter 7.1

Preparation of emulsions

WPI and NaCas emulsions with 1.0, 4.0 and 7.0 % protein content were created using protein solutions prepared with phosphate buffer (0.1 M, pH 6.0) instead of RO water.

Experimental description

The experimental set-up is shown in Figure 3.1. Freshly prepared emulsion (165 ml) was pipetted into a glass flask with three openings and an approximate volume of 200 ml. The two openings on the side were closed by stoppers, the one in the middle left open. The flask was placed into a water bath at 40 °C and the temperature of the emulsion adjusted to 37 °C (thermometer in emulsion). The emulsion was stirred by the use of a magnetic stirrer. In a separate glass beaker, 10 ml of the emulsion was combined with 750 µl AAPH solution (210 g/l AAPH in RO water) or RO water and adjusted to pH 6.0 with NaOH (10 M). To the 165 ml emulsion, the analogous volumes of AAPH solution or RO water (12.4 ml) and NaOH solution (≈ 1.5 ml) were added. After 1 minute, an oxygen probe was set into the centre opening to close the system. After 2 minutes, the first oxygen reading was taken. About 3.5 ml emulsion were taken out through a syringe at various sampling times (2–10 min with AAPH; 2–45 min without AAPH) through one of the stoppers (glass stopper with rubber septum). For the volume taken out, oxygen-free nitrogen streamed into the flask from a nitrogen reservoir. The emulsion taken out was immediately put on ice. It was distributed into 15 ml plastic tubes in triplicate (3 x 1 ml) for lipid hydroperoxide measurements and put into the freezer (–18 °C) within 10 min. Emulsion samples with AAPH (0 min) were prepared separately and not by sampling out of the glass flask.

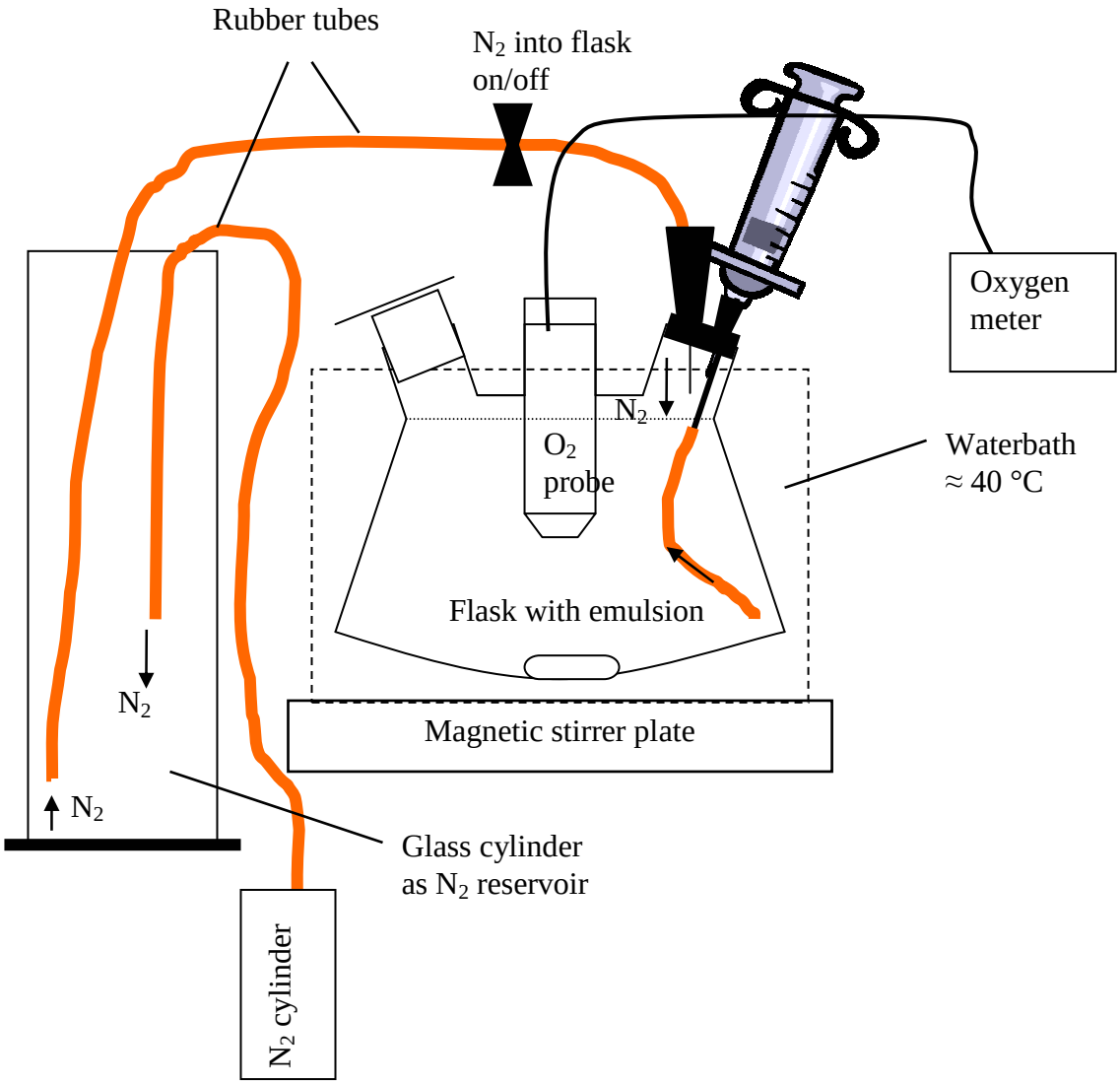


Figure 3.1: Experimental set-up

3.6.4 Methods: Chapter 7.2

Oxygen consumption in protein solutions with AAPH

The experimental set-up was similar to Figure 3.1 in Section 3.6.3, Methods: Chapter 7.1, except that the stopper with the syringe was replaced with a normal plastic stopper. The execution of the experiment was also the same as described in Section 3.6.3, Methods: Chapter 7.1, except for the usage of various aqueous solutions instead of emulsions and that no sampling took place.

Obtaining continuous and cream phase protein solutions for determinations of protein hydroperoxides, amino acids and total sulfhydryls

10 ml or 5 ml of emulsion was added into 50 ml plastic centrifuge tubes respectively. Incubation for various times at 50 ˚C followed. The tubes were then centrifuged with a RC 5C centrifuge from Sorvall at 45,000 g for 60 min at 5 °C. The subnatants (continuous phase of the emulsion) were taken out with 10 ml single use plastic syringes with a blunt steel tip screwed on top. Then the same weight of SDS (2.0 %) solution was added as subnatant had been taken out. Afterwards re-emulsification was done by vortexing with a VM-1000 from Digisystem at the highest vortex speed for 35 s and heavy manual shaking for 10 s. Then the tubes were centrifuged again and also subnatants (cream phase protein solution of the emulsion) taken out as described above.

The cream phase protein solution contained SDS whereas the continuous phase protein solution did not. The cream phase protein was therefore denatured and the continuous phase protein was not. However, in the subsequent methods, both proteins were heavily exposed to denaturants anyway, to SDS (determination of total sulfhydryls), to strong acid or base (amino acid determination) or to trichloroacetic acid and an organic solvent mixture (protein hydroperoxide determination). Therefore the initial difference in the status of the proteins in either fraction was thought to be irrelevant.

Protein hydroperoxide determination

Protein solution (1000 µl) with continuous or cream phase protein was transferred into a 50 ml plastic centrifuge tube. Trichloroacetic acid (10 % weight/volume in RO water)

(5000 µl) plus 5000 µl organic solvent mixture (ethanol/ethyl acetate/n-hexane, 1:1:1 (V/V/V)) were added for protein denaturation/precipitation and the extraction of residual lipid. The mixture was vortexed, centrifuged and the supernatant decanted. To the remaining protein precipitate, which was in pellet form, 5000 µl sulphuric acid (25 mM) was added. The protein was then suspended with a glass rod. Afterwards 280 µl xylenol orange solution (23.34 mM xylenol orange) and 280 µl ferrous iron solution (2.80 mM ferrous sulphate 7-hydrate) based on sulphuric acid (25 mM) were added and the mixture was vortexed. For controls, 5000 µl sulphuric acid (25 mM), 280 µl xylenol orange solution and 280 µl ferrous iron solution were added in triplicate into test tubes. The tubes were then put in the dark for 30 min reaction time at room temperature. Afterwards they were centrifuged again. The supernatant was transferred into 4 ml photometric plastic cells and the absorption at 560 nm was measured against sulphuric acid (25 mM). All samples in this experiment were centrifuged using a RC 5C centrifuge from Sorvall at 45,000 g for 60 min at 5 °C. The absorption obtained in the assay was converted into micromoles of hydroperoxides by means of a hydrogen peroxide standard curve. The hydrogen peroxide curve was created by adding 4970 µl of sulphuric acid (25 mM) plus 30 µl of hydrogen peroxide solution of various concentration levels (based on sulphuric acid (25 mM)) plus 280 µl of xylenol orange and ferrous iron solution into test tubes. The procedure already described above was followed without centrifugation. The assay was adapted from the instructions of Gieseg et al. (2000) and Gay et al. (1999).

Principle of the chemical reaction:

$$Fe^{2+} \xrightarrow{\text{protein hydroperoxides}} Fe^{3+}$$

$$Fe^{3+} + \text{xylenol orange} \longrightarrow \text{coloured complex (absorption maximum at 560 nm)}$$

Total sulfhydryl determination

Protein solution (500 µl) or RO water for the controls, was pipetted into test tubes; 100 µl phosphate buffer solution (pH 8.1), 1100 µl SDS solution (5.0 %, weight/volume in RO water) and 500 µl DTNB solution were added. The tubes were placed in the dark for

30 min at room temperature and afterwards the absorption at 412 nm was measured against RO water. Also the absorption at 540 nm was measured against RO water to take a slight turbidity into account and the reading deducted from the absorption values at 412 nm. The absorption readings at 540 nm were low (≤ 0.020). There were four samples of each sample type which were each measured with one replicate. A standard curve was made for quantification of total sulfhydryls by using RO-water-based cysteine solution of various concentration levels instead of protein solution in the assay. The method was adapted from Taylor and Richardson (1980b).

Principle of the chemical reaction (Guingamp et al. 1993):

SH + 5,5`-dithio-bis(2-nitrobenzoic acid) $\xrightarrow[\text{basic}]{\text{mild}}$ 2-nitro-5-mercaptobenzoic acid

pH

yellow

(absorption maximum at 412 nm)

Preparation of the phosphate buffer solution (pH 8.1)

Tris(hydroxymethyl)methylamine (1 M) and trisodium phosphate-12-hydrate (1 M) were dissolved in RO water and the pH was adjusted to pH 8.1 with phosphoric acid (85 %).

Preparation of the DTNB solution

Sodium acetate-3-hydrate (50 mM) and DTNB (2 mM) were dissolved in RO water.

Chapter 4

Factors affecting lipid oxidation in milk-protein-based O/W emulsions

This chapter examines how some of the basic characteristics of milk-protein-based O/W emulsions influence lipid oxidation. These include change of the droplet size, protein type, protein concentration and the role of the continuous phase protein. In contrast to previous studies, these effects were followed over a wider protein concentration range, leading to a more complete picture of how various factors interact. The comparison of the two milk protein fractions whey protein isolate (WPI) and sodium caseinate (NaCas) was especially interesting.

The oxidation of linoleic acid, which was employed as emulsion lipid, was used to investigate the influence of the various factors. Oxidation indicators were lipid hydroperoxides for the earlier stage and hexanal – specific to linoleic acid oxidation – for the advanced stage of lipid oxidation.

In the previous research, contradictory results were obtained in studies that looked at the influence of the droplet size; some studies found more lipid oxidation for small droplets (Gohtani et al. 1999; Jacobsen et al. 2000; Lethuaut et al. 2002) others for large droplet sizes (Let et al. 2007b; Nakaya et al. 2005). The antioxidative behaviour of whey proteins and fractions (Allen and Wrieden 1982; Djordjevic et al. 2004; Elias et al. 2005; Hu et al. 2003a; Hu et al. 2003b; Tong et al. 2000a; Tong et al. 2000b) as well as of caseins (Allen and Wrieden 1982; Diaz and Decker 2004; Diaz et al. 2003; Djordjevic et al. 2004; Hu et al. 2003b) was studied. Faraji et al. (2004) and Hu et al. (2003b) studied the effect of varying protein concentration and the effect of continuous phase protein was examined by Elias et al. (2005), (2007) and Faraji et al. (2004).

4.1 Effect of droplet size on lipid oxidation in NaCas-based linoleic acid emulsions

In a preliminary experiment, the effects of various droplet sizes on lipid oxidation in a milk protein based emulsion with only one protein type and concentration (1.0 % NaCas) were examined.

The experimental plan is depicted in Figure 4.1. More details about its execution can be found in Chapter 3: Materials and methods.

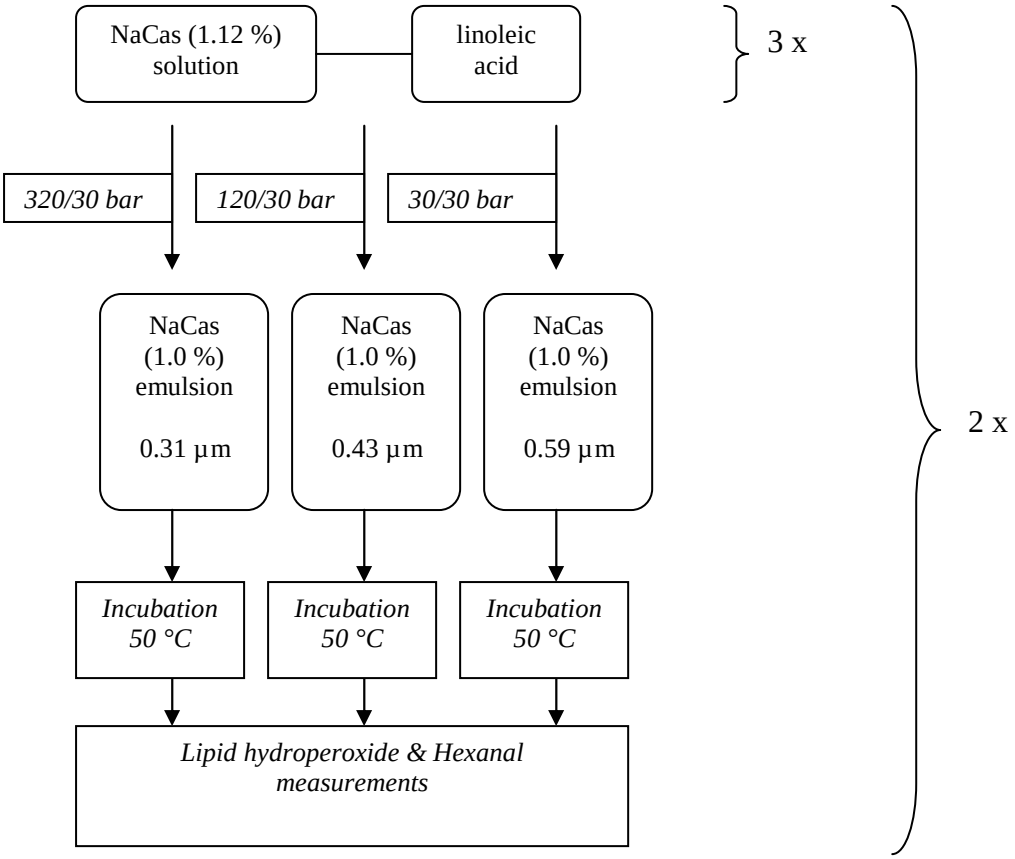


Figure 4.1: Flow chart of the experimental plan

The stability of the droplet sizes over time was important in this experiment to allow a valid reference to droplet sizes and enable meaningful comparisons. Table 4.1 shows the mean droplet sizes (d_{32}) measured after 0, 4, 12 and 24 h incubation time at 50 °C. The mean droplet sizes were 0.31 µm, 0.43 µm and 0.59 µm. They were calculated by combining the measurements of all times for the respective homogenisation pressure from both experimental replicates. The droplet sizes were mostly stable over the incubation time. The greatest change occurred for large droplet sizes from 12 h (0.59 µm) to 24 h (0.68 µm).

Table 4.1: Emulsion droplet sizes (d_{32} (µm)) after 0h, 4 h, 6 h, 12 h and 24 h incubation at 50 °C with standard error of the mean value* (SEM)

Incubation time (h)		Homogenisation pressure		
		30/30 bar	120/30 bar	320/30 bar
0 h	Mean value	0.58	0.41	0.29
	SEM	0.01	0.01	0.01
4 h	Mean value	0.59	0.44	0.31
	SEM	0.01	0.01	0.01
12 h	Mean value	0.59	0.44	0.32
	SEM	0.01	0.01	0.00
24 h	Mean value	0.68	0.42	0.33
	SEM	0.06	0.01	0.01

* four replicates

Figure 4.2 and Figure 4.3 show the lipid hydroperoxide and hexanal development over time.

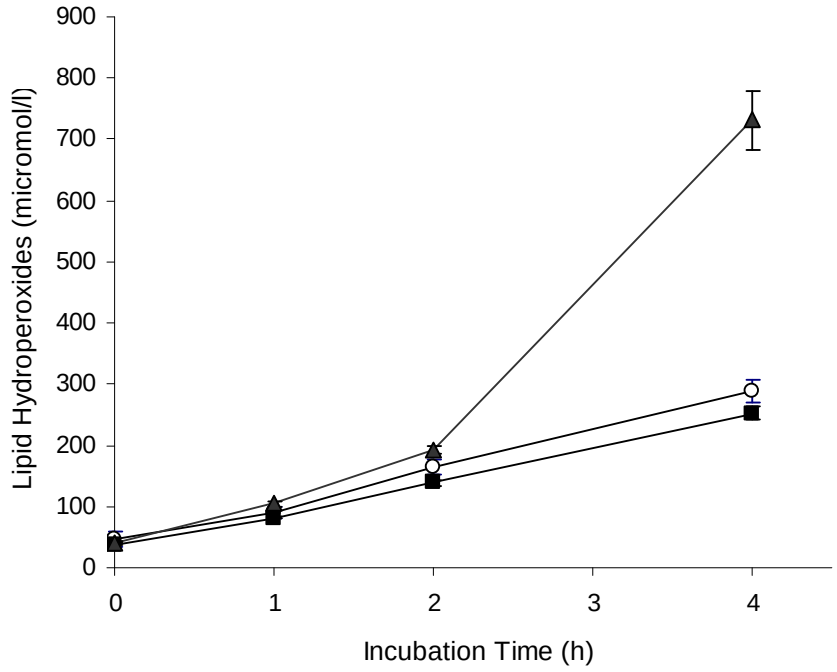


Figure 4.2: Effect of droplet size on lipid hydroperoxide production in NaCas-stabilised linoleic acid emulsions. Emulsions produced at different homogenisation pressures led to droplets sizes d_{32} of 0.31 µm (○), 0.43 µm (■) or 0.59 µm (▲).

From the beginning up to 4 h the two smaller droplet sized emulsions showed an increase in lipid hydroperoxides at about the same rate and reached 290 (0.31 µm) and 253 µmol/l (0.43µm) after 4 h (Figure 4.2). The 0.59 µm emulsion showed a similar increase up to 2 h as the other two, but increased sharply between 2 and 4 h compared with the smaller droplet sized emulsions. At 4 h an average of 732 µmol/l was measured.

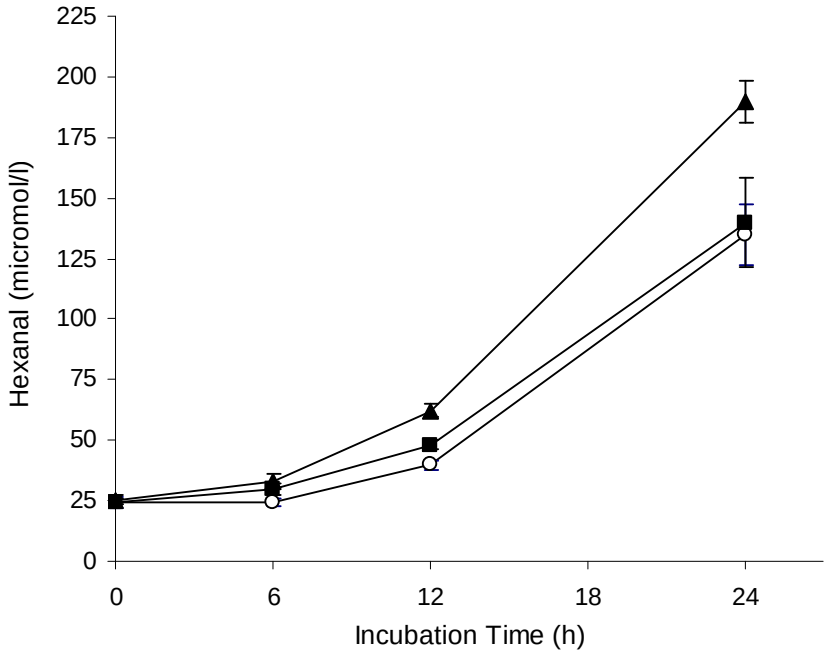


Figure 4.3: Effect of droplet size on hexanal production in NaCas-stabilised linoleic acid emulsions. Emulsions produced at different homogenisation pressures led to droplets sizes d_{32} of 0.31 µm (○), 0.43 µm (■) or 0.59 µm (▲).

The hexanal concentration increased more rapidly in emulsions with larger droplet sizes up to 12 h and even more noticeably from 12 to 24 h. After 24 h, the hexanal concentration for 0.31 µm, 0.43 µm and 0.59 µm emulsions had risen to 135, 140 and 190 µmol/l respectively (Figure 4.3). At 24 h, only the hexanal concentrations of 0.31 µm and 0.59 µm were significantly different ($p < 0.05$ in two sample t-test).

ANOVAs were carried out for comparisons over the whole incubation time range (Appendix Chapter 4, Table 4A). No significant difference in lipid hydroperoxide and hexanal generation could be detected between 0.31 and 0.43 µm emulsions and the 0.59 µm emulsion was significantly different from the two smaller droplet sized emulsions.

The results indicate that lipid oxidation was more inhibited in small rather than large droplet sizes in linoleic acid/NaCas-based O/W emulsions of 1.0 % protein. The greater oxidative stability could have been due to the antioxidant activity of NaCas that might

deploy better with small droplet sizes. Caseins and other proteins have exhibited antioxidant characteristics in many cases (Allen and Wrieden 1982; Diaz and Decker 2004; Diaz et al. 2003; Djordjevic et al. 2004; Elias et al. 2005; Hu et al. 2003a; Hu et al. 2003b; Tong et al. 2000a; Tong et al. 2000b). The radius of each droplet – if regarded as a sphere – is proportional to the ratio between its volume and surface area (Equation 2 (Nakaya et al. 2005)). Therefore, the ratio between the volume of the lipid core and the surface covered by caseins decreases with decreasing droplet size or in other words more NaCas covers less lipid per droplet. At the same time the total droplet surface increases and overall more NaCas can interact with the emulsion lipid.

$$r \propto \frac{V}{A_S} \qquad \text{(Equation 2)}$$

where r is the radius of a sphere , V is the volume and A_S is the surface area.

In smaller droplets, free radicals and metal ions present in the lipid phase have a shorter distance for the diffusion from the lipid core to the interface. Therefore, the likeliness of molecular collision with caseins increases so that free radical scavenging and chelation of metal ions can take place. Nakaya et al. (2005) found small droplets to be more stable towards lipid oxidation than large ones in O/W emulsions based on triglycerides and sucrose lauryl ester or decaglycerol lauryl ester. They suggested a greater proportion of the emulsifier per droplet that resulted in an increased ratio of saturated to unsaturated lipids per droplet. They also mentioned a so called “wedge effect” that could be detected using NMR spectrometry. The lauryl fatty acid chains reaching into the inner of the droplet reduced their mobility. This could also have limited the motions of unsaturated triglycerides in proximity to the interface. In contrast, a large part of the total lipid volume of a larger droplet is situated in the inner core and would not have been influenced by this effect. Lipophilic moieties of caseins also could have partly reached into the lipid phase interacting with linoleic acid, although probably not forming a structure of wedges as in the above mentioned case.

In some studies, decreasing droplet size also resulted in more oxidative stability, but the effect could not be allocated unequivocally to the droplet size as its change had only

been a side effect to the change of another factor, like addition of EDTA (Jacobsen et al. 2001), addition of propyl gallate (Jacobsen et al. 1999), protein concentration (Kiokias et al. 2006), homogenisation temperature or conformational change of protein (Let et al. 2007b).

In other studies, small droplets were less stable towards lipid oxidation and this could have been due to the fact that no effective antioxidants were present in the continuous phase and/or interface or the effect was outweighed by pro-oxidants. Lethuaut et al. (2002), for example, remarked that although small droplets were more susceptible towards oxidation than large ones in bovine-serum-albumin-stabilised emulsions the susceptibility was partly cancelled out by the antioxidative activity of the protein. Coupland et al. (1996) mentioned that the generation of free radicals is common in food processing and particularly in the continuous phase of O/W emulsions. Such free radicals, oxygen and other pro-oxidants, like metal ions, of the continuous phase also have a larger surface to interact with the smaller droplets, thereby antagonising oxidative stability. In that respect, the role of the interface to act as a physical barrier becomes prominent to effectively prevent pro-oxidants from intruding into the lipid phase.

Kiokias et al. (2006) pointed out that the use of low-molecular weight emulsifiers, like Tween 20 and SDS, can increase the total surface area as the droplet size decreases and may result in more lipid oxidation. In a study of Jacobsen et al. (2000), mayonnaise with small droplets showed greater oxidative susceptibility. This was attributed to the iron-containing egg yolk protein, phosvitin, which would constitute a large part of the interface. Rampon et al. (2001) found a greater fluorescence in small rather than large droplet sized bovine serum albumin emulsions. The fluorescence was caused by the reaction of interface protein with lipid oxidation products. For the measurement of direct lipid oxidation indicators no difference was found for the two droplet sizes. It appeared as if a stronger lipid oxidation in small droplets might have been offset by the larger contact surface with bovine serum albumin, allowing more antioxidant activity of the protein.

In summary, it appears that the effect of droplet size on lipid oxidation cannot be regarded independently of other emulsion factors. A decreasing droplet size and therefore a larger total interfacial area can result in inhibition or promotion of lipid oxidation. In future work, systematic studies could help to enlighten the influence of certain factors in connection with increase or decrease of the droplet size. Systematic combinations of the following factors are seen as particularly relevant: emulsifier and lipid type as well as concentration, pH and the metal ion concentration. The investigations should comprise at least two different droplet sizes. Particularly when proteins are used as emulsifiers, the homogenisation temperature should also be controlled as it can influence the unfolding of the protein and therefore its interaction with the emulsion lipid (Let et al. 2007b).

The next set of experiments examined the effects of the protein type – namely WPI and NaCas – over a wide concentration range on oxidation of small and large droplets.

4.2 Effect of droplet size, protein type and protein concentration on lipid oxidation in linoleic acid emulsions

In previous research, Faraji et al. (2004) investigated milk-protein-based menhaden O/W emulsions from 0.25 % to 1.5 % at pH 7.0 but only used WPI, whereas Hu et al. (2003b) used a variety of proteins to compare their antioxidative effect but only used protein concentrations of 0.5 and 1.5 % at pH 3.0. Sun and Gunasekaran (2009) examined the oxidative stability of WPI-stabilised menhaden O/W emulsions (pH 6.7–7.0) with protein concentrations of 0.2 % to 2.0 %. Kiokias et al. (2006) produced NaCas-stabilised cottonseed O/W emulsions (pH 7.0) at protein concentrations of 0.5 % to 2.0 % and investigated their oxidative stability.

In this experiment WPI and NaCas were used over a much larger range of protein concentrations at pH 6.0 and emulsions of two different droplet sizes were examined.

The experimental plan is depicted in Figure 4.4. WPI and NaCas emulsions with protein concentrations of 0.5 to 10.0 % and droplet sizes (d_{32}) of 0.3 and 0.6 µm were produced. More experimental details are given in Chapter 3, Materials and methods.

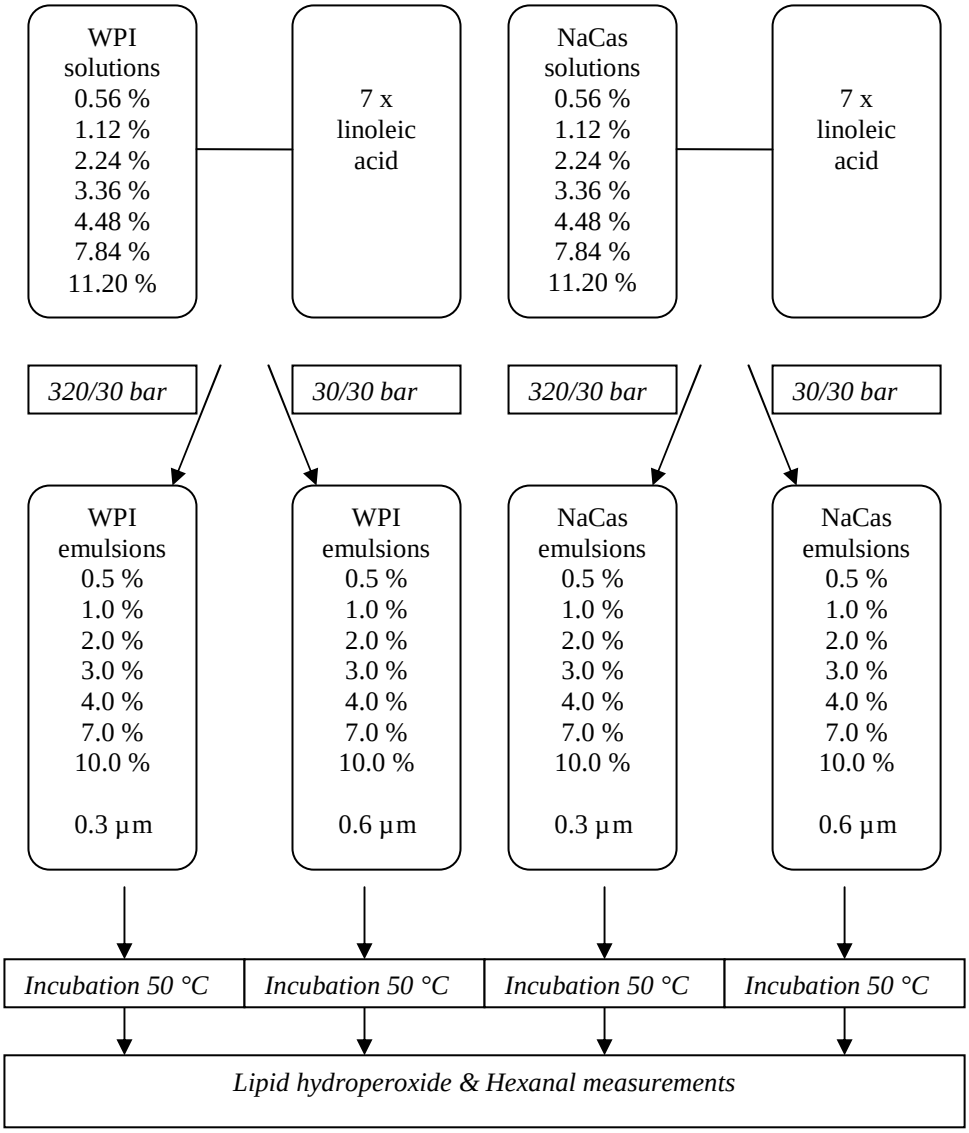


Figure 4.4: Flow chart of the experimental plan

Some studies about lipid oxidation in emulsions have shown that the pH is an important factor. A constant pH value for all sample types and over time was therefore regarded as an essential parameter. The pH was monitored over 24 h and measured after 0, 4, 12 and

24 hours of incubation time. Two independent sample containers were observed over time for all types of emulsion. For all samples the pH was found to be within the range 6.0 ± 0.2 at all times.

The d_{32} values measured for WPI and NaCas emulsions at various times are given in Table 4.2. The overall average for small droplet sized emulsions was 0.31 µm and for large droplet sized emulsions 0.65 µm (determined by averaging mean droplet sizes including all protein types, protein concentrations and incubations times at one pressure stage). After 24 h, some of the emulsions showed creaming (WPI 1.0 % and 3.0 %, large droplets) or aggregates had been formed and irreversible phase separation had occurred (WPI 0.5 %, large and small droplets; NaCas 0.5 %, large droplets). Lipid hydroperoxide and hexanal measurements were still carried out for these samples.

Table 4.2: Mean droplet sizes (d_{32} (µm)) over all protein concentrations after 0 h, 4 h, 12 h and 24 h of incubation time at 50 °C (omitting emulsions with irreversible physical instability) with standard error of the mean value* (SEM)

Incubation time (h)		Protein type			
		WPI		NaCas	
		Homogenisation pressure 30/30 bar	Homogenisation pressure 320/30 bar	Homogenisation pressure 30/30 bar	Homogenisation pressure 320/30 bar
0 h	Mean value	0.65	0.31	0.63	0.30
	SEM	0.01	0.01	0.01	0.01
4 h	Mean value	0.65	0.32	0.63	0.32
	SEM	0.02	0.01	0.01	0.01
12 h	Mean value	0.64	0.32	0.63	0.32
	SEM	0.03	0.01	0.01	0.01
24 h	Mean value	0.74	0.31	0.67	0.32
	SEM	0.05	0.01	0.02	0.01

* 12-14 replicates

Figure 4.5 and Figure 4.6 show lipid hydroperoxide and hexanal concentrations after incubation for 4 and 24 hours, respectively.

(i) Concentrations of lipid hydroperoxides

In emulsions made with 10.0 % protein, the lipid hydroperoxide levels of all emulsion types were very similar and ranged between 10 and 40 µmol/l.

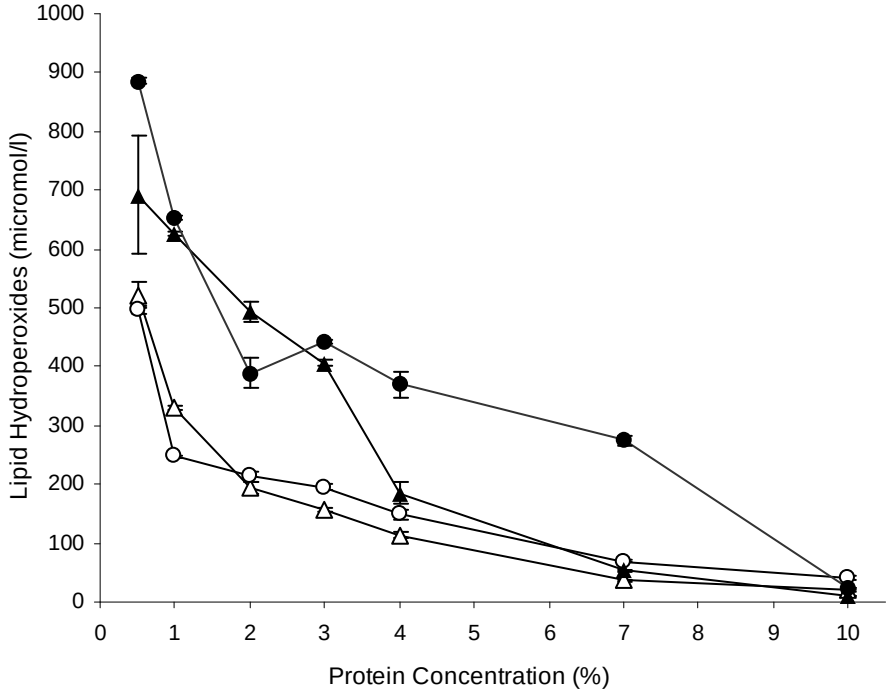


Figure 4.5: Lipid hydroperoxide concentration after 4 h for all emulsion types: WPI 0.65 µm (●), NaCas 0.65 µm (▲), WPI 0.31 µm (○) or NaCas 0.31 µm (△)

For large droplet sizes more lipid hydroperoxides were formed in WPI emulsions for protein concentrations from 4.0 % upwards compared with NaCas emulsions. The mean values measured for WPI emulsions at 4.0, 7.0 and 10.0 % were 370, 275 and 20 µmol/l as compared with only 185, 55 and 10 µmol/l in NaCas emulsions. Below 4.0 % protein, the hydroperoxides were fairly similar for both protein types: with increase in protein concentration from 0.5 to 3.0 %, the lipid hydroperoxides decreased from about 800 to 400 µmol/l.

The lipid hydroperoxide concentrations in small droplet sized emulsions were generally lower than for large droplet sized emulsions. Only large droplet sized NaCas emulsions

made with ≥ 4.0 % protein reached similarly low lipid hydroperoxide levels. Also the lipid hydroperoxide levels in small droplet sized emulsions were more similar for the two protein types over the whole protein concentration range. At 0.5 and 2.0 % protein content, there was no significant difference between the two proteins. At 1.0 % protein content, the lipid hydroperoxide concentration was higher in NaCas emulsions, but at all protein concentrations ≥ 3.0 % it was lower than in WPI emulsions. NaCas therefore appeared to be the better antioxidant at ≥ 3.0 % protein content.

(ii) Concentrations of hexanal

The hexanal levels in 10.0 % protein emulsions were similar (around 25 µmol/l), only the large droplet sized WPI emulsion showed a higher mean value of 125 µmol/l.

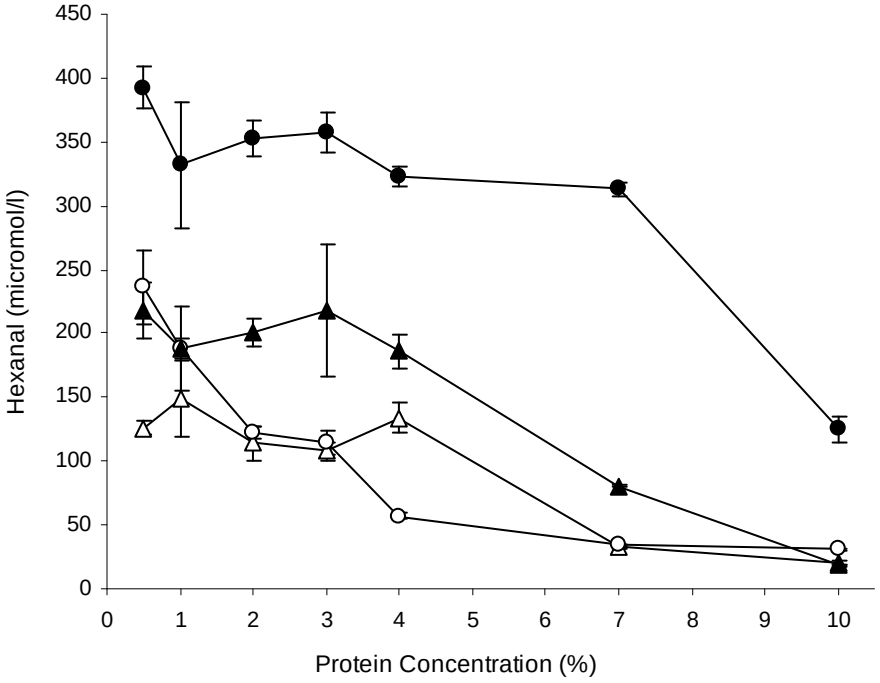


Figure 4.6: Hexanal concentration after 24 h for all emulsion types: WPI 0.65 µm (●), NaCas 0.65 µm (▲), WPI 0.31 µm (○) or NaCas 0.31 µm (△)

The hexanal values of large droplet sized WPI emulsions were the highest of all emulsions at all protein concentration levels. In WPI emulsions, the hexanal level

ranged between 300 and 400 µmol/l at protein levels between 0.5 to 7.0 %, but above 10 % WPI the hexanal concentration markedly decreased to 125 µmol/l. For NaCas emulsions, there was a similar behaviour for protein levels from 0.5 to 4.0 %, where the hexanal concentration seemed to be independent of the protein concentration. The hexanal values ranged from 190 to 220 µmol/l. At higher protein levels, there was a great decrease in hexanal concentration with increasing protein content. Mean values decreased from 190 to 80 to 20 µmol/l for NaCas levels of 4.0, 7.0 and 10.0 %, respectively.

In small droplet sized NaCas emulsions, from 0.5 to 4.0 % protein content, the hexanal concentration ranged between 110 and 150 µmol/l, but decreased to 35 and 20 µmol/l at 7.0 and 10.0 % protein, respectively. For WPI emulsions, the trend was different. With increasing protein level above 0.5 %, the hexanal generation decreased from 235 µmol/l (0.5 % WPI) to 55 µmol/l (4.0 % WPI). At higher WPI concentration, only a slight further decrease could be observed (34 µmol/l at 7.0 % WPI; 31 µmol/l at 10.0 % WPI).

The probability for the difference in lipid hydroperoxide and hexanal levels of WPI and NaCas emulsions is given in Appendix Chapter 4, Table 4B. There were no significant differences for hexanal production in small droplet sized NaCas emulsions between 0.5 to 4.0 % protein content, but there was a significant decrease, at the 0.05 level of probability, for small droplet sized WPI emulsions between 0.5 to 4.0 % protein content (Appendix Chapter 4, Table 4C).

ANOVA results

The mean values for lipid hydroperoxides over all protein concentration levels are depicted in Figure 4.7. The values decreased from 340 µmol/l at the 0.5 % protein level to 40 µmol/l at the 10.0 % protein level.

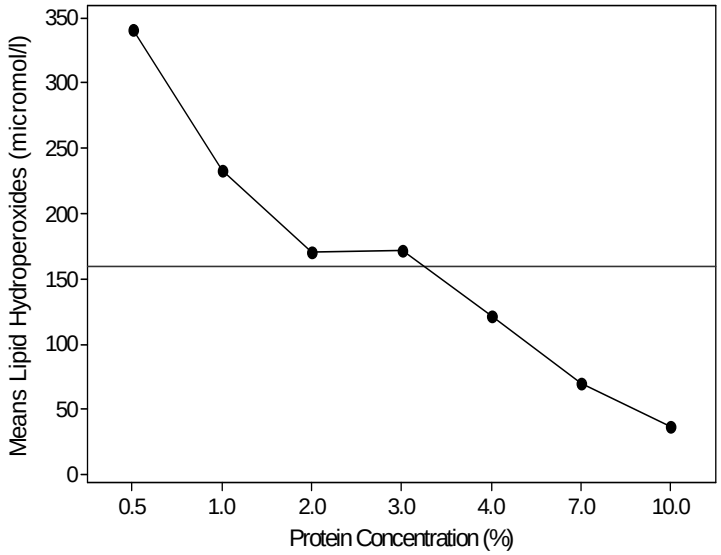


Figure 4.7: Means of lipid hydroperoxides averaging protein type, droplet size and incubation time over the whole protein concentration range ($p < 0.01$)

The corresponding data for hexanal is shown in Figure 4.8. The hexanal mean values decreased with increasing protein concentrations from 120 µmol/l at 0.5 % protein to 30 µmol/l at 10.0 % protein.

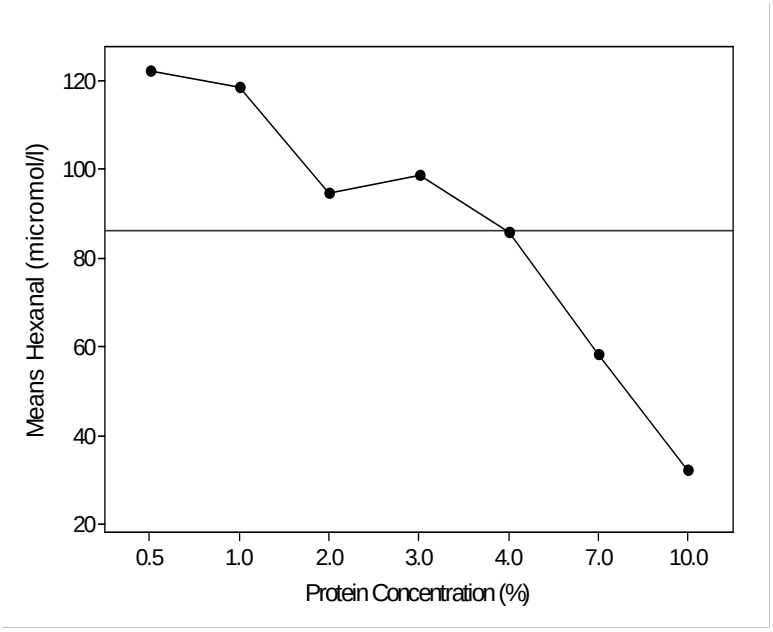


Figure 4.8: Means of hexanal averaging protein type, droplet size and incubation time over the whole protein concentration range ($p < 0.01$)

The general trend, previously discussed with the original data, that increasing protein contents lowered the lipid oxidation rate was confirmed by the ANOVA.

It was also shown that the effect of the difference in droplet size became less important for the production of lipid hydroperoxides with increasing protein concentration. This characteristic was not observed for hexanal. For WPI emulsions made with 0.5 to 10.0 % protein, the differences between the lipid hydroperoxide mean values of the two droplet sizes were slightly different than for NaCas emulsions (Figure 4.9 and Figure 4.10). A summary of the differences is given in Table 4.3.

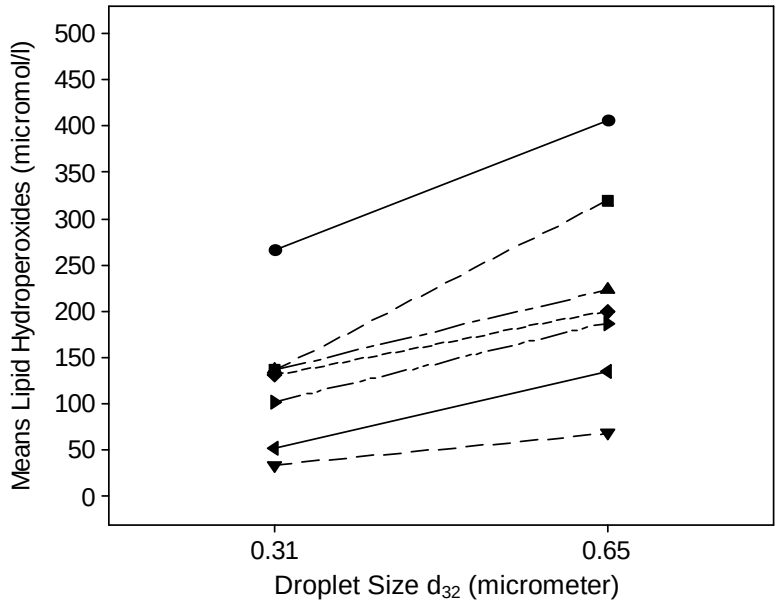


Figure 4.9: Mean lipid hydroperoxide concentrations (0–4 h) at d_{32} = 0.31 and 0.65 µm from 0.5 to 10.0 % WPI: 0.5 % (●), 1.0 % (■), 2.0 % (◆), 3.0 % (▲), 4.0 % (►), 7.0 % (◄) or 10.0 % (▼)

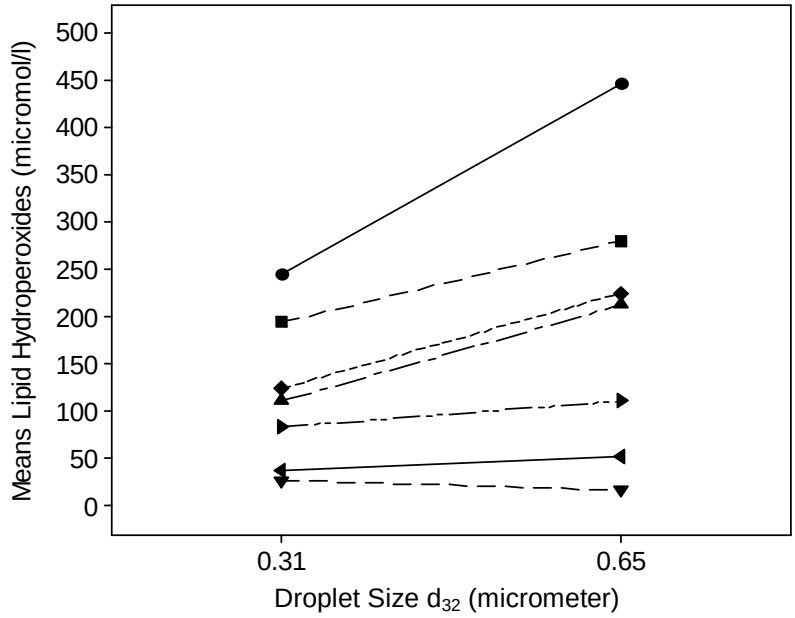


Figure 4.10: Mean lipid hydroperoxide concentrations (0–4 h) at d_{32} = 0.31 and 0.65 µm from 0.5 to 10.0 % NaCas: 0.5 % (●), 1.0 % (■), 2.0 % (◆), 3.0 % (▲), 4.0 % (►), 7.0 % (◄) or 10.0 % (▼)

Table 4.3: Differences in mean lipid hydroperoxide concentrations (µmol/l) between large and small droplet sized emulsions

	Protein concentration (%)						
	0.5	1.0	2.0	3.0	4.0	7.0	10.0
	Difference in mean lipid hydroperoxide concentration between large and small droplet sized emulsions (µmol/l)						
WPI emulsion	140	184	70	87	84	83	34
NaCas emulsion	200	86	101	102	28	14	- 11

In 10 % protein WPI emulsions and NaCas emulsions from 4.0 to 10.0 % protein content, the droplet size had only little influence on lipid hydroperoxide generation. In WPI emulsions from 2.0 to 7.0 % and NaCas emulsions from 1.0 to 3.0 % protein content, the droplet size had a greater influence. In WPI emulsions of 0.5 and 1.0 % and the NaCas emulsion of 0.5 % protein content, the droplet size had a strong impact on lipid hydroperoxide production.

These results confirmed that for NaCas and also for WPI emulsions, small droplet sizes suppressed lipid oxidation more than the large ones. Emulsions with small droplet size, high protein level and prepared with NaCas were in general more physically stable. Lipid oxidation did not seem to be influenced by creaming or the formation of aggregates after 24 hours as the hexanal concentrations were not significantly different to adjacent protein concentrations where it had not occurred. The initial droplet size seemed to be more important for the oxidative stability.

Lipid oxidation was in general lowered by an increase of the protein concentration. This general trend was expected as proteins had shown antioxidative potential in O/W emulsions before. Furthermore an increase of protein concentration led to a decrease in the difference in lipid hydroperoxide production between large and small droplet sized emulsions. The antioxidative effect of the protein concentration appeared to offset the effect of droplet size.

The antioxidative activity of each protein was dependent on the protein concentration and the droplet size. It could not simply be said that NaCas exhibited greater

antioxidative activity than WPI. Other researchers had found that NaCas protected emulsion lipids better from oxidation than WPI (Faraji et al. 2004; Hu et al. 2003b), but in this study the results were more complex. NaCas was the better antioxidant in large droplet sized emulsions of approximately 0.6 µm. Less hexanal was formed and lipid hydroperoxide generation was more inhibited. In small droplet sized emulsions NaCas only inhibited lipid hydroperoxide generation significantly better at protein concentrations of 3.0 % and higher. At lower protein concentration more lipid hydroperoxides were produced in the NaCas emulsion. In large droplet sized WPI emulsions, the hexanal level was constant and independent of the protein concentration up to 7.0 % but dropped at higher protein concentrations. In contrast, in small droplet sized WPI emulsions, the hexanal level decreased depending on the WPI content from lowest protein levels upwards. In large as well as small droplet sized NaCas emulsions, no significant change in the hexanal level could be observed up to 4.0 % protein but it decreased at higher protein concentrations. In small droplet sized emulsions the increased incorporation of WPI limited hexanal and therefore off-flavour production whereas NaCas inhibited hexanal independently of concentration. At low protein concentrations, NaCas showed more potential to inhibit hexanal formation than WPI.

The inhibition of lipid oxidation by proteins in O/W emulsions has been shown to be mostly due to free radical scavenging and metal ion chelation. Each protein exhibits its own specific "portfolio" of antioxidative potential which is likely to depend on the conditions of the system where it is employed. For most but not all conditions, NaCas showed a greater antioxidative potential than WPI. Generally speaking, the specific antioxidative feature of caseins is considered to be their metal chelating capacity because of their phosphoseryl groups (Baumy and Brule 1988; Bennett et al. 2000; Gaucheron et al. 1996) and for WPI it is its free radical scavenging activity due to free sulfhydryl groups (Faraji et al. 2004; Hu et al. 2003a; Kiokias et al. 2007; Kiokias et al. 2006; McClements and Decker 2000; Ostdal et al. 1996; Tong et al. 2000b). In whey protein, β-lactoglobulin contains one free sulfhydryl group (Hu et al. 2003a). Nevertheless both characteristics – phosphoseryl and free sulfhydryl groups – are not solely contributing to the total antioxidative capacity of the respective protein. It has been shown that the dephosphorylation of α-, and β-casein only partially suppressed their antioxidative activity in a liposome system (Cervato et al. 1999) and

caseinophosphopeptides high in phosphoseryl groups did not show the greatest antioxidative capability in a corn O/W emulsion compared with other casein-derived protein fractions (Diaz et al. 2003). When sulfhydryls of the high molecular weight fraction of whey were blocked with NEM in aqueous solution its AAPH-radical-scavenging activity was only reduced by 20 % (Tong et al. 2000b) and when skim milk was treated with $NaBH_4$ the concentration of sulfhydryls strongly increased but the oxygen uptake was only moderately lowered in an oxidation trial (Taylor and Richardson 1980b).

Moreover, each of the two milk proteins features both mechanisms, metal chelation and free radical scavenging. For example, a high molecular weight fraction of whey chelated ferrous iron in the continuous phase of a bovine serum albumin/hexadecane emulsion (Tong et al. 2000b) and WPI exhibited ferric iron binding capacity in aqueous solution, although not as much as NaCas, which bound about five fold more (Faraji et al. 2004). Caseins and peptides from tryptic hydrolysis of casein showed AAPH-radical-scavenging activity in a linoleic acid/Triton X-100 emulsion (Rival et al. 2001a).

Besides the question what molecular characteristics caused the antioxidative function of WPI and NaCas, it is also relevant to question where the antioxidative mechanisms predominantly took place. In the next chapter therefore the role of each protein in the different phases, interfacial and continuous, of the emulsion has been examined.

4.3 Role of unadsorbed protein in lipid oxidation

In section 4.2, it was observed that an increase in the protein concentration led to more oxidative stability of the emulsion. In O/W emulsions, only a limited amount of emulsifier can be adsorbed to form the interface of the emulsion droplets. The rest remains as excess in the continuous phase of the emulsion. This leads to the question what role the unadsorbed protein plays in oxidative stability. To investigate the role of continuous phase protein, a protein saturated and stable interface is required. It has been shown that the interface in WPI-based O/W emulsions (5 % oil) was saturated when the WPI concentration reached 0.2 % (Donnelly et al. 1998; Hu et al. 2003a). Dalgleish et

al. (1995) found stable interfacial layers of constant thickness for NaCas-based soy O/W emulsions between 0.5 to 2.0 % protein and 20 % lipid content. In the present experiments, the protein concentration (WPI, NaCas) was at least 1.0 % and the lipid content 10.6 %. At this protein-to-lipid ratio, protein surface coverage of the droplets can be assumed. Furthermore a constant droplet size of d_{32} = 0.31 µm was measured, indicating droplet stability. The surface protein concentration was determined as 1.1 mg/m^2 (WPI) and 1.5 mg/m^2 (NaCas), calculated according to the method of Srinivasan et al. (1996) (more about the calculation in Chapter 3, Materials and methods).

In the first experiment, the aim was to determine the importance of the continuous phase protein in lipid hydroperoxide and hexanal generation. Therefore the continuous phase of the emulsions was replaced with either RO water or the same protein solution used to prepare the emulsion. For the preparation of the emulsion, a concentration of 4.0 % was chosen as this was in the middle of the protein concentration range and up to which large changes in lipid hydroperoxide and hexanal production had already occurred. In the second experiment, 1.0 % protein emulsions were prepared and the continuous phase protein level was varied up to 4.0 %. For all emulsions, the small droplet size was chosen, as this provided a greater physical stability.

The experimental plan is depicted in Figure 4.11. The emulsions (4.0 % protein) where the continuous phase was substituted with protein solution (4.48 % protein) had a slightly higher total protein concentration than the original emulsion, as no adjustment for the amount of protein absorbed in the interface was made in the replacement protein solution. More experimental details can be found in Chapter 3, Materials and methods.

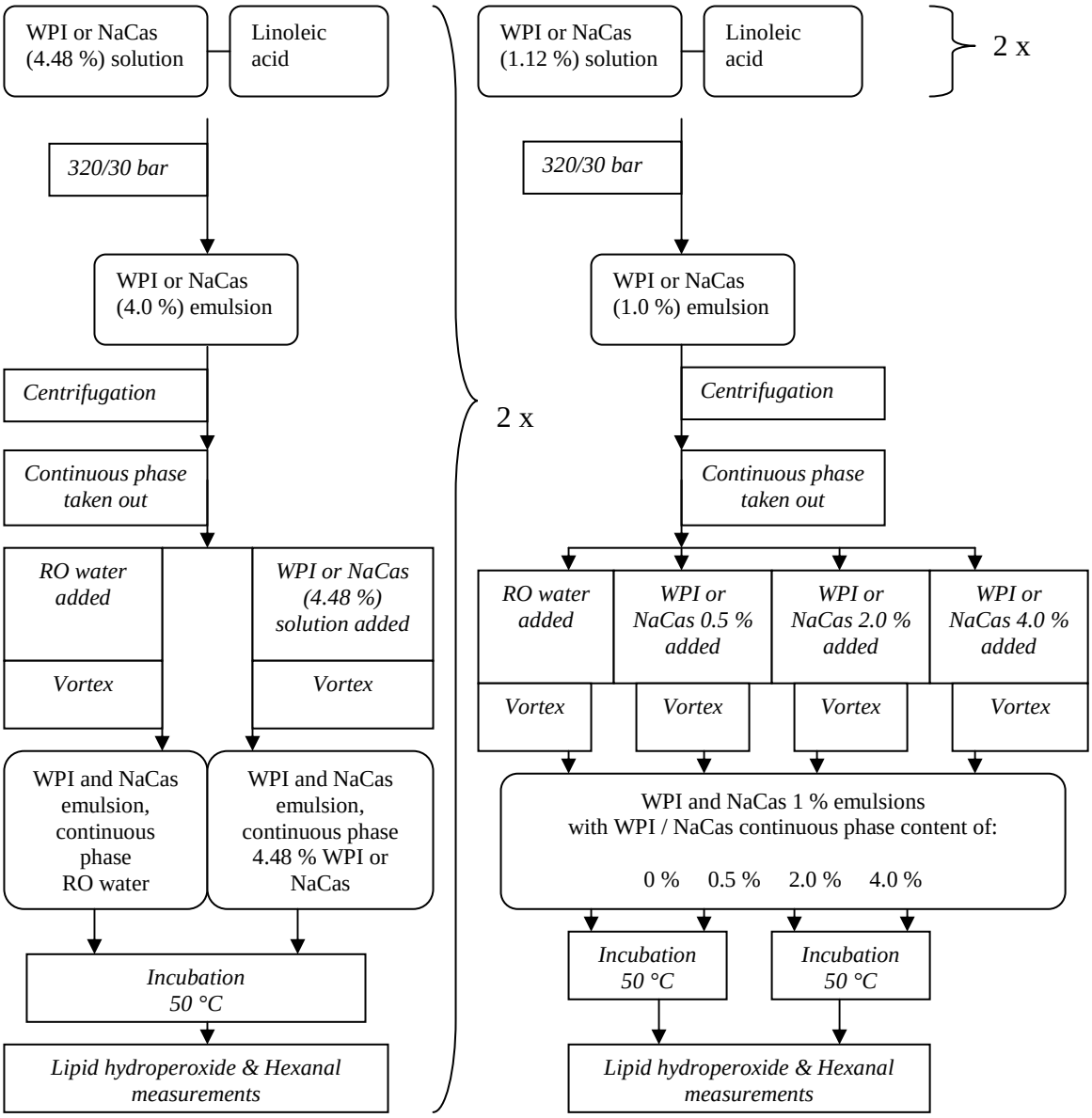


Figure 4.11: Flow chart of the experimental plans

The droplet size was determined as d_{32} in WPI and NaCas emulsions (4.0 % protein) and ranged for all sample types from 0.28 to 0.32 µm within the 24 h incubation period. In Figure 4.12 and Figure 4.13, the generation of lipid hydroperoxides and hexanal is shown. In Appendix Chapter 4, Table 4D it is shown which samples were significantly different.

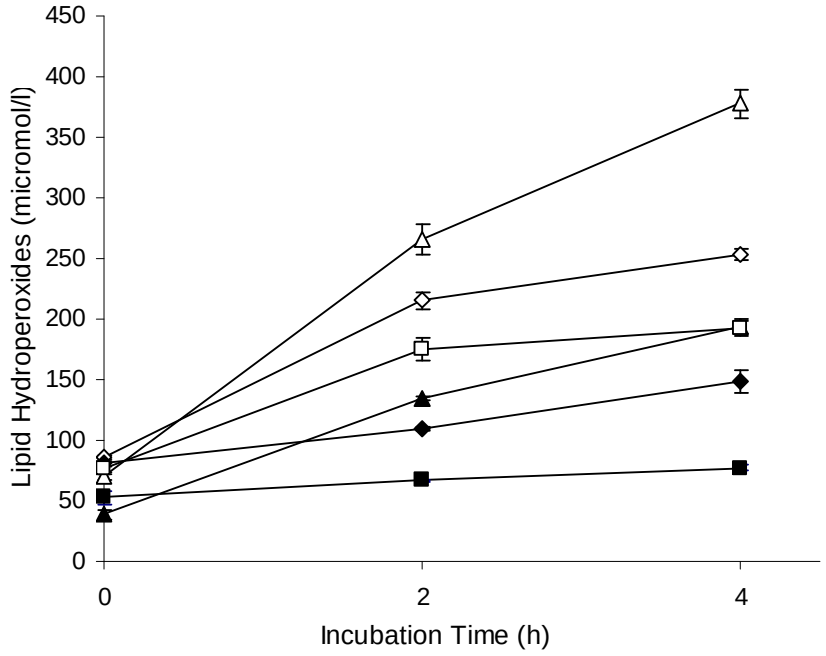


Figure 4.12: Lipid hydroperoxide development over 4 h in milk protein (4.0 %) emulsions with replaced continuous phase: WPI emulsion where continuous phase was replaced with RO water (△), **WPI control emulsion** (◇)**, WPI emulsion where continuous phase was replaced with WPI (4.48 %) solution** (□)**, NaCas emulsion where continuous phase was replaced with RO water** (▲)**, NaCas control emulsion** (◆) **or NaCas emulsion where continuous phase was replaced with NaCas (4.48 %) solution** (■)

After 4 h, the lipid hydroperoxide level of the WPI control emulsion rose to 250 µmol/l but only to 150 µmol/l in the NaCas control. When the continuous phase was replaced with RO water the lipid hydroperoxides rose to 380 µmol/l in the WPI and to 190 µmol/l in the NaCas emulsion. When the continuous phase was replaced with protein solution the lipid hydroperoxides were reduced to 190 µmol/l in the WPI and to 75 µmol/l in the NaCas emulsion. The same tendency was observed for WPI as well as NaCas emulsions i.e. the replacement of the continuous phase with RO water increased the lipid hydroperoxide production and the replacement with protein solution lowered it compared with the control emulsion. Furthermore, the lipid hydroperoxide concentration was lower in NaCas emulsions than in WPI emulsions.

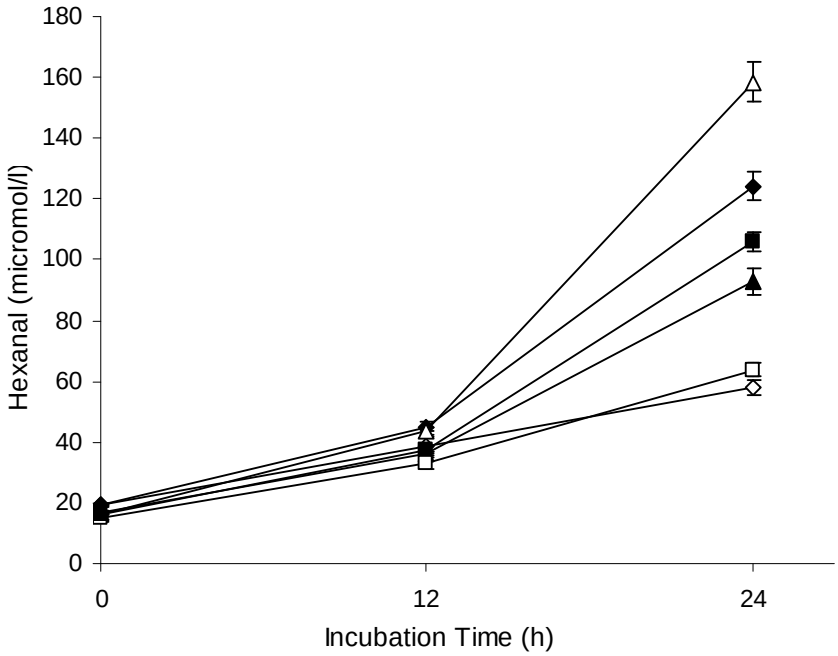


Figure 4.13: Hexanal development over 24 h in milk protein (4.0 %) emulsions with replaced continuous phase: WPI emulsion where continuous phase was replaced with RO water (△), **WPI control emulsion** (◇)**, WPI emulsion where continuous phase was replaced with WPI (4.48 %) solution** (□)**, NaCas emulsion where continuous phase was replaced with RO water (▲), NaCas control emulsion (◆) or NaCas emulsion where continuous phase was replaced with NaCas (4.48 %) solution (■)**

After 24 h the hexanal level of the WPI control emulsion rose to 60 µmol/l and to 125 µmol/l in the NaCas control. These values were very similar to the hexanal results found in the previous section (WPI and NaCas, 4.0 %, 0.3 µm, 24 h). When the continuous phase was replaced with RO water the hexanal level rose to 160 µmol/l in the WPI but only to 95 µmol/l in the NaCas emulsion. When the continuous phase was replaced with protein solution the hexanal level was at 65 µmol/l in the WPI and at 105 µmol/l in the NaCas emulsion after 24 h.

The hexanal production in WPI and NaCas emulsions was different for different treatments. The hexanal level was very similar and not significantly different (Appendix Chapter 4, Table 4D) when the continuous phase was replaced with WPI solution (65 µmol/l) compared with the WPI control (60 µmol/l). However, the hexanal level was much higher when RO water was in the continuous phase (160 µmol/l). For NaCas there

was not much difference – although significant – in all three emulsion types: NaCas control emulsion (125 µmol/l), replacement with NaCas solution (105 µmol/l) and replacement with RO water (95 µmol/l). It is believed that the random variation of hexanal levels observed for NaCas emulsions with 0.5 to 4.0 % protein content (between 100 to 150 µmol/l hexanal) – see Figure 4.6 – is also mirrored in these results. This may indicate a low influence of continuous phase NaCas on hexanal generation.

The droplet size in WPI and NaCas emulsions (1.0 % protein) ranged for all sample types from d_{32} = 0.28 to 0.34 µm within the 24 h incubation period. Figure 4.14 and Figure 4.15 show lipid hydroperoxide and hexanal concentrations with various continuous phase protein levels after 4 and 24 h respectively.

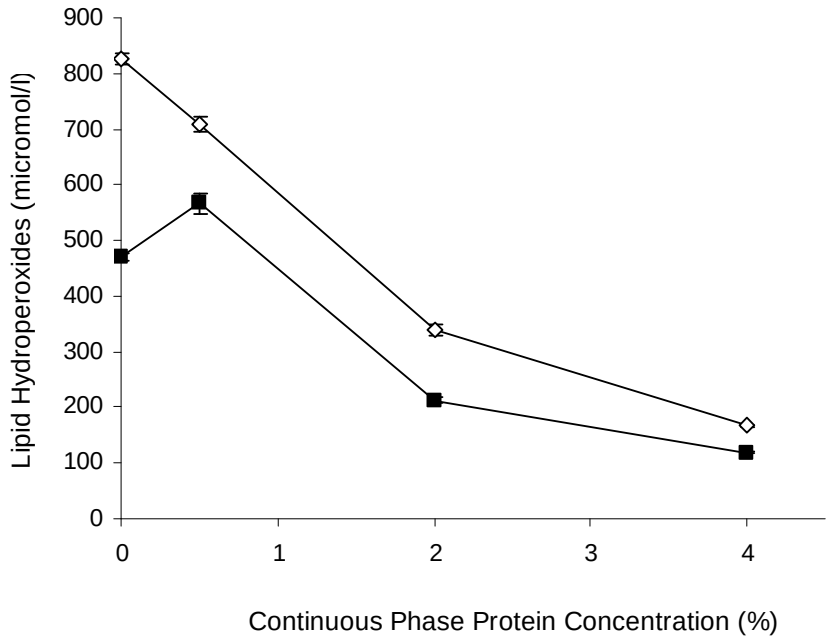


Figure 4.14: Lipid hydroperoxide concentration after 4 h in milk protein emulsions with various continuous phase protein levels: WPI (◇), NaCas (■)

The lipid hydroperoxide concentration was significantly lower for NaCas compared with WPI emulsions over the whole protein concentration range (Figure 4.14 and Appendix Chapter 4, Table 4F). Generally there was a decrease in lipid hydroperoxide concentration with increasing continuous phase protein level. For WPI emulsions, it

decreased from 825 µmol/l (0 %) to 710 µmol/l (0.5 %) to 340 µmol/l (2.0 %) and 165 µmol/l (4.0 %). For NaCas emulsions, there was first an increase in lipid hydroperoxides from 470 µmol/l (0 %) to 565 µmol/l (0.5 %). Thereafter it dropped to 215 µmol/l (2.0 %) and 120 µmol/l (4.0 %). The lipid hydroperoxide mean values were significantly different within one protein type (Appendix Chapter 4, Table 4E).

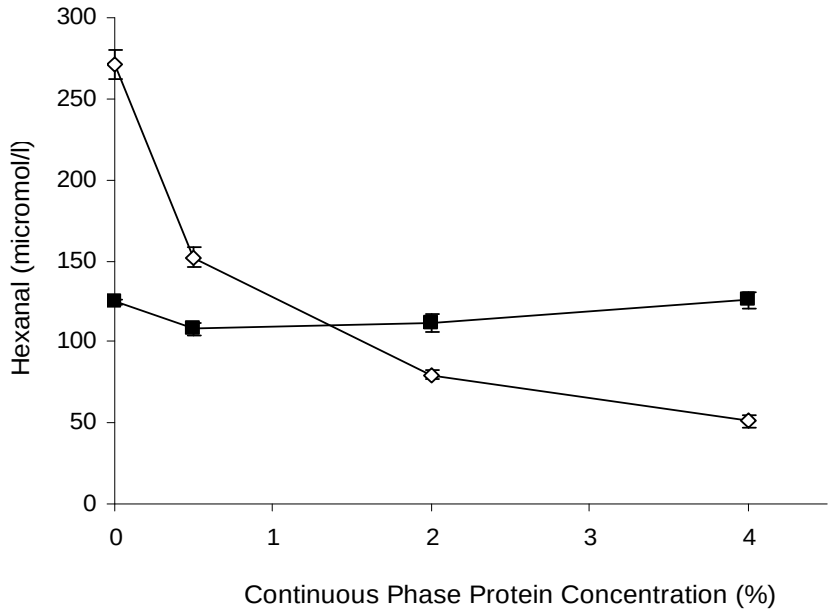


Figure 4.15: Hexanal concentration after 24 h in milk protein emulsions with various continuous phase protein levels: WPI (◇), NaCas (■)

The hexanal concentration decreased from 270 µmol/l (0 %) to 150 µmol/l (0.5 %) to 80 µmol/l (2.0 %) to 50 µmol/l (4.0 %) in WPI emulsions (Figure 4.15). In contrast, the hexanal concentration in NaCas emulsions (0.5 to 4.0 % protein) did not change significantly with protein concentration (125 µmol/l, 108 µmol/l, 112 µmol/l, 126 µmol/l with increasing protein level). The hexanal values were significantly different for all continuous phase WPI levels but not for most NaCas levels (Appendix Chapter 4, Table 4E).

4.4 Addition of milk protein solutions to milk-protein-based emulsions and the influence on lipid oxidation

In the previous results, NaCas was particularly antioxidative towards lipid hydroperoxide formation and delivered lower hexanal levels at low protein concentrations. In contrast, the inhibition of hexanal generation in WPI emulsions was concentration dependent, and hexanal levels were higher at low protein concentrations. The continuous phase protein level contributed largely to these effects. The next experiment was carried out to determine whether the positive characteristics of both proteins could be combined. The emulsions were diluted with milk protein solutions to find out if the low hexanal level in NaCas emulsions (1.0 % protein) could be further lowered by dilution with WPI solution, and if the lipid hydroperoxide production in WPI emulsions could be decreased by dilution with NaCas solution.

Whether or not similar results could be obtained by using different protein concentrations was also tested. If 2.0 % WPI or NaCas solution was added to a 1.0 % WPI or NaCas emulsion this would increase the continuous phase protein concentration, and the lipid hydroperoxide production should be inhibited for either protein type. Also, less hexanal generation might be expected in the case of WPI but not NaCas addition, as only an increase of continuous phase WPI lowered the hexanal generation (up to 4 % protein). If the emulsions were diluted with RO water (i.e. a decrease of the continuous phase protein concentration) it would be expected that more lipid hydroperoxides would be formed for both protein types. Also more hexanal would be produced in WPI than NaCas emulsions diluted with RO water as a change of continuous phase WPI caused more changes in hexanal production.

The experimental plan is depicted in Figure 4.16. The diluents were added to the emulsions in the lipid hydroperoxide and hexanal measurement containers. The same amount of linoleic acid was therefore present in the measurement containers with diluted and undiluted emulsions. In diluted emulsions, the new lipid concentration was 7.1 % and the new protein concentration 0.7 % (dilution with RO water) and 1.3 % (dilution with protein solution).

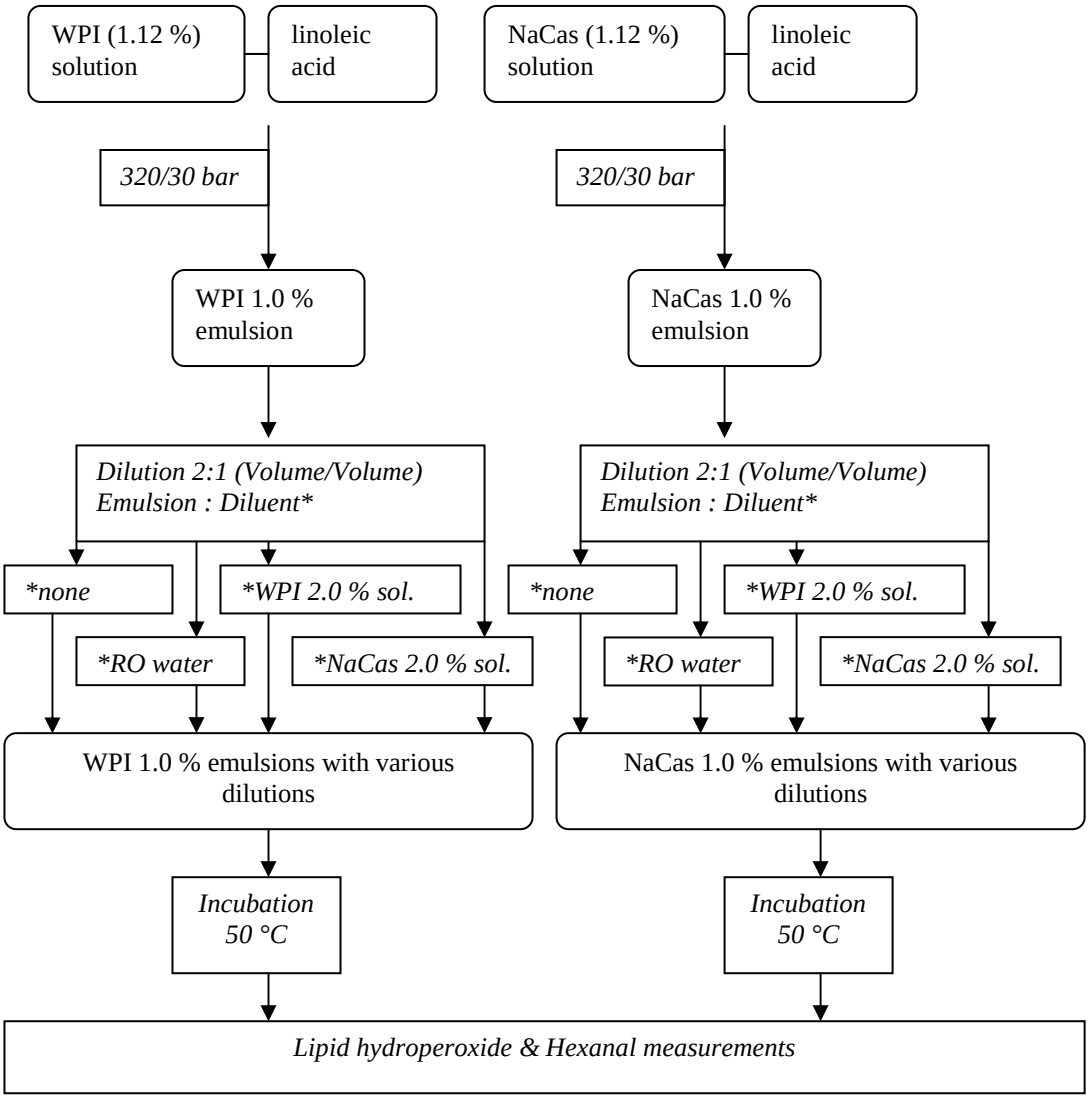


Figure 4.16: Flow chart of the experimental plan

The development of lipid hydroperoxides and hexanal is shown in Figure 4.17 and Figure 4.18. The significance of the difference between samples is shown in Appendix Chapter 4, Table 4G.

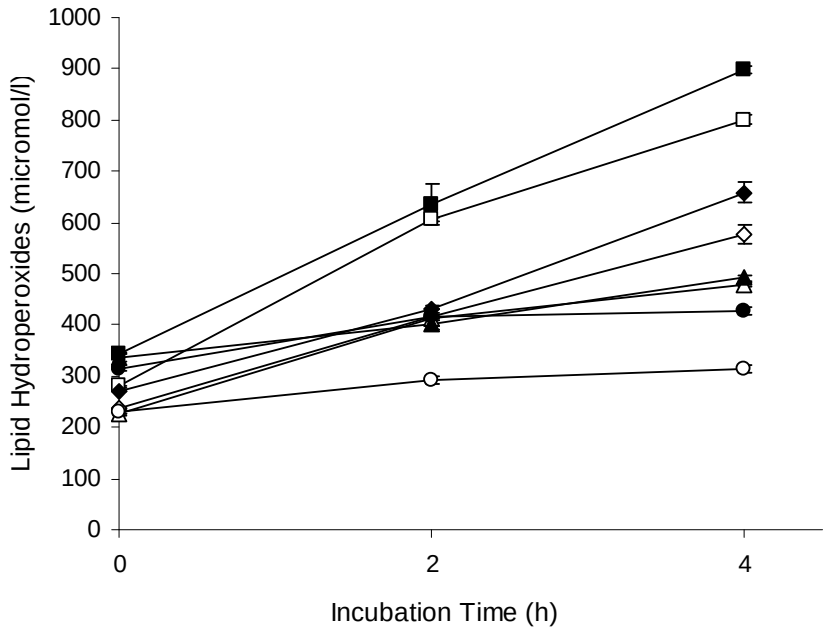


Figure 4.17: Dilution of emulsions with protein solutions and the effect on lipid hydroperoxide generation: WPI (1.0 %) emulsion (◇), **WPI (1.0 %) emulsion + RO water (2:1)** (□), **WPI (1.0 %) emulsion + WPI (2.0 %) solution (2:1)** (△), **WPI (1.0 %) emulsion + NaCas (2.0 %) solution** (○), **NaCas (1.0 %) emulsion** (◆), **NaCas (1.0 %) emulsion + RO water (2:1) (■), NaCas (1.0 %) emulsion + NaCas (2.0 %) solution (2:1) (▲) or NaCas (1.0 %) emulsion + WPI (2.0 %) solution (2:1) (●)**

The dilution of both emulsion types with RO water led to the highest lipid hydroperoxide values of all samples (Figure 4.17). The mean values for lipid hydroperoxides were 800 µmol/l and 900 µmol/l for WPI and NaCas emulsions diluted with RO water, respectively, after 4 h. For the undiluted NaCas emulsion and the undiluted WPI emulsion, these values were 660 and 575 µmol/l respectively. The NaCas emulsion diluted with NaCas solution exhibited 490 µmol/l lipid hydroperoxides. It was not significantly different from the WPI emulsion diluted with

WPI solution which gave 475 µmol/l. The NaCas emulsion diluted with WPI solution showed a slightly lower mean value of 425 µmol/l. The lowest of all mean values (315 µmol/l) was found in the WPI emulsion diluted with NaCas solution.

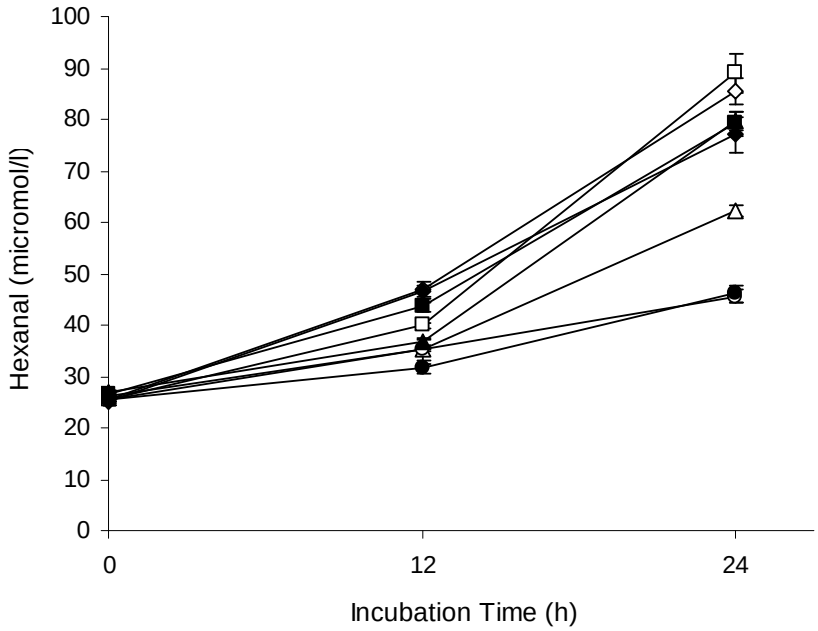


Figure 4.18: Dilution of emulsions with protein solutions and the effect on hexanal generation: WPI (1.0 %) emulsion (◇), **WPI (1.0 %) emulsion + RO water (2:1)** (□), **WPI (1.0 %) emulsion + WPI (2.0 %) solution (2:1) (△), WPI (1.0 %) emulsion + NaCas (2.0 %) solution (2:1) (○), NaCas (1.0 %) emulsion (◆), NaCas (1.0 %) emulsion + RO water (2:1) (■), NaCas (1.0 %) emulsion + NaCas (2.0 %) solution (2:1) (▲) or NaCas (1.0 %) emulsion + WPI (2.0 %) solution) (2:1) (●)**

The hexanal concentrations of several samples were fairly similar after 24 h and not significantly different (Figure 4.18 and Appendix Chapter 4, Table 4G). The values ranged between 75 and 90 µmol/l. The WPI emulsion diluted with WPI solution gave a lower hexanal concentration of 60 µmol/l. When both proteins were combined the hexanal level was the same and overall the lowest (45 µmol/l).

Table 4.4: Absolute values and relative values of lipid hydroperoxides and hexanal compared with the undiluted control emulsion

Sample Type	Lipid hydroperoxides after 4 h (µmol/l)	Hexanal after 24 h (µmol/l)	Difference in lipid hydroperoxides after 4 h compared to control (µmol/l)	Difference in hexanal after 24 h compared to control (µmol/l)
WPI control*	577	85.4	0	0
WPI + RO water*	801	89.0	+ 224	+ 3.4
WPI + WPI*	477	72.1	- 100	- 13.3
WPI + NaCas*	315	45.6	- 262	- 39.8
NaCas control*	659	76.9	0	0
NaCas + RO water*	898	79.1	+ 239	+ 2.2
NaCas + NaCas*	492	79.6	- 167	+ 2.7
NaCas + WPI*	427	46.1	- 232	- 30.8

* Explanation of the samples: WPI control = WPI (1 %) emulsion, WPI + RO water = WPI (1 %) emulsion + RO water (2:1), WPI + WPI = WPI (1 %) emulsion + WPI (2 %) solution (2:1), WPI + NaCas = WPI (1 %) emulsion + NaCas (2 %) solution (2:1), NaCas control = NaCas (1 %) emulsion, NaCas + RO water = NaCas (1 %) emulsion + RO water (2:1), NaCas + NaCas = NaCas (1 %) emulsion + NaCas (2 %) solution (2:1), NaCas + WPI = NaCas (1 %) emulsion + WPI (2 %) solution (2:1)

An overview about the actual effects in terms of increase or decrease in the lipid oxidation rate compared with the undiluted control emulsion of the respective protein type is given in Table 4.4. More lipid hydroperoxides were generated in both WPI and NaCas emulsions diluted with RO water but there was no significant increase in hexanal generation in either type of emulsion. In WPI emulsion diluted with WPI solution, considerably less lipid hydroperoxides were produced, and in NaCas emulsions diluted with NaCas solution there was an even stronger decrease in lipid hydroperoxides. The decrease in hexanal production was much smaller and not significant for the NaCas emulsion diluted with NaCas solution (compared with the WPI emulsion diluted with WPI solution). NaCas caused a stronger lipid hydroperoxide decrease in the WPI emulsion than WPI in the NaCas emulsion and NaCas was much more effective in reducing hexanal in the WPI emulsion than WPI added to the NaCas emulsion. The combination of both milk proteins had a stronger antioxidative effect than a single protein alone.

These experiments have shown how the presence or absence of continuous phase protein influenced lipid oxidation. The initial protein concentration used to prepare the emulsions appeared to have influenced lipid oxidation in terms of altering the continuous phase protein concentration. This was indicated by similar values of lipid hydroperoxides (4 h) and hexanal (24 h) over the protein and continuous phase protein concentration (comparison of data in Section 4.2 and present data). The lower the continuous phase protein concentration of either milk protein type was, the more lipid hydroperoxides were produced. With increasing continuous phase protein concentration the hexanal production decreased in the WPI, but not in the NaCas emulsion where it remained constant.

The dilution of milk protein emulsions (2:1, volume emulsion/volume diluent) with RO water or the protein solution used to prepare the emulsion led, for most sample types, to the expected effects. In comparison to their control, WPI and NaCas emulsions showed higher lipid hydroperoxide levels with decreased continuous phase protein content. Furthermore, neither increase nor decrease of continuous phase NaCas level led to a change of the hexanal concentration in the NaCas emulsion. However, an increase of continuous phase WPI led to the expected decrease of hexanal in the WPI emulsion. When both milk proteins were combined, a synergistic antioxidative effect could be observed that was much stronger than when any of the two proteins was used alone. Especially when WPI emulsion was enriched with NaCas solution, lipid oxidation was markedly inhibited. A positive antioxidative interaction of the two proteins appears to have taken place.

4.5 General discussion

McClements and Decker (2000) pointed out that the driving factor in lipid oxidation of O/W emulsions is the decomposition of lipid hydroperoxides in the presence of transition metals. Higher concentrations of lipid hydroperoxides may be present at the interface due to their partially polar character. Benjelloun et al. (1991) have shown that conventionally processed food oil contained traces of iron and copper and that the

treatment with chelators markedly slowed down lipid oxidation. Although there might be only traces of metals in the oil phase of an emulsion, the immediate proximity to oxidisable substrate increases their significance. In protein-stabilised emulsions, it also appears likely that they would associate with anionic protein moieties on the inner side of the interface due to their positive charge. There might be a lower metal concentration in the non-polar lipid core of droplets. In the present study there is a possibility that an antioxidative mechanism took place where interfacial proteins bound trace metal ions from the oil phase, preventing their interaction with lipids and lipid hydroperoxides. Furthermore, some of those interfacial protein molecules might have taken part in a dynamic exchange process with protein molecules from the continuous phase.

A study of Cho et al. (2003) greatly supports the theory of transfer of iron from the lipid to the continuous phase. With increasing concentrations of chelators (EDTA, sodium tripolyphosphate or citrate) in the continuous phase of an O/W emulsion (emulsifier: polyoxyethylene 10-lauryl ether), they observed a stronger depletion of ferric iron from the lipid phase. As a consequence lipid oxidation in the emulsion was more inhibited.

In the present study a small proportion of the total metal content may be incorporated into the emulsion system via the proteins and the aqueous solution. A possible external source of metal ions could have been the homogenisation process where the emulsion is exposed to stainless steel surfaces on the pre-blender and homogeniser. Some metal ions probably entered the system in association with proteins. Protein bound metal ions do not necessarily take part in the lipid oxidation process if they are strongly bound into the protein structure. In addition, proteins can chelate externally added metal ions.

Tong et al. (2000b) reported that the addition of ferrous iron to a bovine-serum-albumin-stabilised emulsion turned the initially negative zeta-potential to a positive value. When EDTA and also a high molecular weight fraction of whey protein were added to the continuous phase the value was lowered again below the level before iron addition. When EDTA and that whey protein fraction were added to a Tween 20 emulsion, a lower lipid oxidation rate was observed. This indicated that the whey protein and EDTA could partly have had the same antioxidative mechanism i.e. the removal of metal ions from the droplet surface into the continuous phase and thereby

preventing iron from further interaction with lipids and lipid hydroperoxides. In a similar study, Mei et al. (1998a) found that ferrous and ferric iron associated with the surface of negatively charged droplets of a SDS-stabilised n-hexadecane emulsion, as indicated by an increase of the zeta-potential. In emulsions with neutral and cationic surface charge, this was not observed. Chelators (EDTA, phytate) depleted the metal ions from the surface of the SDS-stabilised droplets into the continuous phase depending on their concentration. In the presence of chelators also, lipid oxidation in a SDS-stabilised salmon O/W emulsion was inhibited. In the study of Hu et al. (2003a) WPI-based salmon O/W emulsions were more oxidatively stable at pH 3 than at pH 7. They attributed the finding to the positive charge of WPI on the droplet surface at pH 3, repelling metal ions into the continuous phase. Carboxy and amino groups are either protonated (low pH, net positive charge below the pI) or deprotonated (high pH, net negative charge above the pI). A net negative charge causes more cation attraction than repulsion and for a net positive charge it is the other way around (Remondetto et al. 2004). These findings underline the significance of iron located at the interface of emulsion droplets for the lipid oxidation process.

In the present study, proteins of WPI and NaCas, located at the droplet surface and in the continuous phase, were presumably negatively charged as the pH value (pH 6) was above the pI of the proteins (pI whey protein: pH 5.2 (Ju and Kilara 1998), pI NaCas: pH 4.6 (Chen et al. 1999)). Metal ions would therefore have been attracted to the droplet surface as well as to the proteins in the continuous phase. On the other hand, the proportion of negatively charged proteins present in the continuous phase increased with increase in protein concentration in the system, whereas the negative charge on the droplets remained at the same level. Therefore, the removal of positively charged iron from the droplet surface into the continuous phase appears more likely to occur at the higher continuous phase protein concentrations. The relative affinity of various milk proteins for ferric iron was found to decrease in the following order: α_{s1}-casein > β-casein > bovine serum albumin > κ-casein > β-lactoglobulin > α-lactalbumin. (Hekmat and McMahon 1998). In α_{s1}-, α_{s2}- and β-caseins, phosphorylated side chains of serine are located in the polar moieties of the proteins, which exhibit chelation capacity for ferric iron. Furthermore, the Fe^{2+}/Fe^{3+} equilibrium is thereby affected and this can promote the conversion of ferrous to ferric iron. Since ferric iron is less pro-oxidative,

this process would be advantageous (Elias et al. 2008). Ferrous iron is required for the reaction of lipid hydroperoxides to secondary oxidation products via alkoxyl radicals (Frankel 1998b; McClements and Decker 2000).

For whey proteins, bovine serum albumin can chelate one cupric ion per molecule and inhibited copper-catalysed oxidation of low density lipoproteins (Elias et al. 2008; Schnitzer et al. 1997). The copper concentration in food is generally lower than the iron concentration but copper is also deemed more pro-oxidative (Elias et al. 2008). A prominent iron chelator in whey is the minor whey protein lactoferrin. It also contains iron in its native state and has about 78 to 85 % more iron binding capacity (Satue-Gracia et al. 2000; Steijns and van Hooijdonk 2000). Lactoferrin can bind two ferric ions per molecule (Nielsen et al. 2004). It has also shown antioxidative capacity added to milk, mayonnaise and an infant formula (Nielsen et al. 2004; Satue-Gracia et al. 2000). On the other hand, heat processing is expected to change the structure of lactoferrin irreversibly leading to reduced activity (Brisson et al. 2007). Lactoferrin as part of the WPI used in the present experiments might therefore have had limited chelation capacity as WPI production involves an elevated temperature in the spray drying process. Besides the specific metal chelation abilities of certain milk proteins, general binding mechanisms exist as amino acid side chains of aspartic acid, glutamic acid, histidine, phosphorylated serine, threonine, arginine and lysine (Arcan and Yemenicioglu 2007; Elias et al. 2008) can bind metal ions. Most of these amino acids are commonly found in proteins and would also account for some of the metal binding capacity of WPI and NaCas.

In combination with pro-oxidative transition metals, free radicals are the other driving force in lipid oxidation. One free radical source in O/W emulsions is the lipid phase. Because of the susceptibility of unsaturated fatty acids towards oxidation, at least a low level of free radicals is generally present in the raw material. Homogenisation, a process with a high energy input, contributes to the formation of free radicals that will be located in continuous phase, interface and oil phase. Continuous-phase-born free radicals that have been created during homogenisation can possibly migrate to the lipid phase (Coupland et al. 1996). In the presence of oxygen, peroxyl radicals are formed from lipid radicals leading further to hydroperoxides. Lipid hydroperoxides are

commonly located at the interface of the emulsion droplets. They are either produced there or diffuse to there from the non-polar droplet core (Nuchi et al. 2002). With the decomposition of lipid hydroperoxides by ferrous and ferric iron, alkoxyl- and peroxyl radicals are created respectively. They are a vital element for the propagation of lipid oxidation (McClements and Decker 2000). Interference at this stage of lipid oxidation by free radical scavengers is therefore an important antioxidative mechanism.

A high concentration of continuous phase protein, as in the present system, increases the potential for free radical scavenging in continuous phase, interface and lipid phase. An imaginable antioxidative mechanism could have been the scavenging of alkoxyl and peroxyl radicals by interfacial protein and continuous phase protein, which accessed interfacial regions with high levels of those radicals. The access would partly have depended on the property of the interface to act as physical barrier. Particularly caseins can form thick interfacial layers of $\leq$ 10 nm and for whey proteins 1–2 nm thick interfacial layers have been reported (Dalgleish 1993; Dalgleish et al. 1995; Fang and Dalgleish 1993; Mackie et al. 1991). In spite of this, low molecular weight compounds of WPI and NaCas, with possible free-radical-scavenging activity, might have entered those interfacial areas from the continuous phase.

On the other hand, the access of oxygen and metal ions from the continuous phase could have been restricted by a thick interfacial barrier, leading to more oxidative stability. This aspect might have played a role for large droplet sized emulsions of 4.0 and 7.0 % protein content as much less lipid hydroperoxides were generated in NaCas than in WPI emulsions. The transfer of oxygen might have been more limited across thicker casein than whey protein interfaces. However, as continuous phase protein appeared to greatly influence the oxidative stability of the emulsions, interaction between larger molecules than oxygen with susceptible lipids and lipid hydroperoxides did probably occur. A restriction of the oxygen transfer across the protein interfaces seems therefore not so likely. As metal ions have a somewhat similar size like oxygen molecules – compared with larger organic molecules of the protein fractions – the protein interfaces would probably also not inhibit metal ion migration by means of acting as physical barrier.

Whey proteins have been shown to exhibit free-radical-scavenging activity via free sulfhydryls and other mechanisms (Elias et al. 2006; Pazos et al. 2006; Tong et al. 2000b). Also caseins were found to scavenge free radicals (Diaz and Decker 2004; Rival et al. 2001a). The free sulfhydryl groups of β-lactoglobulin represent a specific molecular feature in whey protein that causes free radical scavenging ability. Although this feature has been referred to many times, there are also other free-radical-scavenging mechanisms in whey proteins and proteins in general.

Elias et al. (2008) points out that all 20 amino acids can form new radicals if the energy level of the radical they get in contact with is high enough, for example hydryoxyl radicals. But only if the new protein radical is on a low energy level with little reactivity that restricts the passing on of the free radical to susceptible materials like unsaturated lipids, the free-radical-scavenging process is antioxidative. In that respect, the three dimensional structure of the protein plays a role as free radicals can be directed to the proteins` interior where they are locally separated and not harmful to other substrates. An example are the findings of Ostdal et al. (1999) who found that tyrosyl radicals in the interior of bovine serum albumin were stable and long lived unlike reactive and short lived free tryrosine radicals.

Besides low reactivity of protein radicals, an important antioxidative feature is that they are easily generated in the first place, thereby allowing interference with the radical chain reactions in lipid oxidation. Elias et al. (2005), for example, found that free sulfhydryls and tryptophan residues of continuous phase β-lactoglobulin oxidised before they could detect lipid oxidation in a Brij-stabilised menhaden O/W emulsion. In general, the ability of a protein to interact with or scavenge free radicals can be indicated by the oxidation of amino acid side chains. Their modification suggests that they are a preferred target and marks them as a reaction partner of free radicals in competition with unsaturated fatty acids. In this way, unsaturated fatty acids are protected from oxidation.

Free radicals as part of esterified unsaturated fatty acids probably also led to the oxidation of free sulfhydryls (first) and tryptophan (second) but not methionine residues of continuous phase β-lactoglobulin in a Brij-stabilised menhaden O/W emulsion (Elias

et al. 2005). In whey protein-stabilised O/W emulsions, tryptophan side chains oxidised on the interface (Rampon et al. 2001; Viljanen et al. 2005). Another example where the free sulfhydryl level decreased was given by Tong et al. (2000b) who added whey protein to the continuous phase of a Tween 20/salmon O/W emulsion. However, in regard of sulfhydryl groups also pro-oxidative activity has been found. Hu et al.(2003a) prepared salmon O/W emulsions with WPI and found that the blockage of free sulfhydryls with N-ethylmaleimide (NEM) did not change lipid hydroperoxide generation but propanal production was more inhibited. Wong and Kitts (2003) found that the capacity of whey to reduce ferric to ferrous iron correlated with the free sulfhydryl content. If free sulfhydryls can generate more ferrous iron, they could therefore act as pro-oxidants under certain conditions.

The findings of Hernandez-Ledesma et al. (2005) support the idea that free-radical-scavenging activity of a protein is probably not the sum of the free-radical-scavenging activity of its amino acid side chains. They found that specific peptides from hydrolysates of β-lactoglobulin and α-lactalbumin scavenged free radicals better or less effective, but not equally like the equimolar mixtures of their constituting amino acids. This indicates that the free-radical-scavenging activity of proteinaceous material (proteins, peptides and amino acids) probably depends on the interaction of the single compounds or moieties in the case of a protein.

Also the nature of the free radical, e.g. the polarity of its host molecule and its reactivity, and where it contacts the protein (polar/non-polar regions, structural accessibility) will determine the effectiveness of the free-radical-scavenging process. Sharp et al. (2004) found a clear correlation between the accessibility of amino acid residues on lysozyme and β-lactoglobulin A by hydroxyl radicals and their oxidisability. For the reaction with hydroxyl radicals, the reactivity of various amino acid side chains was shown to decrease in the following order: cysteine > tryptophan, tyrosine > methionine > phenylalanine > histidine > isoleucine > leucine > proline (Sharp et al. 2004).

As mentioned before, WPI and NaCas had shown a synergistic antioxidative activity. This effect still can not be easily explained. It should be remarked that whey proteins

and caseins also coexist as continuous phase proteins in their natural environment, milk, and might fulfil collective functions like the inhibition of milk fat oxidation. A synergistic antioxidative effect also could have played a role in the findings of Faraji et al. (2004). In a WPI-stabilised menhaden O/W emulsion, NaCas and soy protein were found to be more antioxidative in the continuous phase than WPI. But the effect was attributed to the single proteins present in the continuous phase and not to a possible interaction between continuous phase and interfacial protein. An example of positive and negative synergy of protein-derived material is given in the findings of Hernandez-Ledesma et al. (2005) who found that equimolar amino acid mixtures of peptides exhibited lower or higher free-radical-scavenging activity (AAPH-derived radicals) than the peptides themselves. Although no direct parallel can be drawn to the present system, the example illustrates that interactions can have a synergistic effect contributing to the antioxidative potential.

During the autoxidation of linoleic acid, hydroperoxides develop at the C_9 and C_{13} atoms via the reaction of lipid radicals with oxygen (Frankel 1998a). They subsequently break down into a variety of products. Hexanal is one of the breakdown products and stems from the C_{13} hydroperoxide (Frankel 1998b). However, it is not fully understood yet how various factors, such as the reaction medium and pro-/antioxidants, influence the generation of secondary oxidation products (Kamal-Edin 2003). For the autoxidation of linoleic acid, homolytic cleavage on the unsaturated side of C_{13} leads from the C_{13} hydroperoxide to hexanal. The reaction requires mild acidic conditions (Frankel 1998b).

In the present experiments, C_9 and C_{13} hydroperoxides were presumably formed. The formation was inhibited by higher protein levels. Possible mechanisms were free radical scavenging and chelation of pro-oxidative metal ions. In WPI emulsions (0.31 µm), lipid hydroperoxides and hexanal showed a similar logarithmic or linear decrease over the WPI concentration (see Figure 4.5/Figure 4.6 and Figure 4.14/Figure 4.15). Therefore, hexanal development might have been largely dependent on the lipid hydroperoxide level. WPI has probably not interfered in the reaction from C_{13} hydroperoxide to hexanal.

In NaCas emulsions (0.31 µm), lipid hydroperoxides and hexanal showed a different development over the NaCas concentration, as lipid hydroperoxide concentrations decreased similar to the development in WPI emulsions, but hexanal concentrations remained fairly constant up to 4 % protein concentration (see Figure 4.5/Figure 4.6 and Figure 4.14/Figure 4.15). Hexanal development seemed more independent of the lipid hydroperoxide level.

It seems as if a part of NaCas suppressed the reaction pathway from the C_{13} hydroperoxide to hexanal and that it was already effective at a lower NaCas concentration. A further increase of that factor did not bring more inhibition over a wide NaCas level range.

In a study of Faraji et al. (2004) it was proposed that a further increase of WPI in the continuous phase of a WPI-stabilised menhaden O/W emulsion might not have led to a greater inhibition of propanal production as lower protein levels were sufficient to bind the available metal ions. A parallel may be drawn to the present study, where metal ions might have been bound effectively already at lower protein concentrations by NaCas but not by WPI, as the caseins may have better chelation abilities (Hekmat and McMahon 1998). The chelation of metal ions, particularly of ferric iron by phosphoseryl residues, near the reaction site of homolytic cleavage could have led to a shift of the Fe^{2+}/Fe^{3+} equilibrium and a lower Fe^{2+} concentration. As ferrous iron is required for the reaction of the C_{13} hydroperoxide to the C_{13} alkoxyl radical, and the C_{13} alkoxyl radical leads via homolytic cleavage to hexanal, the generation of hexanal would have been inhibited. Another possible explanation could be that that NaCas fraction locally depleted H^+ by its buffer activity; H^+ is required for the homolytic cleavage reaction. Third, the NaCas fraction could have hindered the homolytic cleavage reaction sterically by blocking the reaction site.

In conclusion, the findings of the present chapter showed the importance of factors, such as droplet size, protein type and concentration as well as the amount of protein in the continuous phase on the oxidative stability of O/W emulsions. The single factors appeared to have influenced the impact of other factors on oxidative stability. For real food emulsions this may, for example, mean that incorporated unsaturated lipids may be

stable and no off-flavours are produced if certain homogenisation conditions are applied using NaCas or WPI at a high protein level. In contrast, this may only be the case for NaCas but not WPI if a lower homogenisation pressure was applied.

In the next chapter the influence of some compositional characteristics of WPI and NaCas on the oxidative stability of emulsions will be explored. As both milk protein fractions are known to bind metal ions such as iron and copper (Chapter 2, Section 2.8.2), they may already contain a certain level of these compounds, potentially contributing to lipid oxidation in the emulsions.

Chapter 5

Influence of low molecular weight (LMW) compounds on lipid oxidation

5.1 Influence of the removal of LMW compounds of WPI and NaCas by dialysis and the addition of metal ion chelators on lipid oxidation

Metal ions, particularly iron and copper cations, are strong pro-oxidants in the lipid oxidation process. This has also been shown for O/W emulsions (Ahn and Kim 1998; Cho et al. 2003; Donnelly et al. 1998; Frankel et al. 2002; Nuchi et al. 2001). Potential metal sources are the emulsifier and the water it is dissolved in. The RO water, used in this study, showed an iron concentration of <0.02 mg/l and a copper concentration of <0.0005 mg/l. The milk proteins employed as emulsifiers had an iron concentration of 5 mg/kg (WPI) and 6 mg/kg (NaCas). The copper concentration was 0.28 mg/kg (WPI) and 0.6 mg/kg (NaCas). Therefore, the proteins could be seen as a potential source of pro-oxidants, although some metal ions might not take part in the lipid oxidation process if they are firmly bound to the proteins or buried within their molecular structure. To remove free metal ions from the protein solutions used to prepare emulsions, they were dialysed against RO water. Also chelators (EDTA, citrate) were added to the protein solutions to bind free metal ions as well as metal ions bound to the proteins. These protein solutions were dialysed as well to remove chelator–metal ion complexes, excess chelator and/or free metal ions. By this process, emulsions with even lower metal ion content and basically free of chelators were presumably obtained.

The experimental plan is depicted in Figure 5.1. Further details can be found in Chapter 3, Materials and methods.

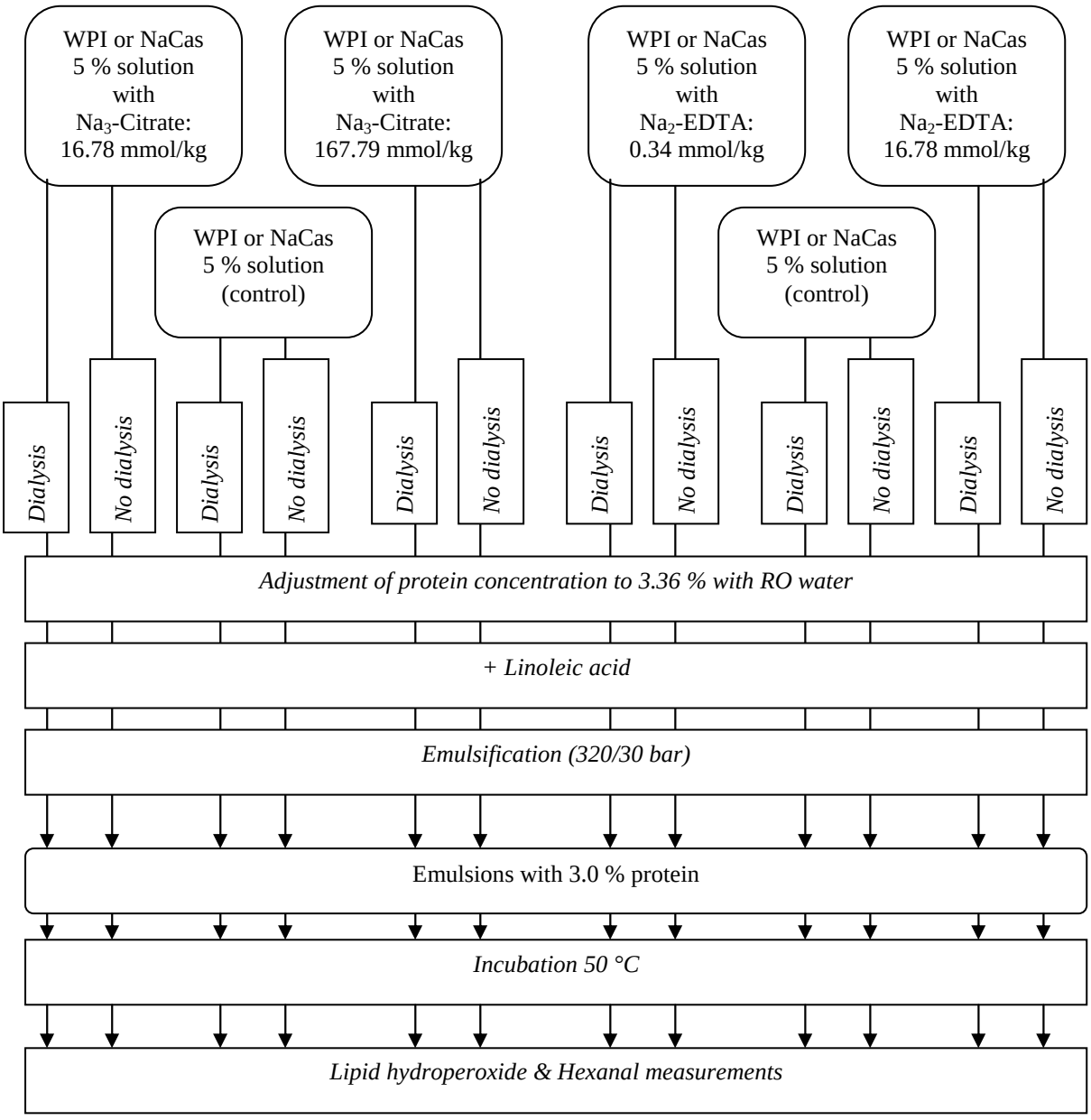


Figure 5.1: Flow chart of the experimental plan

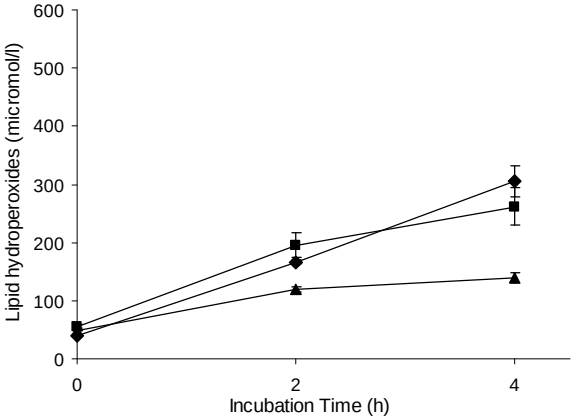
WPI and NaCas solutions with 5 % protein were prepared. Various amounts of different chelators were incorporated. One part of each solution was used for a dialysis process against RO water (molecular weight cut-off 12000-14000), the other part was left unprocessed. To compensate for the dilution during dialysis, all dialysed – and not-dialysed – solutions were adjusted to 3.36 % protein with RO water. These solutions were then used for the preparation of emulsions with 3.0 % protein. The emulsions then were further processed as usual. When the protein solutions had not been dialysed, the final chelator concentrations in the emulsions were 0 mmol/kg (controls), 0.3 mmol/kg EDTA, 15 mmol/kg EDTA, 15 mmol/kg citrate and 150 mmol/kg citrate. The legal EDTA concentration for many O/W food emulsions in the USA is 75 to 100 ppm (0.3 mmol/kg) (FDA 2007). A relatively high EDTA level (50 fold higher) was chosen to see if this would make a difference when dialysis was applied as well as for the control emulsions where no dialysis was involved.

To compare the efficacy of the two chelators and because citrate is the weaker chelator, 15 mmol/kg was also chosen for the lowest citrate concentration and 150 mmol/kg for the highest citrate concentration. Table 5.1 gives an overview about the chelator types, their incorporation levels into the protein solutions and their concentration in the emulsions when the protein solutions were not dialysed. Also the code names of the chelators/chelator levels used in the results section are shown.

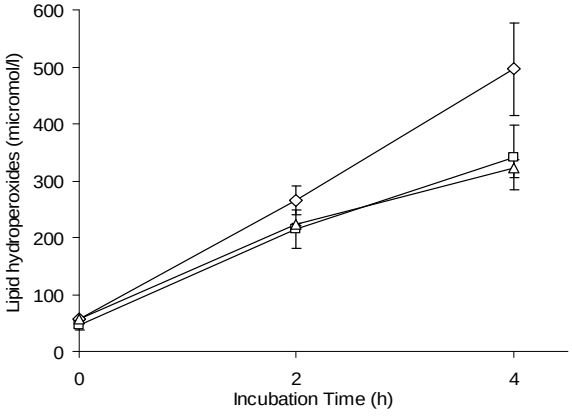
Table 5.1: Chelator types, incorporation levels and respective code names

Chelator type	Chelator concentration		Code name of chelator level
	in protein solutions without dialysis (mmol/kg)	in emulsions without dialysis (mmol/kg)	
Control (no chelator)	0	0	control
Trisodium Citrate	16.78	15.0	low citrate
	167.79	150	high citrate
Disodium EDTA	0.34	0.3	low EDTA
	16.78	15.0	high EDTA

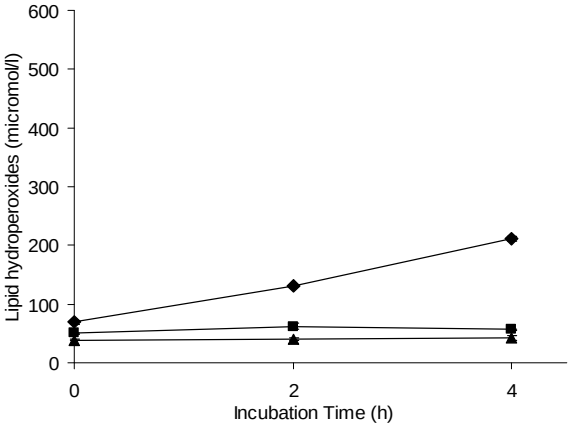
Figure 5.2 and Figure 5.3 show the lipid hydroperoxide development for emulsions prepared with chelator-treated, dialysed and not-dialysed protein solutions. The overall values after 4 hours are given in Table 5.2.



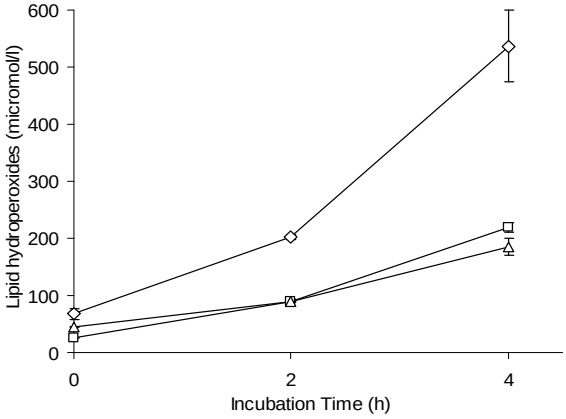
(a) WPI, not-dialysed: control (◆), low citrate (■) or high citrate (▲)



(c) WPI, dialysed: control (◇), low citrate (□) or high citrate (△)

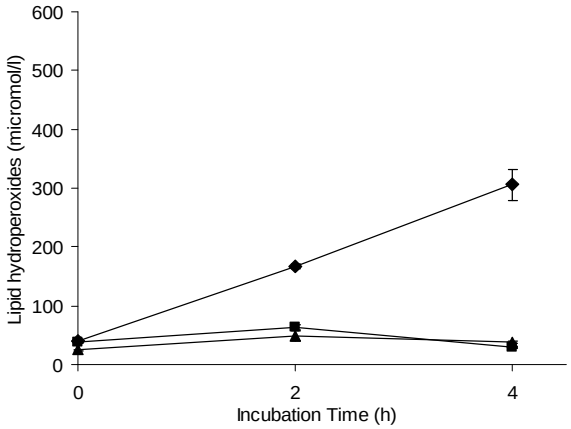


(b) NaCas, not-dialysed: control (◆), low citrate (■) or high citrate (▲)

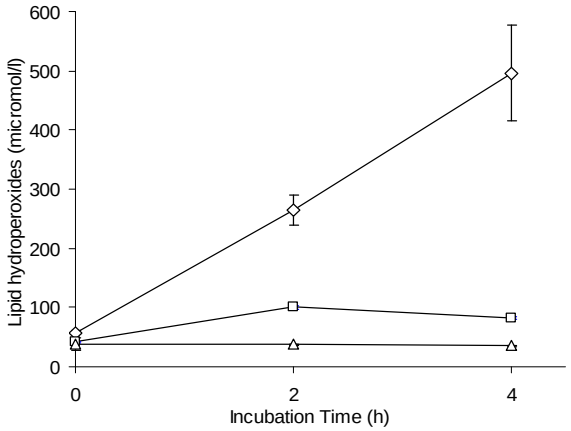


(d) NaCas, dialysed: control (◇), low citrate (□) or high citrate (△)

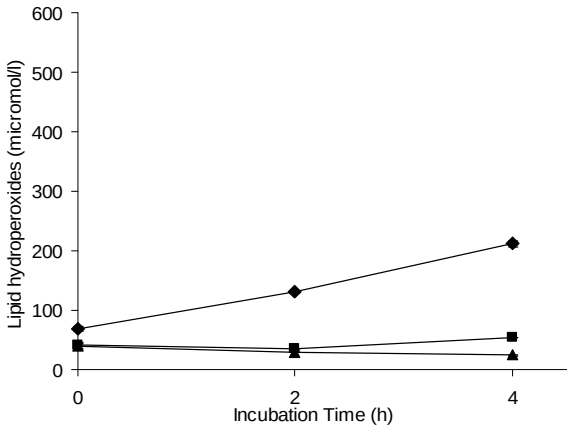
Figure 5.2: Lipid hydroperoxide development in emulsions prepared with citrate-treated, dialysed and not-dialysed protein solutions



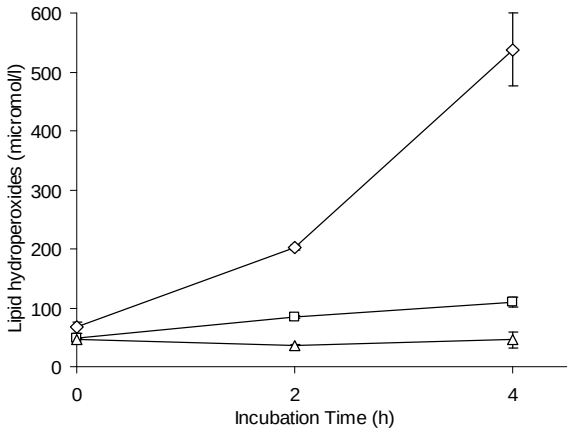
(a) WPI, not-dialysed: control (◆), low EDTA (■) or high EDTA (▲)



(c) WPI, dialysed: control (◇), low EDTA (□) or high EDTA (△)



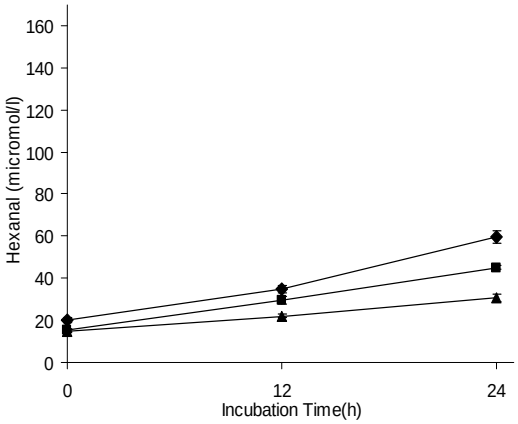
(b) NaCas, not-dialysed: control (◆), low EDTA (■) or high EDTA (▲)



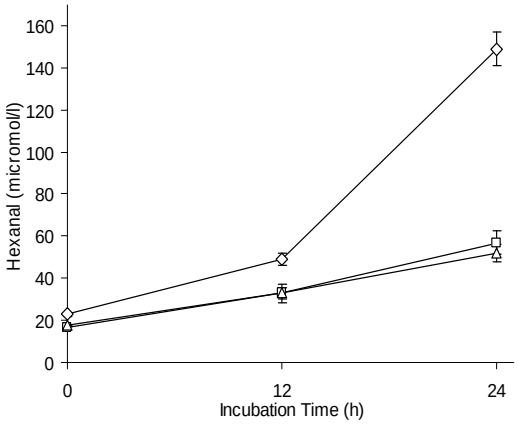
(d) NaCas, dialysed: control (◇), low EDTA (□) or high high EDTA (△)

Figure 5.3: Lipid hydroperoxide development in emulsions prepared with EDTA-treated, dialysed and not-dialysed protein solutions

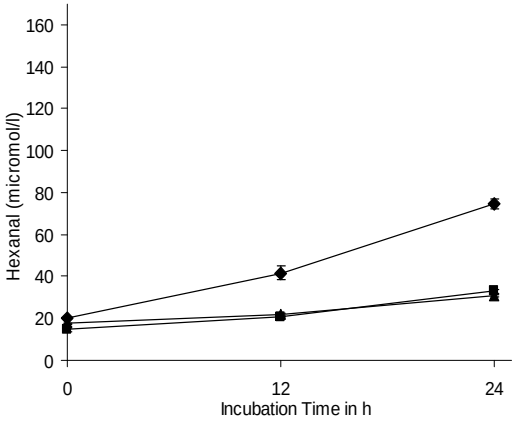
Figure 5.4 and Figure 5.5 show the hexanal development for emulsions prepared with chelator treated, dialysed and not-dialysed protein solutions. The overall values after 24 hours are given in Table 5.2.



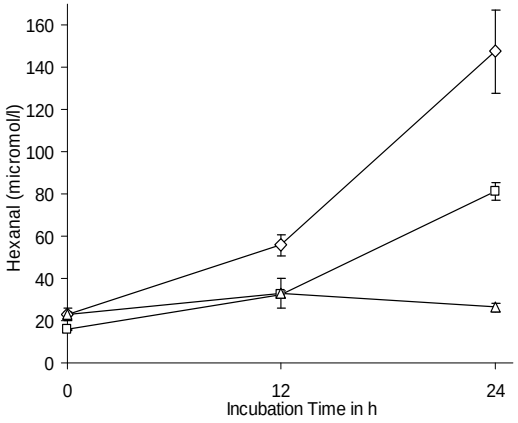
(a) WPI, not-dialysed: control (◆), low citrate (■) or high citrate (▲)



(c) WPI, dialysed: control (◇), low citrate (□) or high citrate (△)

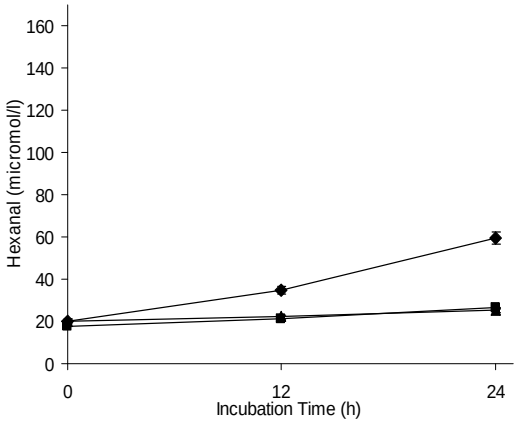


(b) NaCas, not-dialysed: control (◆), low citrate (■) or high citrate (▲)

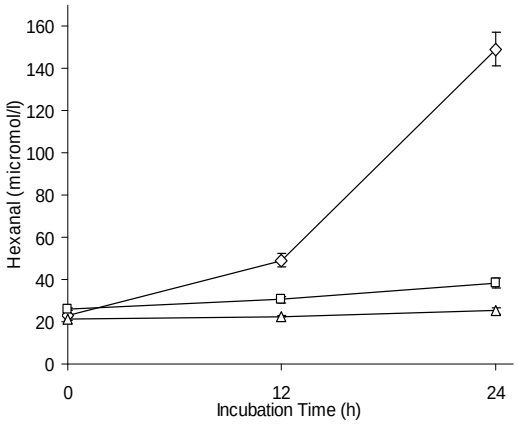


(d) NaCas, dialysed: control (◇), low citrate (□) or high high citrate (△)

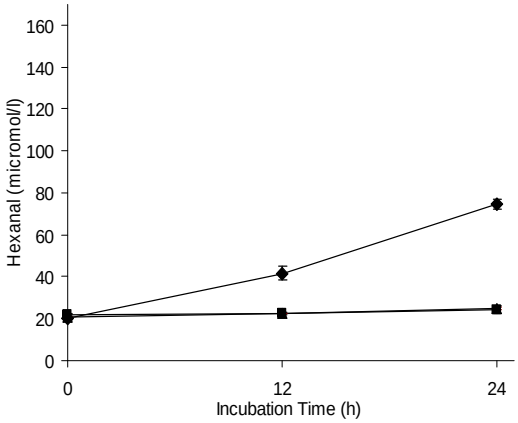
Figure 5.4: Hexanal development in emulsions prepared with citrate-treated, dialysed and not-dialysed protein solutions



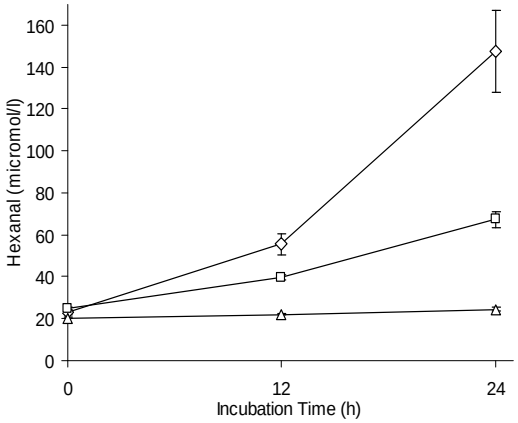
(a) WPI, not-dialysed: control (◆), low EDTA (■) or high EDTA (▲)



(c) WPI, dialysed: control (◇), low EDTA (□) or high EDTA (△)



(b) NaCas, not-dialysed: control (◆), low EDTA (■) or high EDTA (▲)



(d) NaCas, dialysed: control (◇), low EDTA (□) or high EDTA (△)

Figure 5.5: Hexanal development in emulsions prepared with EDTA-treated, dialysed and not-dialysed protein solutions

Table 5.2: Formation of lipid hydroperoxides (after 4 h) and hexanal (after 24 h) for WPI and NaCas emulsions with and without dialysis at the various chelator levels

Lipid oxidation indicator	Sample type	Chelator addition				
		Control no chelator	Citrate low	Citrate high	EDTA low	EDTA high
Lipid hydroperoxides after 4 h (µmol/l)	WPI, not-dialysed	305	260	140	30	40
	WPI, dialysed	495	340	320	80	35
	NaCas, not-dialysed	210	55	40	55	25
	NaCas, dialysed	535	220	185	110	45
Hexanal after 24 h (µmol/l)	WPI, not-dialysed	60	45	30	25	25
	WPI, dialysed	150	55	50	40	25
	NaCas, not-dialysed	75	35	30	25	25
	NaCas, dialysed	145	80	25	65	25

It is clear that dialysis increased the extent of lipid oxidation, as measured by formation of both lipid hydroperoxides and hexanal. This was particularly noticeable for longer incubation times. In control emulsions without dialysis, less lipid hydroperoxides were produced for NaCas emulsions than for WPI emulsions after 4 hours, but there was only a little difference in the level of hexanal after 24 hours, with a lower value for the WPI emulsion. With dialysis, there was very little difference in the extent of oxidation for the controls of either protein type.

The addition of chelators greatly reduced the amount of both lipid hydroperoxides and hexanal.

In the samples without dialysis and with chelator, generally very little lipid hydroperoxides were produced over 4 hours. At all levels of citrate and EDTA, the lipid hydroperoxide concentration had not increased after 4 hours. WPI emulsions in combination with citrate were an exception. At the low citrate level, the lipid hydroperoxide concentration had increased to the same level as for the control. The hexanal concentration had not changed after 4 hours at both EDTA levels for either protein type. At both citrate levels, the hexanal concentration had slightly increased in NaCas emulsions. A similar increase was found in the WPI emulsion at the high citrate level. In the WPI emulsion with the low citrate level, it had increased more notably. Generally speaking EDTA was more effective in inhibiting lipid oxidation than citrate. In NaCas emulsions, citrate exhibited an antioxidative effect similar to EDTA.

In samples with dialysis there was not much difference in lipid hydroperoxide production between the two levels of either chelator. With the high EDTA level, lipid hydroperoxides remained at the initial concentration for both proteins and the values were slightly higher for the low EDTA level. Both citrate levels led to more lipid hydroperoxide production than either EDTA level, particularly in the WPI emulsion. In WPI emulsions, with dialysis, the amount of hexanal was greatly reduced by EDTA. After 24 hours, it had remained at the initial concentration at the high level of EDTA and was only slightly higher at the low level. For both citrate levels, the hexanal concentration was higher and the same after 24 hours. In the NaCas emulsions, with dialysis, the initial hexanal concentration was found at the high citrate and EDTA level after 24 hours. It was higher for the low chelator levels and EDTA inhibited hexanal only slightly better than citrate. In general, EDTA also inhibited lipid oxidation better in emulsions with dialysis. A high level of citrate inhibited the hexanal production in the NaCas emulsion as effectively as EDTA but not in the WPI emulsion.

Generally, samples treated with EDTA gave more similar lipid oxidation values. Also the higher chelator level of either EDTA or citrate gave more similar lipid oxidation values (Table 5.2).

An overview about the similarity/difference of samples is given in Appendix Chapter 5, Table 5A and 5B. The samples were compared in two sample t-tests. In Table 5A, combinations of the protein type and the application of dialysis are compared at certain chelator levels. In Table 5B, varying chelator levels are compared for certain combinations of protein type and application of dialysis.

General trends already observed with the original data could be confirmed by ANOVA tests. The main effects, shown in Figure 5.6 and Figure 5.7, were all significant ($p \leq 0.01$) except for the hexanal means of the protein type.

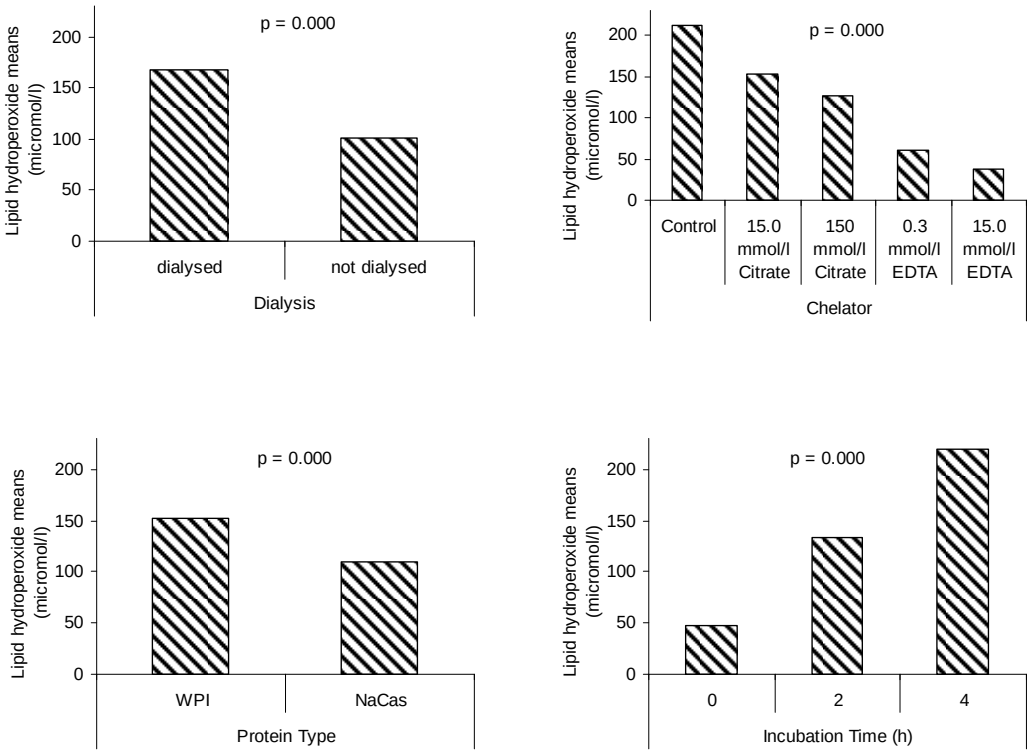


Figure 5.6: Main effects of dialysis, chelator, protein type and incubation time on lipid hydroperoxide generation. Means are shown.

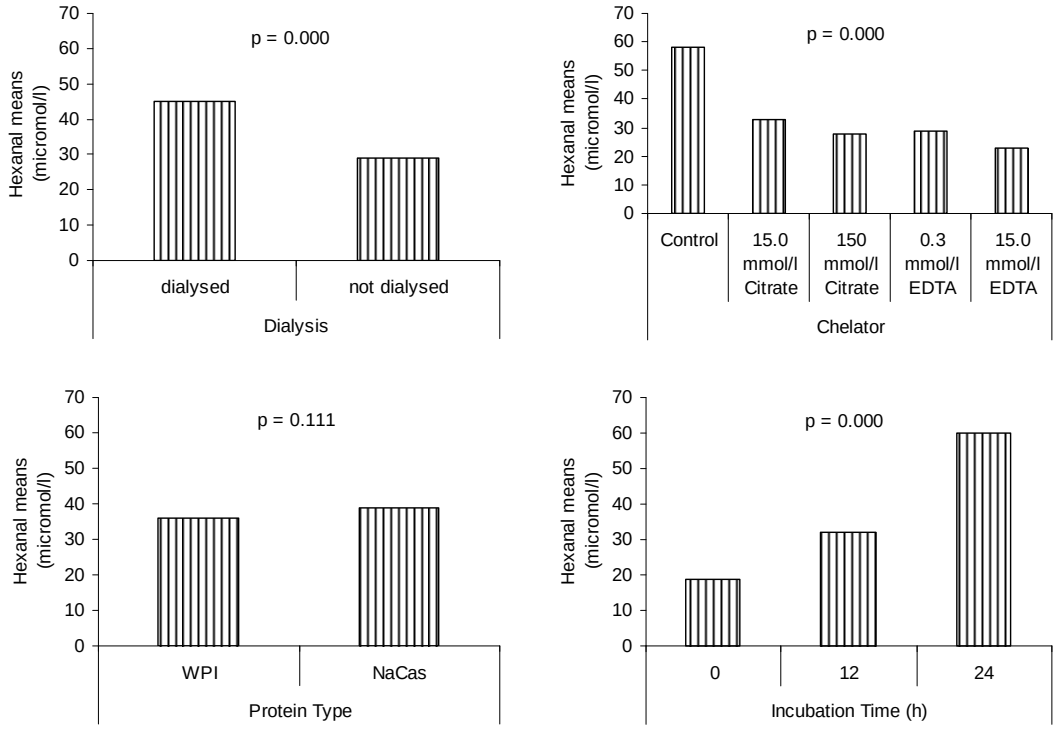


Figure 5.7: Main effects of dialysis, chelator, protein type and incubation time on hexanal generation. Means are shown.

Over the observed incubation times, considerable changes of the lipid hydroperoxide and hexanal levels had occurred. Overall more lipid oxidation occurred in samples that underwent dialysis treatment. EDTA was more effective than citrate and overall less lipid oxidation occurred at higher chelator levels. Overall less lipid hydroperoxides but similar levels of hexanal were generated in NaCas compared with WPI emulsions (Figure 5.6/Figure 5.7).

Dialysis of protein solutions prior to emulsion formation had in general a pro-oxidative influence. This was in contrast to the initial idea that lipid oxidation rates might have been reduced by the removal of pro-oxidative metal ions. It is likely that other LMW compounds present in protein solutions, like peptides, amino acids, lactose, organic acids etc., were also removed. Some of these components may have had antioxidative properties. Tong et al. (2000a), for example, showed that a LMW fraction (molecular weight ≤ 3000) of whey had an antioxidative activity when added to a Tween-stabilised O/W emulsion.

Gaucheron et al. (1996) showed that EDTA and also citrate were able to chelate ferrous iron bound to milk proteins, using NaCas. In the present study, it can be assumed that all available metal ions were chelated at a high EDTA level, including those bound to the proteins. Hence, the lipid oxidation values remained very low. As the emulsions from protein solutions with and without dialysis had similar levels of lipid oxidation, the chelation of metal ions from high molecular weight (HMW) proteins probably played an important role for the oxidative stability. The lipid oxidation level at the high EDTA level for all samples with dialysis was lower than for control samples (no chelator) with no dialysis. In these emulsions, LMW proteins were still present but also metal ions. Therefore, it can be assumed that the removal of metal ions from HMW proteins was more important for the oxidative stability of the emulsions than the presence of LMW proteins. For citrate, it seemed as if its chelation capacity was the limiting aspect. The high citrate level did not lead to the same low lipid oxidation values, especially lipid hydroperoxide levels, when the emulsions were prepared with dialysed or not-dialysed protein solutions. It seemed as if the chelating capacity of citrate could hardly offset the loss of antioxidative LMW compounds.

Possible migration of compounds during dialysis that is likely to enhance or inhibit lipid oxidation in the emulsions is illustrated in Figure 5.8. The protein type is not considered.

In A and B no chelators are added. In B but not A dialysis takes place. In B dialysis leads to the removal of pro-oxidative free metal ions. Also LMW proteins that have bound metal ions (supposedly pro-oxidative) will also be removed. On the other hand LMW proteins where no metals are bound, possibly antioxidative, are also removed. Furthermore, pro-oxidative metals remain in A and B bound to HMW proteins.

In C and D a chelator is added, which binds to metal ions and consequently reduces their pro-oxidative effect. In C metal ion-chelator complexes remain in the system. In D these complexes are removed by dialysis and also LMW proteins that are possibly antioxidative.

In E and F at a high chelator concentration, most metal ions are bound to the chelator. In E the complexes remain in the system. In F the complexes are removed by dialysis and also LMW proteins that are possibly antioxidative.

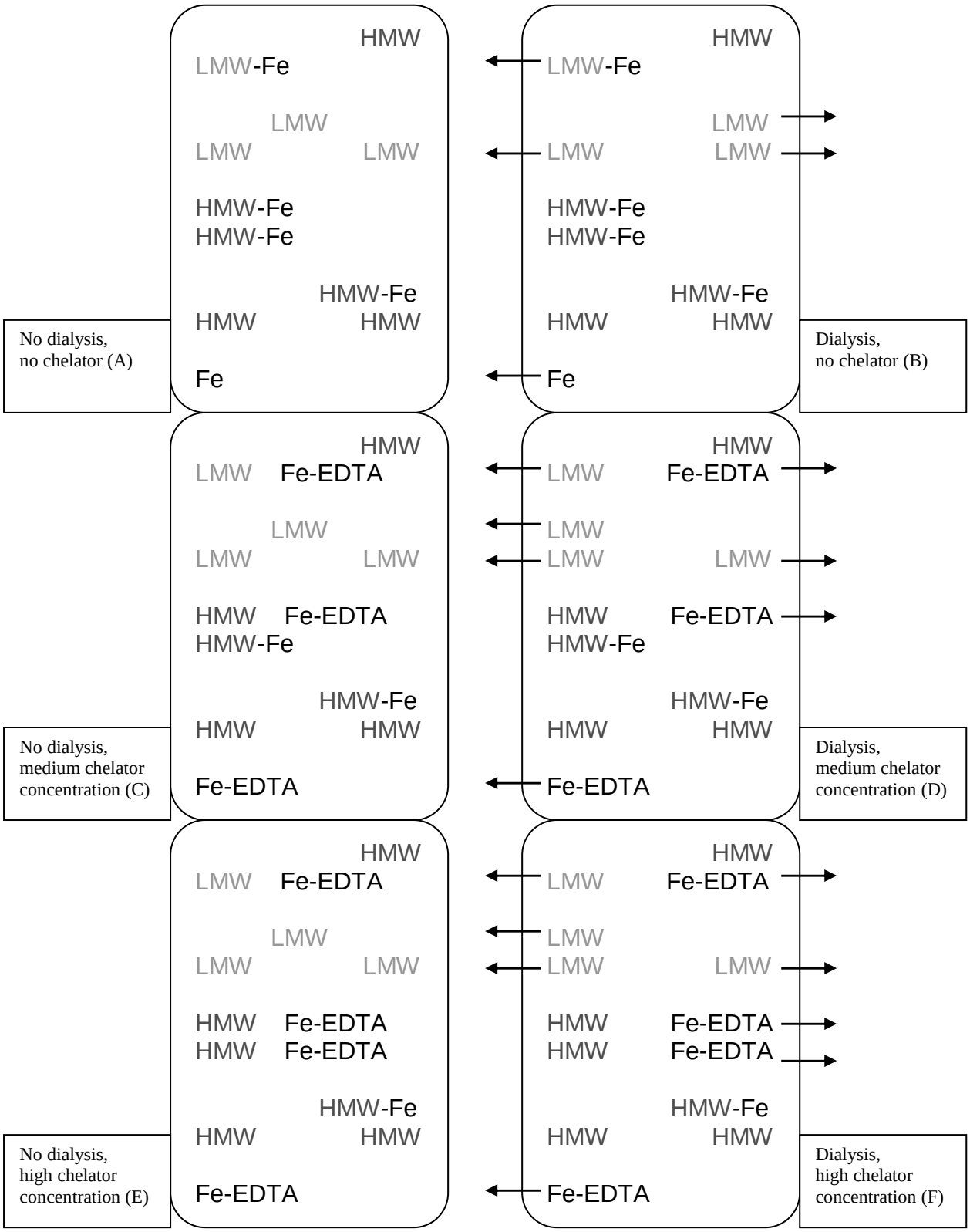


Figure 5.8: Schematic depiction of the dialysis process. Likely migration of compounds is indicated by arrows; LMW = low molecular weight protein fractions, HMW = high molecular weight protein fractions, Fe = iron ions

In other studies, the antioxidative activity of citrate and EDTA had been investigated when the chelators were added after emulsification (Cho et al. 2003; Djordjevic et al. 2004; Hu et al. 2004). In each of the studies, EDTA gave greater oxidative stability than citrate, when applied at the same concentration up to 0.1 mmol/l. Above a certain concentration, more EDTA did not yield greater oxidative stability, which was attributed to the chelation of all available metal ions. Greater antioxidative activity of EDTA over citrate was attributed to a higher binding constant for ferric iron and greater chemical inactivation, making iron less available to take part in reactions. Partly citrate had been found pro-oxidative which was explained by an increase of iron mobility by the chelator and insufficient reduction of its reactivity at the same time. Both citrate and EDTA were able to increase the concentration of ferric iron in the continuous phase that stemmed from the oil phase of an emulsion. As a result of this transfer oxidative stability increased. In summary, various antioxidative mechanisms of chelators can be named: change of metal location, reduction of metal solubility, alteration of redox potential of metals (reducing their reactivity as oxidants) and interference with redox cycles and sterical hindrance of interactions between metals and hydroperoxides (preventing hydroperoxide decomposition and free radical generation).

The loss of LMW proteins by dialysis enhanced lipid oxidation in milk-protein-based O/W emulsions. This pro-oxidative effect could be compensated by the addition of a high level of EDTA before dialysis. In fact, the samples treated with EDTA (metal ions complexed) and also dialysis (LMW proteins removed) were more stable towards lipid oxidation than the controls without chelator treatment (metal ions present) and no dialysis (LMW proteins present). This suggests that the removal of metal ions from HMW proteins was more prominent for the oxidative stability than the presence of LMW proteins. However, emulsions where metal ions were complexed with the chelator and LMW proteins were still present exhibited the greatest oxidative stability and emulsions in which metal ions were not complexed and LMW proteins had been removed were oxidised to the highest level.

5.2 Effects of LMW fractions on oxidative stability

It appears that the LMW fractions of WPI and NaCas were important for the oxidative stability of emulsion lipids. Therefore, it was thought that an increase of their concentration could lead to better oxidative stability. Solutions enriched with LMW fractions were obtained by the dialysis of RO water against protein solutions. These solutions were used instead of RO water to prepare protein solutions for emulsification as usual. Emulsions fortified with LMW-WPI and LMW-NaCas were tested for their oxidative stability. Lipid oxidation was compared with control emulsions prepared with only WPI, NaCas and also NaCas + 100 ppm EDTA. A reasonably high total protein concentration (5 %) was chosen to achieve similar oxidative stability as with the addition of 100 ppm EDTA.

The experimental plans are shown in Figure 5.9 and Figure 5.10. The production of control emulsions (WPI, NaCas and NaCas + EDTA) is not depicted in the experimental plans. Dialysis tubes filled with RO water were placed into WPI or NaCas solutions (5.5 %), and left for about 42 h. The protein solution was replaced every 14 h. The tubes were cut open and the protein content was determined using the Kjehldahl method. The solutions, undiluted and diluted with RO water, were used to prepare 5.60 % WPI and NaCas protein solutions. These were employed as usual for the emulsification with linoleic acid to produce emulsions with 5.0 % protein. More details about the execution of the experiments can be found in Chapter 3, Materials and methods.

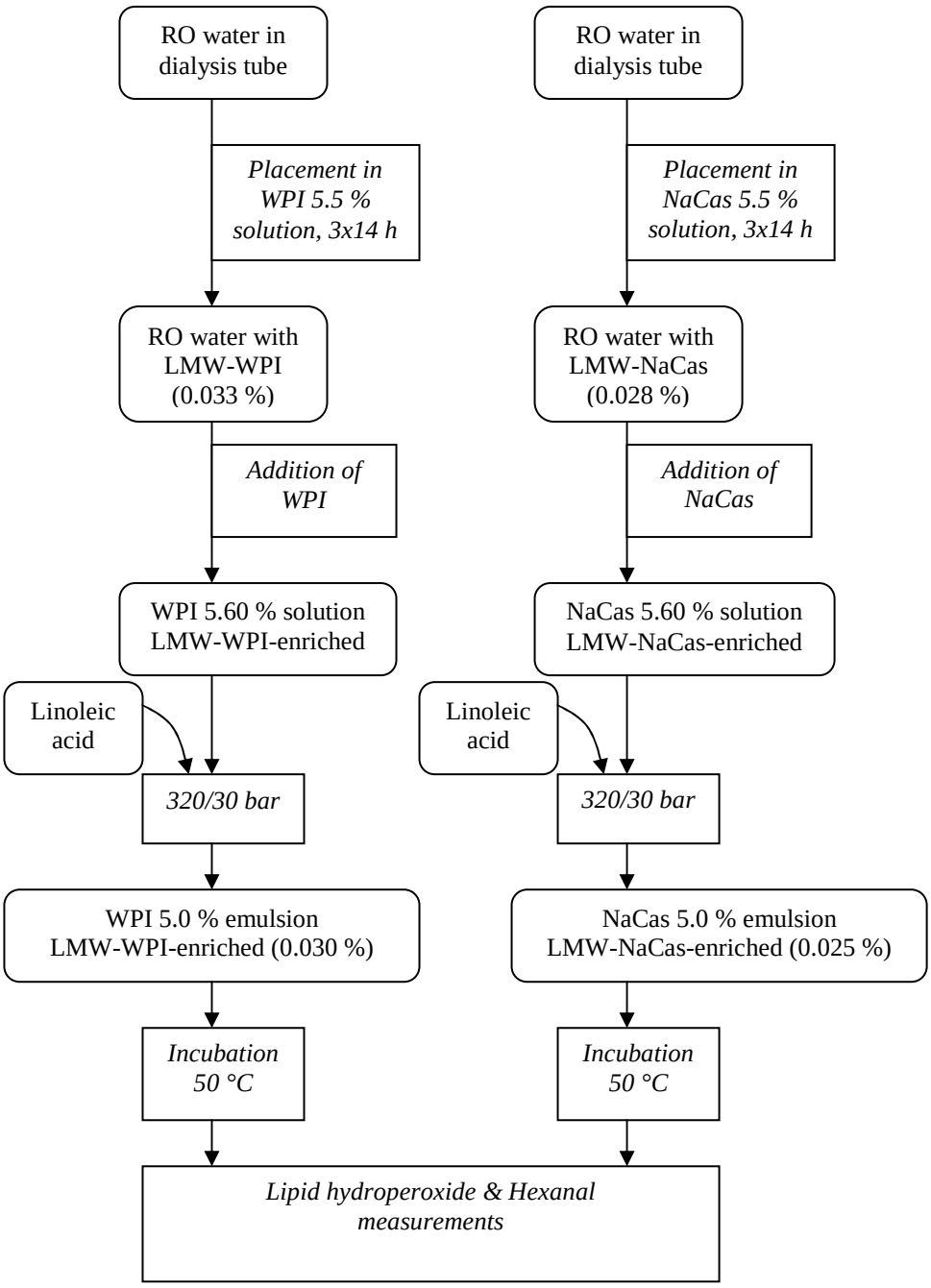


Figure 5.9: Flow chart of the experimental plan (A)

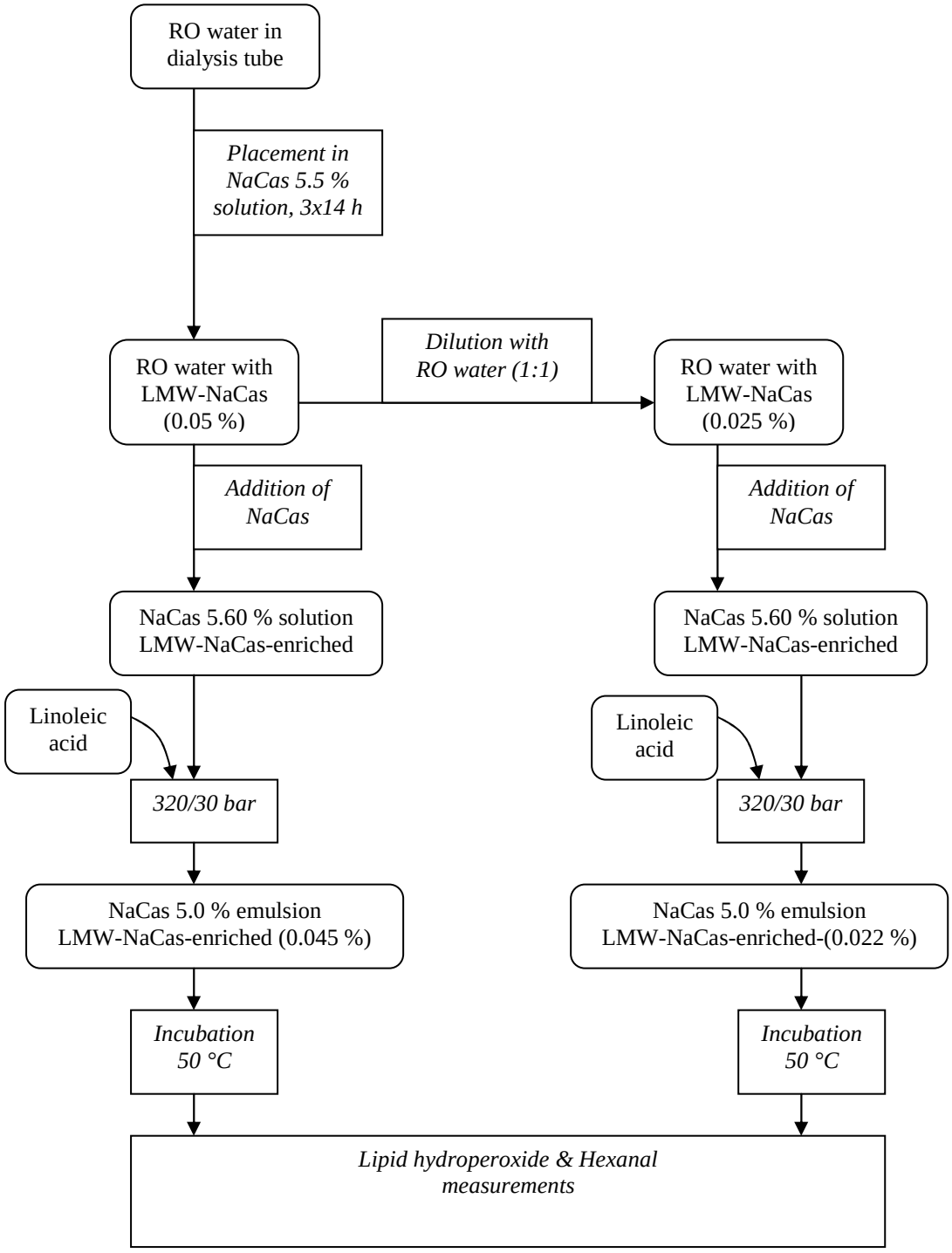


Figure 5.10: Flow chart of the experimental plan (B)

WPI and NaCas emulsions with and without enrichment of their LMW fraction were tested for their oxidative stability (Table 5.3).

The lipid hydroperoxide concentrations of the WPI control and WPI emulsion enriched with LMW-WPI were 180 and 165 µmol/l after 4 h, respectively, but the values were not significantly different. For the NaCas control and NaCas emulsion enriched with LMW-NaCas the lipid hydroperoxide concentrations were 100 and 35 µmol/l after 4 h, respectively (Table 5.3). The values were significantly different.

Table 5.3: Lipid hydroperoxide and hexanal concentrations for emulsions enriched with LMW-WPI and LMW-NaCas

Sample type	Incubation time (h)	Lipid hydroperoxides ± SEM* (µmol/l)	Hexanal ± SEM* (µmol/l)
WPI	0	55 ± 5	105 ± 5
	4	180 ± 5	-
	24	-	305 ± 15
WPI	0	60 ± 5	105 ± 5
+ 0.030 %	4	165 ± 5	-
LMW-WPI	24	-	265 ± 10
NaCas	0	60 ± 5	90 ± 5
	4	100 ± 5	-
	24	-	385 ± 10
NaCas	0	50 ± 5	90 ± 5
+ 0.025 %	4	35 ± 5	-
LMW-NaCas	24	-	300 ± 20

* SEM = standard error of the mean value

The hexanal concentrations of the WPI control and WPI emulsion enriched with LMW-WPI were 305 and 265 µmol/l after 24 h respectively, but they were not significantly different. For the NaCas control and NaCas emulsion enriched with LMW-NaCas, the concentrations of hydroperoxide were 385 and 300 µmol/l after 24 h, respectively (Table 5.3). The values were significantly different. Probability values for the difference between samples were obtained from two sample t-tests. They can be found in Appendix Chapter 5, Table 5C.

NaCas emulsions enriched with LMW-NaCas showed a greater difference in lipid oxidation compared with the NaCas control emulsion, especially in respect of lipid

hydroperoxide production. Therefore the LMW-NaCas fraction was further investigated. The lipid hydroperoxide concentration was followed over a longer time. Also two concentration levels of LMW-NaCas were used and EDTA was added to have an oxidatively very stable emulsion sample as a comparison (Table 5.4).

The development of lipid hydroperoxides was delayed in NaCas emulsions enriched with LMW-NaCas compared to the NaCas control, indicated by the mean values after 8 h (Table 5.4). Higher concentration of LMW-NaCas provided a further delay. The emulsion to which EDTA was added exhibited very low, stable lipid hydroperoxide concentration over time. After 16 h, all emulsions reached similar lipid hydroperoxide levels except for the one with EDTA. All mean values were significantly different from each other after 8 h. After 16 h, the mean value of the control was still significantly higher than the high level of LMW-NaCas. The mean values of both LMW-NaCas levels were not significantly different.

Table 5.4: Lipid hydroperoxide and hexanal concentrations for emulsions enriched with LMW-NaCas at two levels and with EDTA incorporated

Sample type	Incubation time (h)	Lipid hydroperoxides ± SEM* (µmol/l)	Hexanal ± SEM* (µmol/l)
NaCas	0	115 ± 5	65 ± 5
	8	785 ± 30	-
	16	1030 ± 15	-
	24	-	390 ± 10
NaCas + 0.022 % LMW-NaCas	0	80 ± 5	70 ± 5
	8	235 ± 10	-
	16	940 ± 30	-
	24	-	350 ± 20
NaCas + 0.045 % LMW-NaCas	0	90 ± 5	50 ± 5
	8	135 ± 10	-
	16	900 ± 30	-
	24	-	190 ± 5
NaCas + 100 ppm EDTA	0	80 ± 5	50 ± 5
	8	30 ± 5	-
	16	40 ± 5	-
	24	-	80 ± 5

* SEM = standard error of the mean value

The hexanal concentration in the emulsion with the high LMW-NaCas level was significantly lower and only half the value of the control sample after 24 h. On the other hand, this value was still about 120 µmol/l above the level found in the emulsion with EDTA (Table 5.4). The control sample and the emulsion with the low LMW-NaCas level did not exhibit a significantly different hexanal concentration. Probability values for the difference between samples were obtained from two sample t-tests and are shown in Appendix Chapter 5, Table 5D.

These studies confirm that LMW fractions in the milk protein solutions influence lipid oxidation in the emulsions. The oxidative stability increased when LMW fractions were incorporated at a higher concentration level. LMW fractions of NaCas caused a strong decrease in lipid hydroperoxides and a lower hexanal production. An increase of only 0.04 % protein by enrichment with LMW-NaCas reduced the hexanal generation by half, indicating a remarkable antioxidative potential of the LMW fraction of NaCas.

Another aspect that needs to be considered is the actual composition of the LMW fractions. Milk contains several low molecular weight compounds and some of them were probably also present in LMW-WPI and LMW-NaCas. LMW compounds of milk are lactose (4.6 %), minerals (0.65 %), organic acids- citrate, formate, acetate, lactate, oxalate (0.18 %) and vitamins- e.g. A, C, D, thiamine, riboflavin (*http://www.foodsci.uoguelph.ca/dairyedu/chem.html* 2008). Some of the organic acids are known as chelators and might contribute to antioxidative characteristics. Also ascorbic acid is an antioxidant. Lactose was found to form complexes with ferrous iron (Bachran and Bernhard 1980). Hu et al. (2003a) mentioned phosphate as iron-chelating compound in whey. Ferguson (2008) refers to a number of non-protein nitrogen (NPN) compounds in milk with low molecular weight. NPN compounds would comprise 6.0 % of all nitrogen-containing compounds. He denotes that those 6.0 % consist of urea (48.0 %), amino acid N (15.1 %), peptide N (11.0 %), creatine N (8.6 %), orotic acid N (5.2 %), creatinine N (4.1 %), ammonia N (3.1 %), uric acid N (2.7 %) and hippuric acid N (1.4 %). Urate, also naturally occurring in bovine milk, has been shown to reduce light-induced lipid hydroperoxide production in milk by almost 30 % and was twice as effective as ascorbic acid (Ostdal et al. 2000). Hydrolysates of milk proteins containing peptides and amino acids have shown antioxidative potential in emulsions (Diaz et al.

2003; Rival et al. 2001a). It has been shown that they exhibited abilities to scavenge free radicals (Hernandez-Ledesma et al. 2005; Rival et al. 2001a) and to chelate metal ions (Shears et al. 1987; Vegarud et al. 2000). In future studies about the oxidative stability of milk-protein-based emulsions particular attention should also be paid to the anti-/pro-oxidative influence of the various LMW compounds associated with milk proteins.

Chapter 6

Effect of heat treatment of milk protein solutions prior to emulsification on the oxidative stability of O/W emulsions

The major whey proteins, β-lactoglobulin and α-lactalbumin, denature in aqueous systems at elevated temperatures (> 70 ˚C). Their conformation changes via an unfolding process in which the tertiary structure is modified. Sulfhydryl groups and hydrophobic moieties previously situated in the interior of the proteins become exposed to the surrounding environment (Anema 2007; Anema and McKenna 1996). Denatured whey proteins may therefore contribute to improved oxidative stability when used as emulsifier for O/W emulsions. Sulfhydryl groups possess antioxidative potential and greater hydrophobicity could improve the contact between proteins and emulsion lipid, which could also result in a stronger physical barrier against pro-oxidants from the continuous phase. Furthermore, Tong et al. (2000a) reported about the increased exposure of aromatic amino acid residues (tyrosyl, phenylalanyl, tryptophan) upon heat-treatment of whey and associated those residues with free radical scavenging activity.

Other research has looked at the stabilising properties of caseins towards the aggregation of heat-denatured whey proteins in aqueous solutions. α_S-Casein, β-casein and partly κ-casein inhibited the aggregation of whey proteins (WPI, β-lactoglobulin, α-lactalbumin, bovine serum albumin) at temperatures between 70-90 ˚C at pH 6.0-7.1, indicated by decreased turbidity of the protein solutions. Therefore chaperone-like characteristics have been attributed to them (Bhattacharyya and Das 1999; Morgan et al. 2005; O'Kennedy and Mounsey 2006; Yong and Foegeding 2008). In other studies, it was reported that κ-casein and also α_{s2}-casein can form complexes with denatured whey proteins (Anema 2007; Anema and McKenna 1996). However, there is a lack of knowledge how these protein–protein interactions influence lipid oxidation in O/W emulsions.

From these considerations, the effects of heat treatment of pure WPI solutions and mixed solutions of WPI plus NaCas on the oxidative stability of O/W emulsions were studied. A possible correlation between WPI denaturation and lipid oxidation was also investigated.

Pure WPI solutions and mixed solutions of WPI and NaCas (1:1, weight/weight) of 1.12 % and 2.24 % protein content were heated in a water bath at 84 ± 0.3 ˚C for up to 40 minutes. The solutions were then cooled down to 5 ˚C and used to produce emulsions of 1.0 and 2.0 % protein content as before. An overview of the treatments is given in Table 6.1.

The denaturation of WPI in the solutions was followed by measuring the content of β-lactoglobulin by the Native PAGE method. Also the concentration of free and total sulfhydryls in the solutions was followed over time. Lipid hydroperoxide and hexanal concentrations in the emulsions were determined as previously detailed. Further details can be found in Chapter 3, Materials and methods.

Table 6.1: Scheme of protein solution mixtures and heating at 84 °C

Protein type	Protein concentration (%)	Heating time at 84 ˚C (min)
WPI	1.12	0, 10, 20, 30, 40
WPI	2.24	0, 10, 20, 30, 40
WPI + NaCas	1.12	0, 10, 20, 30, 40
WPI + NaCas	2.24	0, 10, 20, 30, 40

For most protein solutions, the denaturation of β-lactoglobulin was linear up to 20 min heating time but levelled off thereafter, reaching a similar degree between 80 and 90 % after 40 min. For WPI + NaCas solution (1.12 % protein), non-linearity began already from 10 to 20 min heating time and the final degree of denaturation was the lowest of all samples (70 %). In the pure WPI solution (2.24 % protein), about 5 % more β-lactoglobulin had denatured after 20 and 30 min than in the solutions with WPI + NaCas (2.24 % protein) and WPI (1.12 %). The latter ones showed almost identical denaturation curves. In summary, at lower protein concentration and in the mixture of WPI + NaCas, β-lactoglobulin denaturation was more inhibited (Figure 6.1).

The concentration of free sulfhydryl groups generally increased through heating and was higher at higher WPI levels. With increasing WPI level, the free sulfhydryl concentration also increased more over the heating time. The free sulfhydryl concentration was higher in the solution with 1.12 % WPI and 1.12 % NaCas in comparison to the solution with only 1.12 % WPI, although both had the same WPI content (Figure 6.2). This effect might be attributed to modified conformational changes of whey proteins at elevated temperatures, due to the formation of complexes with caseins. At the lowest WPI level (solution with 1.12 % protein, WPI/NaCas (1:1)), heating of the protein solution did not alter the free sulfhydryl concentration.

The total sulfhydryl concentration was higher at higher WPI levels and decreased through heat treatments. It decreased strongly from 0 to 10 min heating time and remained constant for the rest of the heating period. The decrease from 0 to 10 min was stronger at higher WPI levels. Solutions with the same WPI content also showed the same total sulfhydryl development over the heating time, indicating that the presence of caseins did not influence the total sulfhydryl concentration (Figure 6.3).

The decrease of the total sulfhydryl concentration by the influence of heat was stronger than the increase in free sulfhydryls. This effect was more pronounced at higher WPI levels.

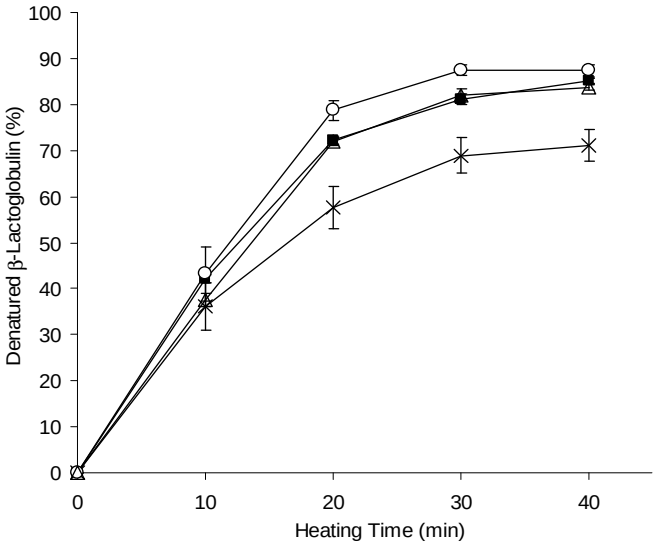


Figure 6.1: Denaturation of β-lactoglobulin on heat treatment of protein solutions at 84 ˚C for various times: WPI solution with 1.12 % protein (■), WPI + NaCas solution with 1.12 % protein (×), WPI solution with 2.24 % protein (○), WPI + NaCas solution with 2.24 % protein (△)

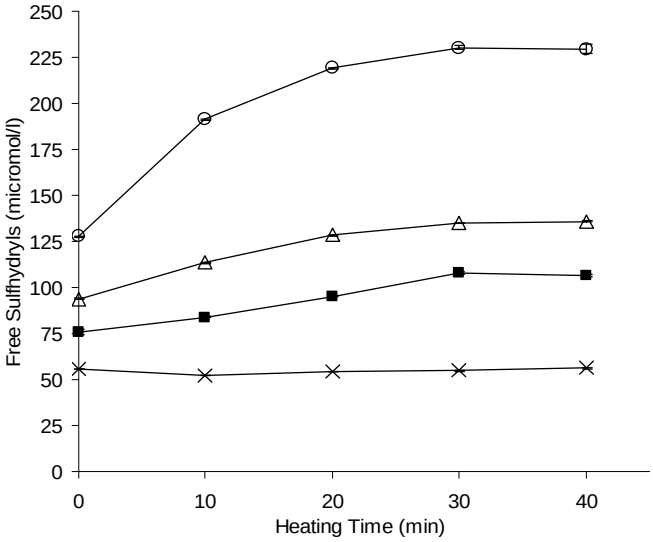


Figure 6.2: Changes in free sulfhydryl content during heat treatment of protein solutions at 84 ˚C for various times: WPI solution with 1.12 % protein (■), WPI + NaCas solution with 1.12 % protein (×), WPI solution with 2.24 % protein (○), WPI + NaCas solution with 2.24 % protein (△)

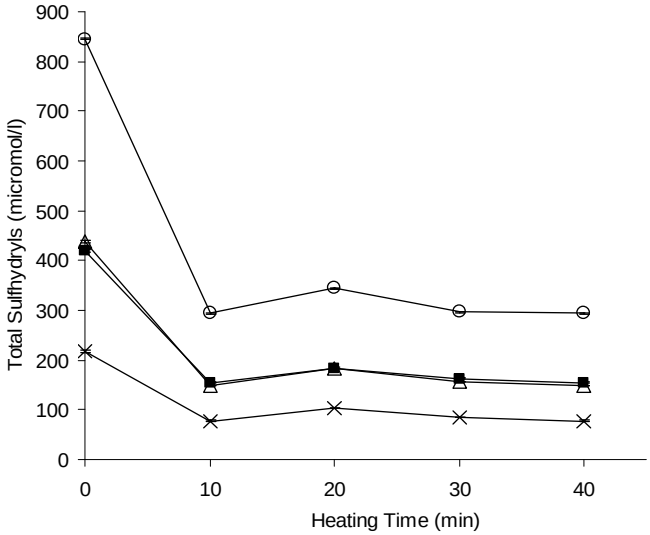


Figure 6.3: Changes in total sulfhydryl content during heat treatment of protein solutions at 84 ˚C for various times: WPI solution with 1.12 % protein (■), WPI + NaCas solution with 1.12 % protein (×), WPI solution with 2.24 % protein (○), WPI + NaCas solution with 2.24 % protein (△)

The lipid hydroperoxide concentration in the emulsions after 4 hours incubation time was not influenced by the heat treatment at 84 ˚C for various times. There was also no correlation between lipid hydroperoxide generation and the degree of β-lactoglobulin denaturation in the protein solutions. The use of WPI only or a WPI/NaCas mixture showed no influence either. At the higher protein level, less lipid hydroperoxides were formed (after 4 hours: 200 to 400 µmol/l at the 2.0 % protein level and 700 to 900 µmol/l at the 1.0 % protein level) (Figure 6.4 and Figure 6.5). The droplet size (Z-average) was stable for all emulsions between 0 h and 4 h (data not shown).

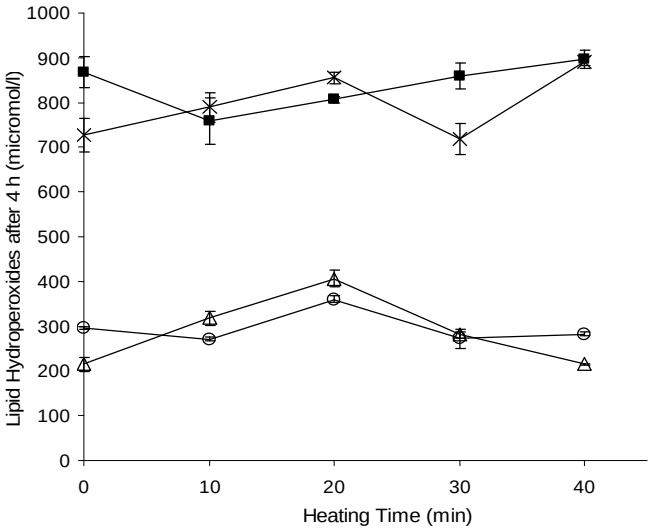


Figure 6.4: Lipid hydroperoxide concentration after 4 h incubation time of emulsions where the protein solution had been heated at 84 ˚C for various times: WPI 1.0 % (■), WPI + NaCas 1.0 % (×), WPI 2.0 % (○), WPI + NaCas 2.0 % (△)

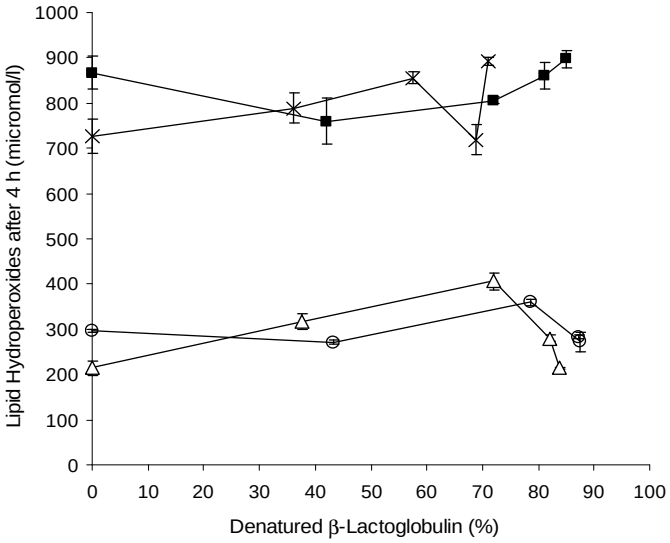


Figure 6.5: Relationship between lipid hydroperoxide concentration and the degree of β-lactoglobulin denaturation after 4 h incubation time of emulsions where the protein solution had been heated at 84 ˚C for various times: WPI 1.0 % (■), WPI + NaCas 1.0 % (×), WPI 2.0 % (○), WPI + NaCas 2.0 % (△)

Heat treatment of protein solutions prior to emulsion formation had in general no effect on hexanal production, with one exception. In WPI emulsions with 1.0 % protein, all emulsions where the protein solution had been heated showed higher hexanal concentrations after 24 h compared with the control (0 min at 84 ˚C), but no consistent development with heating time or β-lactoglobulin denaturation was found. During the 24 h incubation time (including 0, 12, 24 h values) there was significantly ($p \leq 0.01$; ANOVA) less hexanal produced in the unheated control sample compared with all other WPI samples of 1.0 % protein content, where heat treatment had been applied (Figure 6.6 and Figure 6.7). In Table 6.2 the stability of the droplet size (Z-average) is shown for the 24 h incubation period. The average droplet size of WPI emulsions (1.0 % protein content) where the protein solution had been heated for 20, 30 and 40 min had increased to 0.52 – 0.63 µm after 24 h, whereas other emulsions showed a stable droplet size ≤ 0.25 µm. Also aggregates had formed, indicating irreversible physical instability.

The protein concentration also influenced the hexanal generation, although not so strongly as the lipid hydroperoxide generation. Emulsions with the higher protein content showed lower hexanal concentrations after 24 h incubation time. In WPI/NaCas emulsions less hexanal was formed than in pure WPI emulsions, especially at the low protein levels (Figure 6.6 and Figure 6.7).

Table 6.2: Droplet size Z-average (µm) of emulsions at 0 h and 24 h

Heating time of protein solution at 84 ˚C (min)	Emulsion type							
	WPI 1.0 %		WPI/NaCas 1.0 %		WPI 2.0 %		WPI/NaCas 2.0 %	
	Incubation time (h)							
	0	24	0	24	0	24	0	24
	Droplet size Z-average ± SEM* (µm)							
0	0.23±0.00	0.21±0.00	0.24±0.00	0.23±0.00	0.23±0.00	0.20±0.00	0.23±0.00	0.21±0.00
10	0.23±0.00	0.25±0.00	0.24±0.00	0.22±0.00	0.21±0.00	0.19±0.00	0.21±0.00	0.20±0.00
20	0.24±0.00	0.61±0.02	0.23±0.00	0.22±0.00	0.23±0.00	0.21±0.00	0.23±0.00	0.22±0.00
30	0.26±0.00	0.63±0.01	0.25±0.00	0.24±0.01	0.24±0.00	0.24±0.00	0.23±0.00	0.22±0.00
40	0.27±0.01	0.52±0.02	0.24±0.00	0.23±0.00	0.26±0.00	0.24±0.00	0.22±0.00	0.22±0.00

* SEM = standard error of the mean value; three replicate measurements (from one sample container)

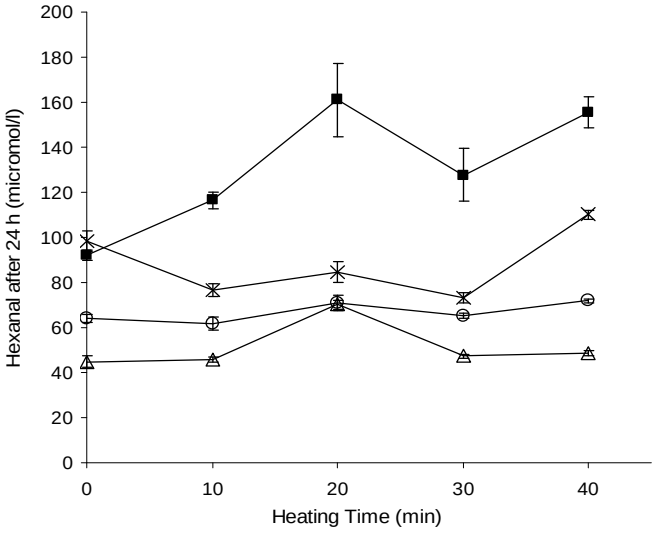


Figure 6.6: Hexanal concentration after 24 h incubation time of emulsions where the protein solution had been heated at 84 ˚C for various times: WPI 1.0 % (■), WPI + NaCas 1.0 % (×), WPI 2.0 % (○), WPI + NaCas 2.0 % (△)

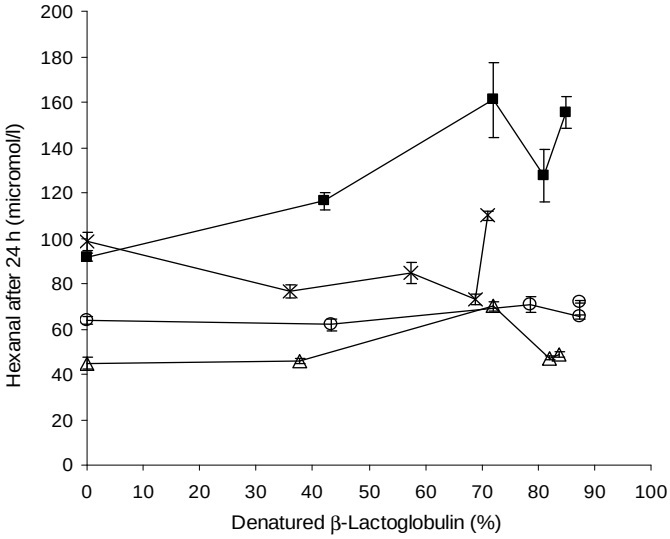


Figure 6.7: Relationship between hexanal concentration and the degree of β-lactoglobulin denaturation after 24 h incubation time of emulsions where the protein solution had been heated at 84 ˚C for various times: WPI 1.0 % (■), WPI + NaCas 1.0 % (×), WPI 2.0 % (○), WPI + NaCas 2.0 % (△)

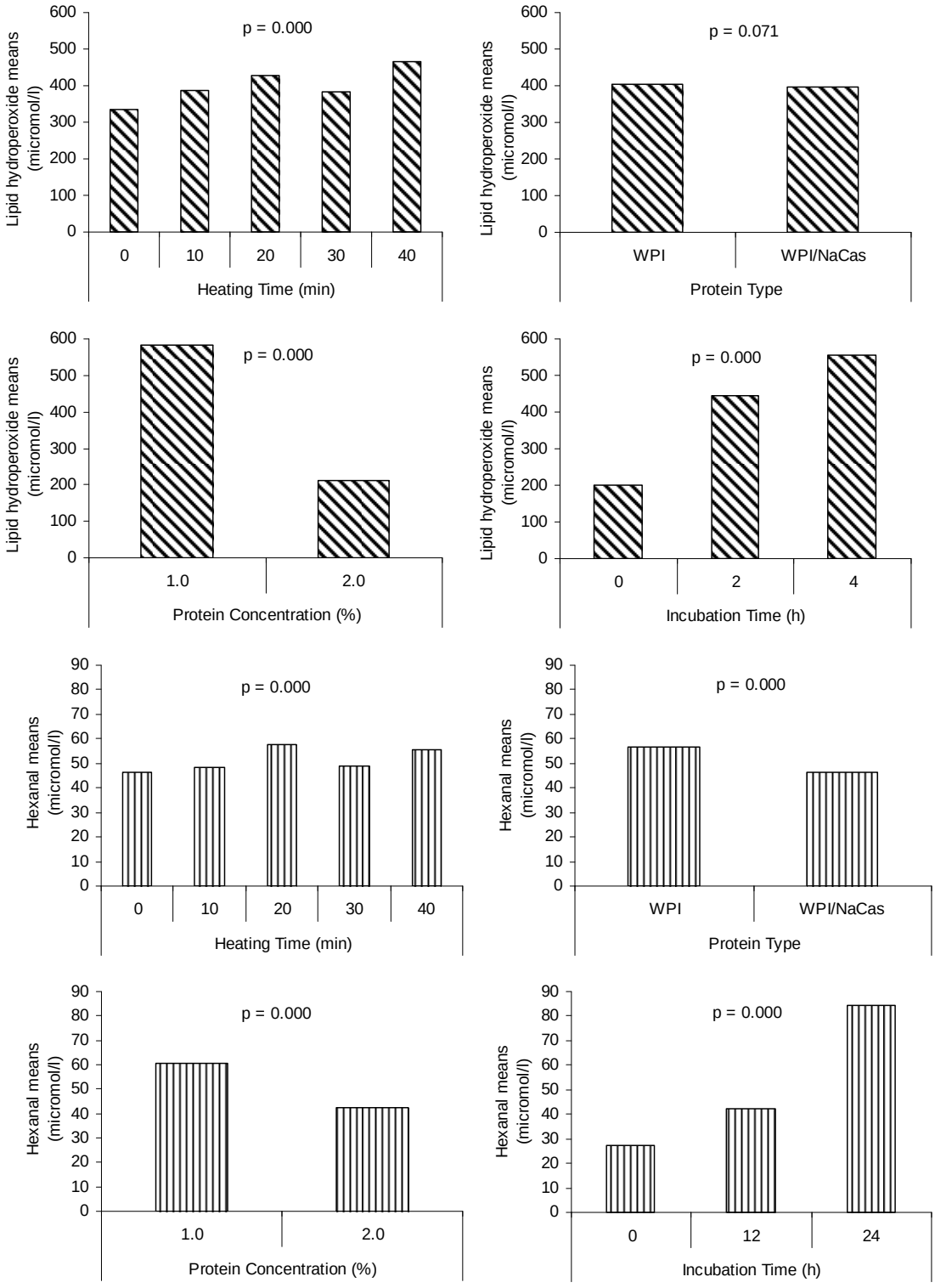


Figure 6.8: Results from ANOVA: main effects of heating time, protein type, protein concentration and incubation time on lipid hydroperoxide and hexanal generation. Means and the significance level for their difference are shown.

ANOVA were carried out to quantify the influence of the factors heating time of the protein solutions, protein type, protein concentration and incubation time of the emulsions on lipid hydroperoxide and hexanal production. The main effects and their significance levels are shown in Figure 6.8.

In summary, lipid hydroperoxide production was neither influenced by the heat treatments of the protein solutions nor by the use of pure WPI solutions or mixed WPI/NaCas solutions for emulsification. On the other hand, the protein concentration had a predominant effect on the inhibition of lipid hydroperoxide production (Figure 6.4). In respect of hexanal generation, the heat treatments only appeared to have influenced the WPI-stabilised emulsion at the low protein level (1.0 %). Higher hexanal levels were measured when heat treatment had been applied, but no consistent development was found with increasing heating time. The physical instability (aggregation, increase in droplet size) of those emulsions (heating for 20, 30 and 40 min) suggests that enhanced hexanal production was related to low physical stability. At the higher protein concentration less hexanal was produced. In WPI/NaCas emulsions, hexanal formation was generally more inhibited than in pure WPI emulsions of the same protein level. In particular, this was observed for the low protein level (1.0 %) with heat treatments, where NaCas did apparently also improve the physical stability of the emulsions (Figure 6.6 and Table 6.2).

There was no correlation found between the denaturation of β-lactoglobulin and lipid hydroperoxide production. In general, β-lactoglobulin denaturation and hexanal generation did not correlate (Figure 6.5 and Figure 6.7). However, for the low protein level (1.0 %) with only WPI, β-lactoglobulin denaturation coincided with higher hexanal levels, but no consistent increase of hexanal was found with increasing degree of β-lactoglobulin denaturation. In a study of Kiokias et al. (2007) the denaturation of β-lactoglobulin and α-lactalbumin reached the same degree in WPC solutions with approximately 3 % protein content after 15 min at temperatures ≥75 ˚C. Both proteins make up about 97 % of WPC (Donnelly et al. 1998; Kiokias et al. 2007), which has a similar protein concentration like WPI. Therefore the degree of whey protein denaturation in general and of β-lactoglobulin seemed not to have influenced the oxidative stability of the emulsions in the present study.

In protein solutions, the free sulfhydryl concentration generally increased but at the same time the total sulfhydryl concentration decreased stronger by the influence of the heat treatments (Figure 6.2 and Figure 6.3). Neither changes of the free nor total sulfhydryl concentration seemed to have influenced the oxidative stability of the emulsions. The increase of free sulfhydryls and decrease of total sulfhydryls in heated whey protein solutions is in accordance with findings of Taylor and Richardson (1980b) and Tong et al. (2000a). At heating temperatures above 80 ˚C, β-lactoglobulin can form intermolecular disulfide bonds, as referred to in Liu et al. (2007). This can explain the decrease in total sulfhydryl concentration through heating.

In a similar study to the present one (Kiokias et al. 2007), a higher degree of whey protein denaturation (β-lactoglobulin and α-lactalbumin) coincided with more oxidative stability (measuring conjugated diene hydroperoxides) in sunflower O/W emulsions (pH 6.78) stabilised with 3 % WPC. WPC solutions had been heated for 15 min at 40, 60, 72, 75 and 85 °C and before cooling down mixed with sunflower oil and sonicated in an ice bath. Thereafter the emulsions were further emulsified with an APV Lab 1000 homogeniser at 300 bar, which appears to be the same homogeniser model and a similar pressure stage as used in the present study (320/30 bar).

Kiokias et al. (2007) elaborated that upon heating and homogenisation whey proteins partially unfold, the hydrophobic parts become exposed enhancing the proteins` emulsifying properties. Native and denatured whey proteins can act synergistically in physically stabilising emulsions as the native whey proteins quickly occupy the oil–water interface and the denatured whey proteins form a thick surrounding layer. The interface could thereby become an effective barrier against pro-oxidants from the continuous phase. It was referred to a study of Kiokias and Bot (2006) that showed whey protein association with the oil phase greatly increased at emulsification temperatures between 65 to 85 ˚C, in WPC-stabilised vegetable fat O/W emulsions with 4 % protein (pH 6.8). Furthermore, Kiokias et al. (2007) pointed out that whey proteins could unfold more by heat influence at neutral pH as there is less interaction between the protein molecules than at low pH. Thereby free sulfhydryl groups with free-radical-scavenging activity become more exposed at increased temperatures, deploying greater antioxidative activity.

All WPI solutions had a temperature of 5 ˚C at the point of emulsification in the present study, whereas the temperature varied in the study of Kiokias et al. (2007). This makes it difficult to allocate the relative contribution of the temperature and the degree of denaturation to oxidative stability. In two other studies the relevance of the emulsification temperature for the oxidative stability of emulsions was also shown, albeit the studied systems were more complex using milk as emulsifier.

In a study of Let et al. (2007b), fresh milk (1.0 % fat) was pasteurised at 72 ˚C and then combined with cod liver oil (0.5 %). The mixture was either cooled down to 50 ˚C or directly homogenised with a two-valve Rannie homogeniser. Lower lipid hydroperoxide and (E)-2-hexenal (a secondary lipid oxidation product) levels were found for the 72 ˚C treatment without cooling to 50 ˚C. This indicated that the emulsification temperature was also important for the oxidative stability of the emulsions and not only the degree of denaturation. The milk proteins probably had the same degree of denaturation in either treatment but only the temperature at the time of emulsification varied. The authors suggested that β-lactoglobulin was more surface active at the higher emulsification temperature, resulting in improved interfacial stability. Also more sulfhydryl groups with free-radical-scavenging activity were exposed at the higher temperature, both resulting in increased oxidative stability.

Sorensen et al. (2007) used the same milk emulsion system as Let et al. (2007b). The higher emulsification temperature (72 versus 50 ˚C) resulted also in greater oxidative stability (measuring lipid hydroperoxides), when the same emulsification procedures were followed. The greater oxidative stability for samples emulsified at 72 ˚C was attributed to greater surface activity of β-lactoglobulin, resulting in more stable interfaces that can act as a physical barrier towards pro-oxidants from the continuous phase, and a higher concentration of free sulfhydryl groups. Also more casein was found in the continuous phase at the higher emulsification temperature. It was referred to another study, where casein exhibited an antioxidative influence because of metal-ion-chelation activity particularly in the continuous phase of a milk–algae oil emulsion (Gallaher et al. 2005). For the present study, however, there was no antioxidative effect found arising from a possible interaction of heat and the presence of caseins.

Although the antioxidative influence of the whey proteins will probably increase upon heat treatments, as their emulsifying property improves by increased hydrophobicity and a greater exposure of free-radical-scavenging amino acid residues, the unfolding of the proteins will partly also occur during the homogenisation process when the proteins are forced onto the droplet interfaces. This may explain why the proteins did not alter the oxidative stability in the present study, although they were denatured to various degrees. On the other hand, denatured whey proteins would also have been present in the continuous phase, but did apparently not notably contribute to oxidative stability.

A higher temperature during the homogenisation process, causing more oxidative stability in the previously mentioned studies, could have increased the reaction rates of proteins with free radicals and metal ions at a point when a high collision rate of all reaction partners prevailed. After emulsification, emulsion systems are more static and the rate of collision between proteins and pro-oxidants is probably much lower. The oxidative stress on susceptible lipids in protein-stabilised O/W emulsions prepared at a higher temperature may therefore be reduced already at the point of emulsion formation. In the present study such an effect could not have occurred, as all emulsions were prepared at the same temperature.

For emulsions prepared with the low WPI concentration (1.0 %), heat treatment of protein solutions impaired physical and presumably oxidative stability. The concentration of denatured whey protein might have been too high for the formation of physically stable emulsions. The amount of native whey protein was lower in the solutions with 1.12 % WPI compared with 2.24 % WPI at a similar degree of denaturation. Low physical stability of whey-protein-stabilised O/W emulsions at low concentrations of native whey protein on the droplet interface has been reported (Kiokias and Bot 2006; Segall and Goff 2002). Higher emulsification temperatures ($\geq$ 70 ˚C) have also led to an increase of the droplet size in whey protein-stabilised vegetable fat O/W emulsions after temperature-cycling, compared with stable droplet sizes at lower emulsification temperatures (Kiokias and Bot 2006). It has also been reported that native and denatured whey proteins would act synergistically in stabilising emulsion droplets as the native whey proteins quickly occupy the oil–water interface and the denatured whey proteins form a thick surrounding layer (Kiokias et al. 2007).

The right proportion of native to denatured whey proteins therefore appears to be an influential factor for the formation of stable droplet interfaces that may act as effective physical barrier to give oxidative stability.

The presence of casein also improved the physical stability at the low protein concentration (1.0 % protein) and resulted in greater oxidative stability at both protein levels. Caseins (from NaCas) and whey proteins adsorbed in equal proportions to the droplet interface in protein-stabilised soy O/W emulsions (pH 7) when the protein concentration was at a low limit to cover the droplet surfaces. At higher protein levels the proportion of caseins on the interface was higher (Dalgleish 2004; Hunt and Dalgleish 1994). In the present study, greater oxidative stability with NaCas might have been directly related to better physical stability, for example because of thicker interfaces, partly formed with caseins. Generally, the interface thickness is about 10 nm for casein-stabilised emulsions compared with 2 nm in whey-protein-stabilised emulsions (Dalgleish 2004; Dalgleish et al. 1995). In addition, other antioxidative mechanisms are possible, e.g. metal ion chelation or free-radical-scavenging activity. A combination of WPI and NaCas also improved the oxidative stability in Chapter 4, Section 4.3, when protein solutions were used to dilute emulsions prepared with the respective other protein type. This shows that the synergistic antioxidative effect of the two protein fractions is not necessarily restricted to the formation of the droplet interface.

In other studies, heat-treated milk protein solutions were added to already prepared emulsions, usually stabilised with small molecule surfactants. The influence of the protein on the oxidative stability of the emulsion would in those cases probably only have related to the anti- or pro-oxidative activity deployed in the continuous phase. In a study of Elias et al. (2007), β-lactoglobulin was heated in aqueous solution (95, 70, 50 and control 20 °C for 15 min). When the continuous phase of a Brij-stabilised menhaden O/W emulsion (pH 7) was replaced with the protein solutions, the oxidative stability increased the higher the temperature of the heat treatment had been. With increasing temperature of the heat treatment, amino acid residues of tryptophan and sulfhydryl groups became more exposed in the protein solution and the free-radical-scavenging activity towards AAPH-derived radicals improved. The highest free

sulfhydryl concentration was found at 70 ˚C and for tryptophan the maximum was at 95 ˚C. Heating also influenced the ability of the protein to chelate metal ions. The lowest chelation ability was reached at the highest heating temperature (95 ˚C). It seems therefore as if the oxidative stability was ruled by the increased exposure of free-radical-scavenging groups, like tryptophan residues.

An increase of the antioxidative capacity of skim milk and skim milk fractions of pH 6.7 (skim milk, sweet whey, acid whey) upon heat treatment (room temperature, 70, 90, 110 and 130 ˚C) was observed by Taylor and Richardson (1980b). The cooled fractions were added to a SDS-stabilised methyl linoleate emulsion, which generally showed greater oxidative stability at the higher temperature of heat treatment. The free sulfhydryl concentration increased and the total sulfhydryl concentration decreased by the influence of elevated temperatures. Casein solution (pH 6.7), obtained by acidification of the skim milk, subsequent washing and pH readjustment, was also heat-treated at the same conditions. When the casein was added to the linoleate emulsion, heat treatment showed no effect on the oxidative stability, except for extreme conditions (110 and 130 ˚C for 30 min), when the oxidative stability decreased compared with the control emulsion.

In a study of Tong et al. (2000a) a high molecular weight fraction of raw whey (molecular weight cut-off 3500) was heated at room temperature (control), 60, 80 and 90 ˚C for 15 min. The samples were added to Tween 20-stabilised salmon O/W emulsions (pH 7) to test their influence on oxidative stability (no information about the temperature at the time of addition). When the control sample was added, slightly higher lipid hydroperoxide and TBARS levels were measured. When the sample heated at 60 ˚C was added, a clearly higher TBARS level and slightly higher lipid hydroperoxide level was measured. In contrast, the heat treatments at 80 and 90 ˚C both led to a considerable and similar decrease in lipid hydroperoxides and TBARS levels.

More aromatic amino acid residues (tyrosyl, phenylalanyl, tryptophan) and free sulfhydryl groups became exposed with increasing heating temperature of the solutions, indicating unfolding of the protein. At 90 ˚C the free sulhydryl concentration decreased again, which was attributed to oxidation at the relatively extreme temperature. The total

sulfhydryl concentration decreased with increasing heating temperature. The antioxidative influence of the solutions heated at 80 and 90 ˚C in the emulsion was attributed to the greater exposure of free-radical-scavenging amino acid residues (aromatic residues and free sulfhydryls), whereas the pro-oxidative influence found for the sample treated at 60 ˚C and the control, could not be explained.

In a study of Tong et al. (2000b), a high molecular weight fraction of raw whey (molecular weight cut-off 3500) was heated at various temperatures (room temperature, 55, 60, 65 and 70 ˚C). It showed decreasing ability to scavenge free radicals derived from AAPH in aqueous solution, the higher the heating temperature had been. The free-radical-scavenging activity was 42 % for the unheated sample compared with the control without protein, whereas only 9 % for the sample treated at 70 ˚C. In comparison, it was 34 % when free sulfhyrdyl groups had been blocked by N-ethylmaleimide (NEM), pointing to a moderate ability of free sulfhydryls to scavenge free radicals. The results also indicated that the free-radical-scavenging activity was considerably impaired by the influence of heat. This was attributed to a possible protein aggregation which could have led to a reduced exposure of hydrophobic amino acid residues with free-radical-scavenging activity, like tyrosyl.

Liu et al. (2007) found decreased antioxidative activity of β-lactoglobulin towards Cu^{2+}-induced oxidation of low density lipoproteins when the protein had been heated in aqueous solution (100 ˚C for 2 min, cooled to 10 ˚C afterwards). This was attributed to a loss of sulfhydryl groups due to disulfide formation, which was measured by SDS-PAGE and Western blot analyses.

Contradictory results have been obtained about the influence of heat on the antioxidative properties of whey proteins. In various studies the heat treatment of whey proteins has improved their antioxidative properties (Elias et al. 2007; Kiokias et al. 2007; Let et al. 2007b; Sorensen et al. 2007; Taylor and Richardson 1980b; Tong et al. 2000a), whereas others reported about pro-oxidative or reduced antixodiative activity upon heat treatment (Liu et al. 2007; Tong et al. 2000a; Tong et al. 2000b). Taylor and Richardson (1980b) also reported about decreased oxidative stability of a linoleate emulsion to which casein was added that had been heated at extreme temperatures ≥ 110

˚C. For the present study, heat treatment showed either no effect or appears to have caused physical instability of the emulsions (at the lower WPI level) and thus possibly oxidative instability.

In future studies about the influence of heat on the anti- or pro-oxidative properties of whey proteins and caseins, the role of other factors needs to be investigated.

Elevated temperatures can increase the surface hydrophobicity of whey proteins and may improve their ability to form thick interfaces that function as effective physical barrier against pro-oxidants from the continuous phase. On the other hand the ratio of native to denatured whey protein or the presence of caseins will influence the interfacial properties and thereby the oxidative stability of an emulsion.

At the point of emulsification, the collision rate of molecules is probably high compared with the situation in already formed emulsions. The probability for collisions of proteins and other compounds with antioxidative properties with metal ions or free radicals seems therefore more likely during emulsification. In combination with the high collision rate of molecules, the temperature at the time of emulsification is likely to influence the oxidative stability of the emulsion lipid as metal ion chelation and free-radical-scavenging reactions are accelerated with increasing temperature. In that respect the temperature at the point of emulsification may play a role for the oxidative stability of emulsions. The oxidative stability may thus not only be influenced by the denaturation of proteins and the consequences thereof. The temperature at the point of emulsification seems therefore a factor that needs to be further elucidated.

At elevated temperatures whey proteins unfold, which also leads to an increased exposure of aromatic amino acid residues and sulfhydryl groups, contributing to free-radical-scavenging activity. On the other hand the chelation ability can be impaired as shown by Elias et al. (2007). In that context the concentration of pro-oxidative metal ions in an emulsion possibly limits the antioxidative influence of heat-treated whey proteins. At low metal ion concentrations, low metal ion chelation ability may not be important and could be outweighed by the antioxidative effect of more exposed free-radical-scavenging groups. At higher metal concentrations, the chelation capacity is

probably a more important factor and crucial for the oxidative stability. It also needs to be considered that free sulfhydryl groups were found to be pro-oxidative under some conditions as they may reduce ferric to ferrous iron which is the stronger pro-oxidant (Marcuse 1960; Marcuse 1962; Pazos et al. 2006; Wong and Kitts 2003). The presence of metal ions is therefore also a factor that needs to be considered in future studies about the influence of heat on the antioxidative properties of whey proteins.

Chapter 7

Effect of free radicals on lipid and protein oxidation

Free radicals are key compounds and potent promoters in the oxidation process of biological materials. The oxidation of lipids is fuelled by the presence of free radicals. Also proteins have been shown to undergo oxidation through free radical activity (Davies 2003; Elias et al. 2008; Hawkins and Davies 2001). It was shown that high protein concentrations limited lipid oxidation in O/W emulsions. As free radicals are known to be an important factor in lipid oxidation, the milk proteins may function as free radical scavengers. This means that the damage to the emulsion lipid could be limited by the accumulation of free radicals on the proteins. In turn this could lead to molecular modifications of the proteins. An oxidation of proteins rather than lipids would have less negative consequences for the sensory properties of the emulsion. In this section, lipid and protein oxidation in the emulsions was investigated with a special focus on the free-radical-scavenging activity of the milk proteins. Oxygen consumption was introduced as a new method to measure oxidation in general.

7.1 Effects of free radicals on oxygen consumption and lipid hydroperoxide production in emulsions

The influence of free radicals on lipid oxidation in emulsions was investigated by the addition of the free radical source 2,2`-azobis(2methylpropion-amidine) dihydrochloride (AAPH). The azo compound AAPH has been used in many free radical studies before. It is water soluble and one AAPH molecule breaks down at a temperature-dependent rate into two smaller molecules with a carbon-centered radical (C•) on each (*http://www.caymanchem.com/app/template/Product.vm/catalog/82235/promo/searchresult.82235/a/z* 2008). The influence of milk protein type and concentration on oxygen consumption and lipid hydroperoxide production was investigated.

The experimental outline is depicted in Figure 7.1. Emulsions were prepared with protein solutions based on phosphate buffer (pH 6.0) as the decomposition rate of AAPH also depends on the pH. More details about the execution can be found in Chapter 3, Materials and methods.

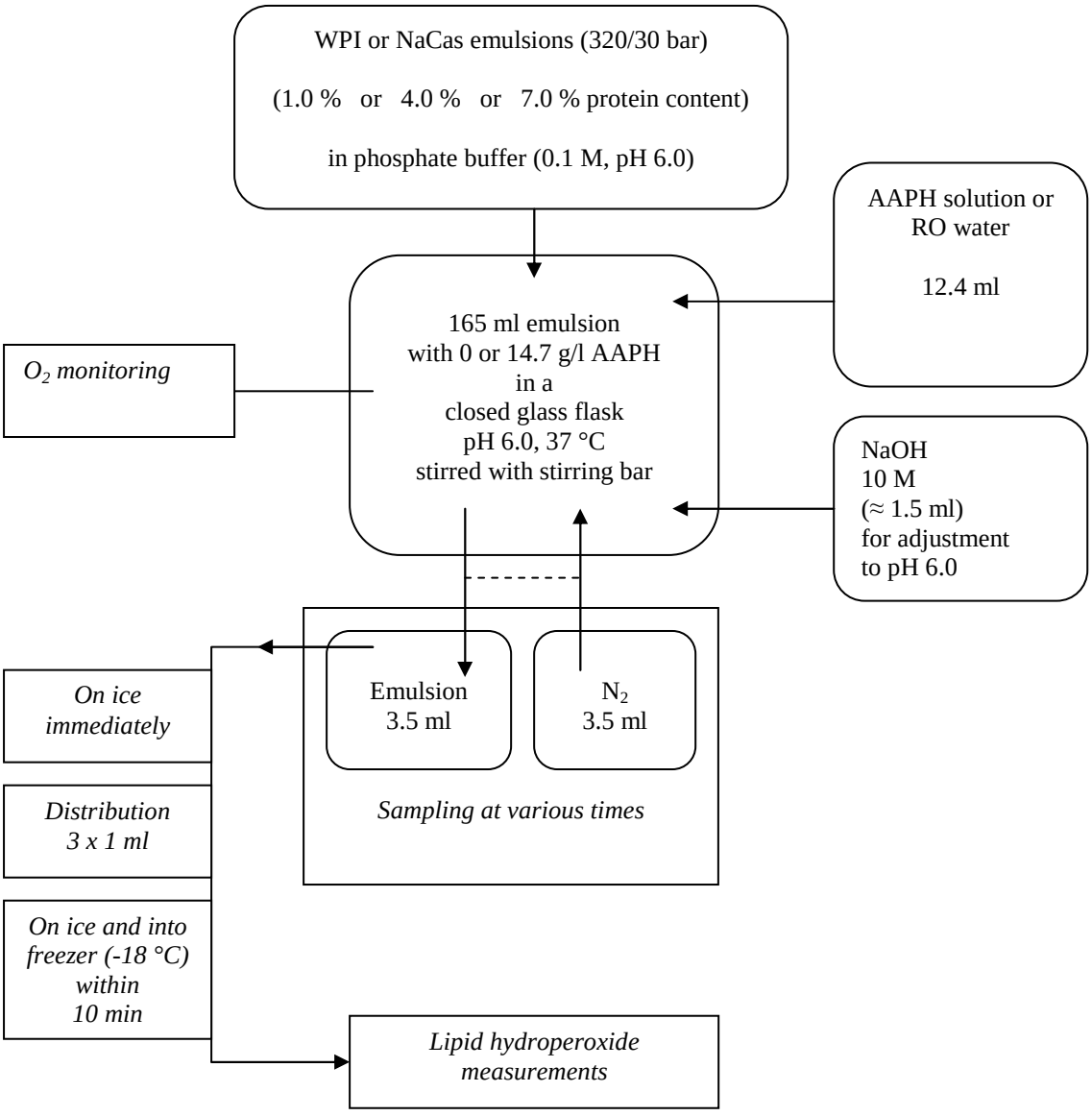


Figure 7.1: Experimental outline: oxygen consumption and lipid hydroperoxide generation in emulsions, accelerated by AAPH

In this experimental set-up, it was difficult to measure the initial oxygen concentrations. After the addition of AAPH, one minute was granted to reach homogeneity and a second minute allowed for equilibration after the system had been closed with a stopper. The first measurements, therefore, took place after two minutes.

The decrease of the oxygen concentration for emulsions with and without AAPH (14.7 g/l) is shown in Figure 7.2.

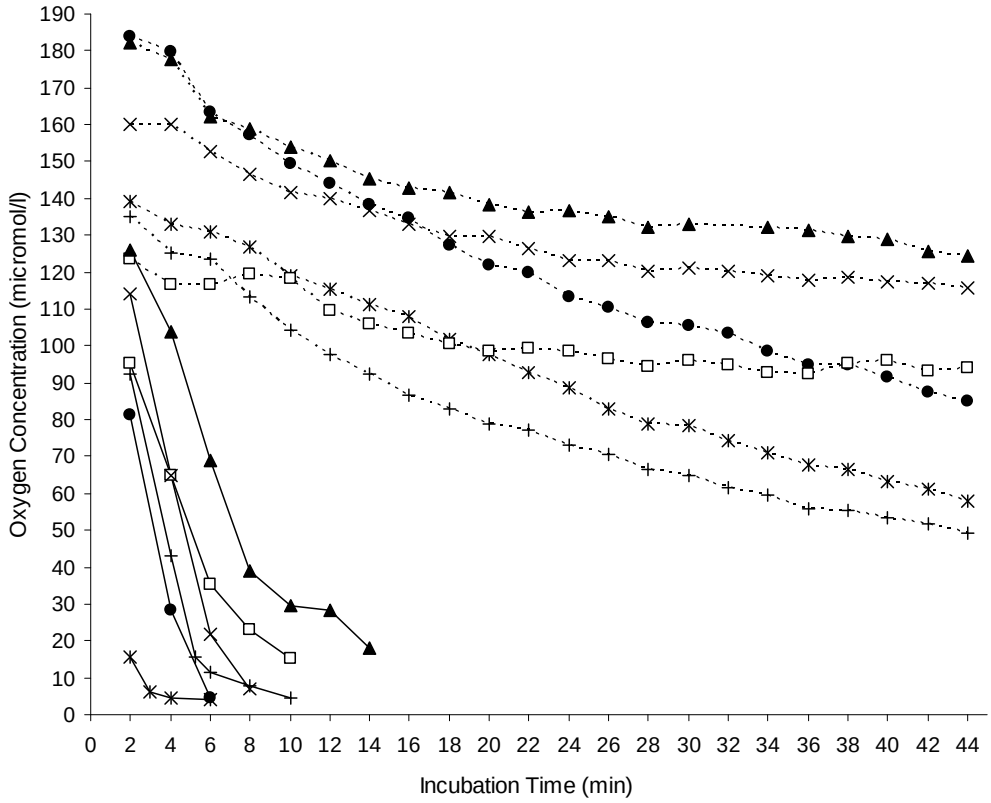


Figure 7.2: Decrease of oxygen concentration in WPI and NaCas emulsions with AAPH (14.7 g/l) (—) and without AAPH (---): WPI 7.0 % (×), WPI 4.0 % (+), WPI 1.0 % (⁎), NaCas 7.0 % (▲), NaCas 4.0 % (□), NaCas 1.0 % (●)

Emulsions with AAPH showed a much faster decrease of the oxygen concentration than those without AAPH. When AAPH was added, the oxygen concentration had dropped to ≤ 20 µmol/l within 15 min, whereas at least 50 µmol/l oxygen was still present after 45 min without AAPH addition.

In NaCas emulsions without AAPH, the oxygen concentration decreased the strongest in the 1.0 % protein emulsion and the decrease was weaker and similar for the 4.0 and 7.0 % protein level. In WPI emulsions without AAPH, the decrease in oxygen concentration was similar for the 1.0 and 4.0 % protein level (also similar to the NaCas 1.0 % protein level) and stronger than for the 7.0 % protein level (which showed a similar decrease as for the NaCas 7.0 % protein level).

The decrease in oxygen concentration as a function of incubation time for emulsions with AAPH was linear at the beginning of the incubation process and reached very low values within a few minutes (Figure 7.3). For emulsions with three or more data points in the area of linear decrease, linear regression curves were created in Microsoft Excel (Table 7.1), so that the initial oxygen concentrations (0 min) could be determined. The values obtained were averaged and the mean value of the initial oxygen concentrations calculated (145 ± 8 (SEM) µmol/l). In emulsions of 1.0 % protein, oxygen decreased very fast and only a few data points lay in the area of linear decrease. For WPI and NaCas emulsions with 1.0 % protein the initial oxygen concentration was estimated to be 145 µmol/l (Figure 7.3).

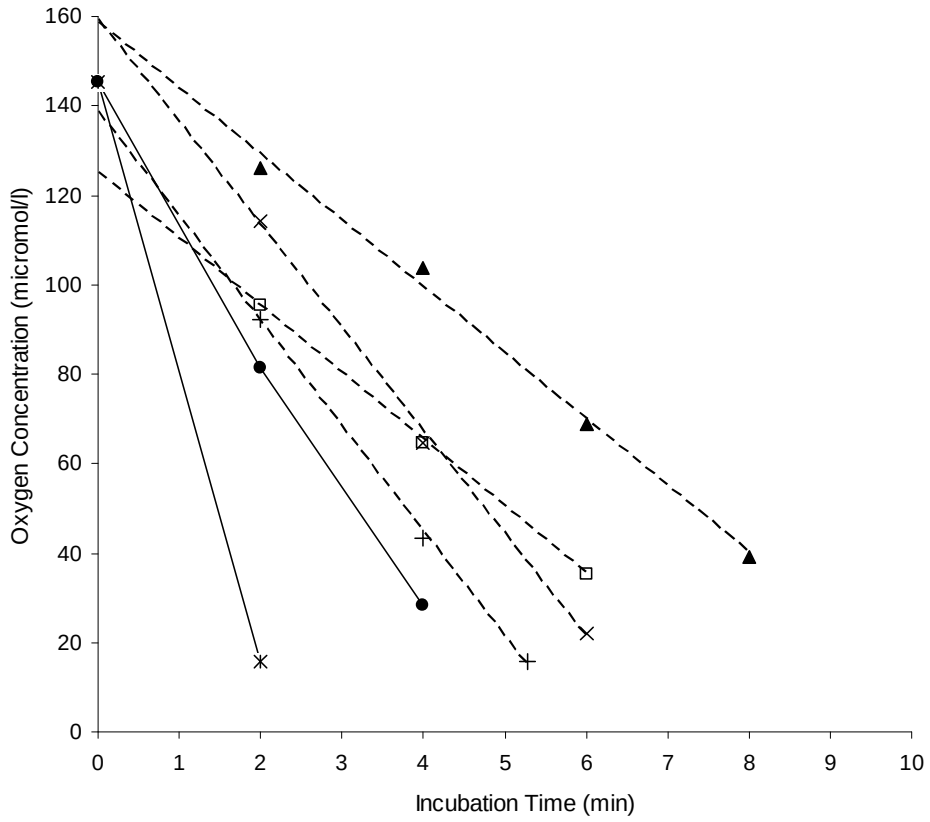


Figure 7.3: Decrease of oxygen concentration in WPI and NaCas emulsions with AAPH (14.7 g/l): linear regression curves (---), connection of data points (—), WPI 7.0 % (×), WPI 4.0 % (+), WPI 1.0 % (✳), NaCas 7.0 % (▲), NaCas 4.0 % (□), NaCas 1.0 % (●)

Table 7.1: Linear regression analysis of data on oxygen concentration in WPI and NaCas emulsions of 4.0 and 7.0 % protein with AAPH (14.7 g/l)

Emulsion type	Equation of the linear regression curve	Coefficient of determination (R^2)
WPI, 4.0 %	y = - 23.536x + 138.78	0.9989
WPI, 7.0 %	y = - 23.069x + 159.22	0.9983
NaCas, 4.0 %	y = - 15.014x + 125.22	0.9999
NaCas, 7.0 %	y = - 14.795x + 158.43	0.9931

In order to follow and compare oxygen consumption over time easier, the oxygen concentration in micromole per litre was converted into micromoles of oxygen consumed (Figure 7.4).

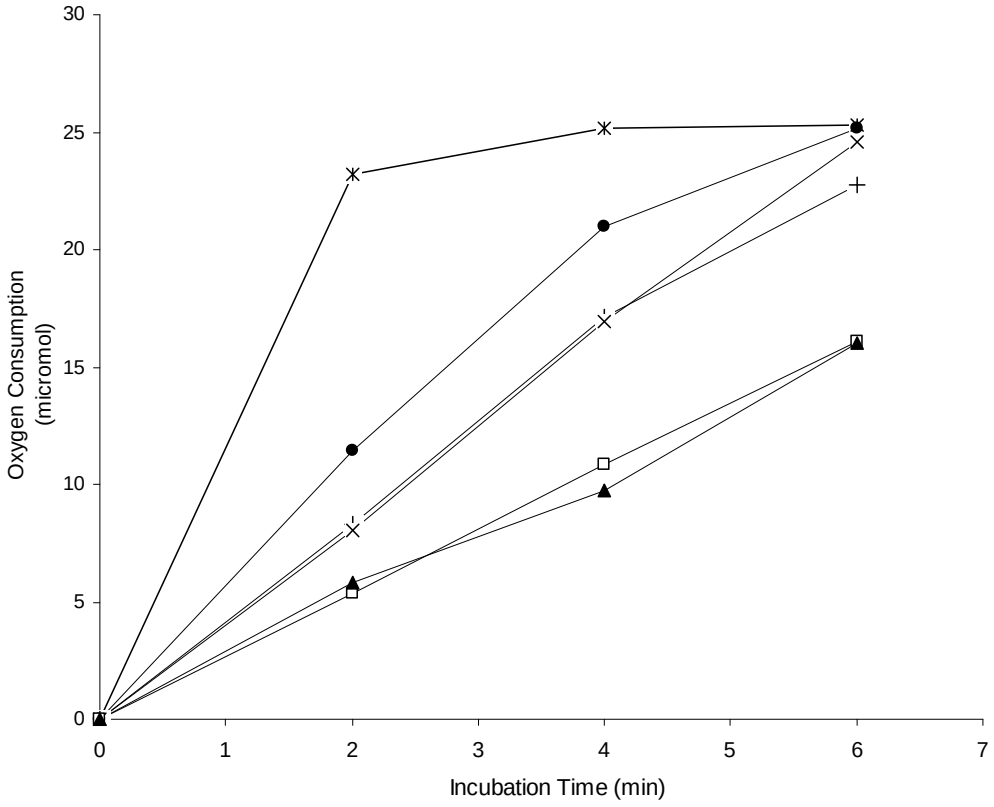


Figure 7.4: Oxygen consumption in micromole in WPI and NaCas emulsions with AAPH (14.7 g/l): WPI 7.0 % (×), WPI 4.0 % (+), WPI 1.0 % (✳), NaCas 7.0 % (▲), NaCas 4.0 % (□), NaCas 1.0 % (●)

At all protein levels, oxygen was consumed faster in WPI than in NaCas emulsions. Within one protein type, oxygen was consumed at the same rate for 4 and 7 % protein emulsions, but consumed faster at the 1 % protein level.

The development of lipid hydroperoxides in the same WPI and NaCas emulsions with and without AAPH (14.7 g/l) is shown in Figure 7.5.

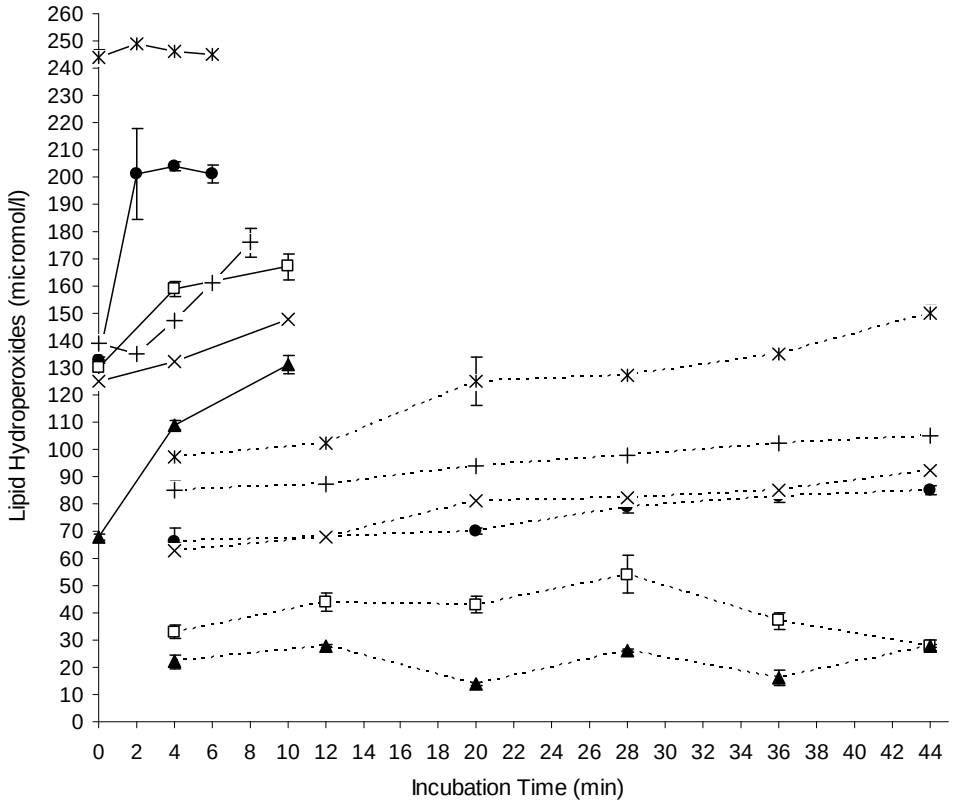


Figure 7.5: Lipid hydroperoxide development in WPI and NaCas emulsions with AAPH (14.7 g/l) (—) and without AAPH (---): WPI 7.0 % (×), WPI 4.0 % (+), WPI 1.0 % (⁎), NaCas 7.0 % (▲), NaCas 4.0 % (□), NaCas 1.0 % (●)

Lipid hydroperoxides developed much faster in emulsions with AAPH. Furthermore, the values of the same type of emulsion were at all times lower when no AAPH was added.

Table 7.2: Lipid hydroperoxide concentrations in WPI and NaCas emulsions of various protein concentrations without AAPH after 4 and 44 min

Emulsion type	Lipid hydroperoxides in emulsion (µmol/l)	
	after 4 min	after 44 min
WPI 1.0 %	97	150
WPI 4.0 %	85	105
WPI 7.0 %	63	92
NaCas 1.0 %	66	85
NaCas 4.0 %	33	28
NaCas 7.0 %	22	28

Less lipid hydroperoxides developed in NaCas emulsions than in WPI emulsions without AAPH. NaCas emulsions of 4.0 % and 7.0 % protein showed no increase in lipid hydroperoxides over 44 min. In general, less lipid hydroperoxides developed with increasing protein concentration (Table 7.2).

When AAPH was added, the highest lipid hydroperoxide levels were found in the WPI (1.0 % protein) emulsion. The values were constant over time (240-250 µmol/l). In the WPI (4.0 % protein) emulsion the lipid hydroperoxide level increased from 140 to 175 µmol/l within 8 minutes and in the WPI (7.0 % protein) emulsion from 125 to 150 µmol/l within 10 minutes. In the NaCas (1.0 % protein) emulsion the second highest lipid hydroperoxide levels were found. The level had increased from 135 to 200 µmol/l after two minutes and remained constant over time thereafter. In the other NaCas emulsions, the lipid hydroperoxide levels increased from 130 to 165 µmol/l (4.0 % protein) and from 70 to 130 µmol/l (7.0 % protein) within 10 minutes.

The same trends were found in emulsions with and without AAPH. The lower the protein concentration, the stronger was the lipid hydroperoxide generation. In NaCas emulsions less lipid hydroperoxides were formed.

As expected, the addition of a free radical source (AAPH) had a strongly pro-oxidative effect on the emulsions. Oxygen was consumed faster and lipid hydroperoxides were produced faster or reached higher levels than in emulsions without AAPH. It was also shown that proteins had an antioxidative effect. With AAPH, the consumption of oxygen occurred at a linear rate in the initial stages. It can be assumed that the rate of free radical production was also linear over time (*http://www.caymanchem.com/app/template/Product.vm/catalog/82235/promo/searchresult.82235/a/z* 2008). This indicates a correlation between the production of AAPH-derived radicals and oxygen consumption. The slope of oxygen consumption varied depending on the protein type and concentration. There was no difference between the rate of oxygen consumption for emulsions with 4.0 and 7.0 % protein within one protein type, but at 1.0 % protein content, oxygen was consumed faster (Figure 7.4). Also, oxygen was consumed faster in WPI than in NaCas emulsions. As oxygen consumption and AAPH radical production appear to be linked, the rate of oxygen consumption is likely to mirror the ability of the proteins to scavenge AAPH-derived radicals.

In emulsions without AAPH, the same antioxidative characteristics were generally observed: greater inhibition towards oxygen consumption and lipid hydroperoxide generation were found for higher protein levels and NaCas. Therefore one might attribute the observed antioxidative performances of these emulsions, to a large extent, to the ability of the incorporated proteins to scavenge free radicals.

Figure 7.6 shows how the proteins possibly intervened in the autoxidation of the emulsion lipid accelerated by AAPH-derived radicals. By the reaction of proteins with those radicals, presumably more stable and less reactive radicals were formed on the proteins (free-radical-scavenging process). Less AAPH-derived radicals were therefore available for reactions with the lipid molecules of the emulsion and lipid oxidation was inhibited. As the AAPH radical production rate was constant, only depending on the temperature and the pH value, and less oxygen was consumed at the higher protein content, it is assumed that the free radicals formed on the proteins did not react with oxygen to a notable extent. Figure 7.6 also illustrates how less oxygen is consumed and lipid hydroperoxides are formed when the protein concentration increases from (a) to (c).

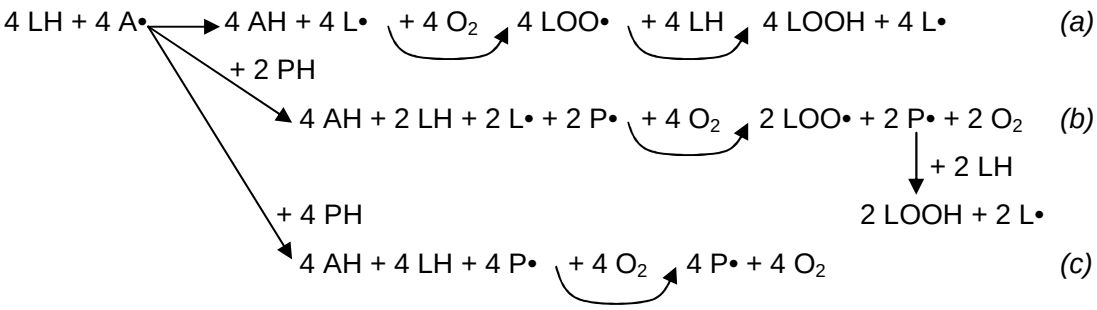


Figure 7.6: Possible antioxidative intervention of proteins in the autoxidation of the emulsion lipid accelerated by AAPH: LH = lipid, L• = lipid radical, PH = protein, P• = protein radical, AH = AAPH derived radical reduced with hydrogen, A• = AAPH derived radical, O_2 = oxygen, LOO• = peroxyl radical, LOOH = lipid hydroperoxide, (a) A• reacts only with lipid, no protein is present, (b) A• reacts with some lipid and protein at a low protein concentration, (c) A• reacts only with protein at a high protein concentration

The proteins (PH) scavenged AAPH-derived radicals (A•) and also lipid radicals (L•) already present in the emulsions, although this is not explicitly shown in Figure 7.6. The focus here was on the strong external pro-oxidant AAPH.

7.2 Effects of free radicals on protein oxidation

It was shown that the milk proteins exhibited free-radical-scavenging abilities. Free radicals were transferred to the protein molecules which presumably led to some molecular damage of these molecules by oxidation. In this section, molecular damage to the proteins and how this might be linked to their antioxidative mechanisms was investigated.

The first intention was to detect free radicals directly on the proteins, but it was abandoned as the most appropriate investigating tool would have been electron spin resonance (ESR) spectrometry and this would have required a major readjustment of the project.

Instead the consumption of oxygen in protein solutions exposed to the influence of AAPH (14.7 g/l) and the development of protein hydroperoxides in emulsions were followed to examine the reactivity of the proteins toward oxygen. Also the generation of protein hydroperoxides was compared with the generation of lipid hydroperoxides in the same emulsion system to get an estimate of the relative reactivity of emulsion lipid (linoleic acid) and protein (WPI) towards oxygen. Elias et al. (2008) mention in their review about the antioxidative activity of proteins and peptides that the relative kinetics of protein and lipid oxidation were not investigated in many studies about protein oxidation, leaving uncertainty if lipid oxidation causes the oxidation of proteins or if proteins oxidise as they are protecting the lipids from oxidation.

Secondly, the loss of amino acids and total sulfhydryls were determined. Amino acid loss and changes of the total sulfhydryl concentration were investigated in a WPI emulsion (1.0 % protein) and also in a WPI solution (1.12 % protein) exposed to the influence of AAPH (14.7 g/l). It was thereby possible to examine differences in the deteriorative effects caused by free radicals from oxidising lipid compared with free radicals derived from AAPH.

RO water, phosphate buffer (pH 6.0, 0.1 M) or protein dispersed in phosphate buffer (pH 6.0, 0.1 M) plus AAPH (14.7 g/l) were incubated in a closed glass flask at 37 ˚C for 70 to 100 min. The decrease of the oxygen concentration was monitored. The experimental plan is depicted in Figure 7.7. More details can be found in Chapter 3, Materials and methods.

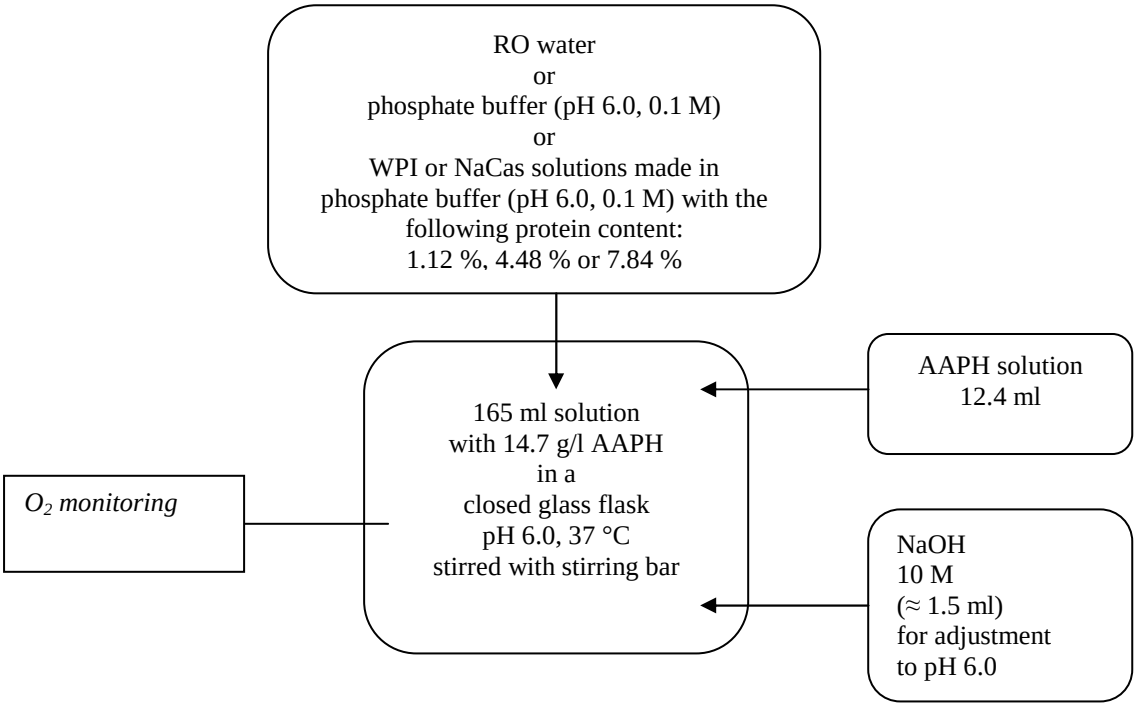


Figure 7.7: Experimental plan: oxygen consumption in protein solutions with AAPH

The development of protein hydroperoxides, in the continuous and cream phase, and lipid hydroperoxides in a WPI emulsion (1.0 % protein) incubated for 4 h at 50 ˚C was followed. Protein hydroperoxides were determined as described as follows. Protein solution from the continuous or cream phase was used in the protein hydroperoxide assay. The amount of hydroperoxides (µmol) in 1 ml of the respective protein solution was determined based on a hydrogen peroxide standard curve. The protein content (g) of 1 ml of the continuous and cream phase solutions of the emulsion incubated for 0 h had been determined in another experiment carried out under the same experimental conditions (Figure 7.9). The two values, the amount of hydroperoxides (µmol) and the protein content (g) in 1 ml protein solution, were divided, giving the hydroperoxide concentration (µmol/g) on the protein. Previously the amount of WPI present in the continuous phase and on the interface of a WPI (1.0 % protein) emulsion had been determined (Chapter 3, Materials and methods, 3.3.3 Methods: Chapter 4.3). The protein hydroperoxide concentration (µmol/g) on the protein found for the respective phase and incubation time was multiplied with the amount (g) of protein in the continuous phase and on the emulsion interface of 1 ml of a WPI (1.0 % protein) emulsion. The amount of hydroperoxides (µmol) obtained was multiplied by 1000, resulting in the protein hydroperoxide concentrations (µmol/l) in the emulsion. The presentation of the values in µmol/l of emulsion allowed a direct comparison with the development of lipid hydroperoxides (µmol/l). The experimental plan is depicted in Figure 7.8. More details about the execution can be found in Chapter 3, Materials and methods.

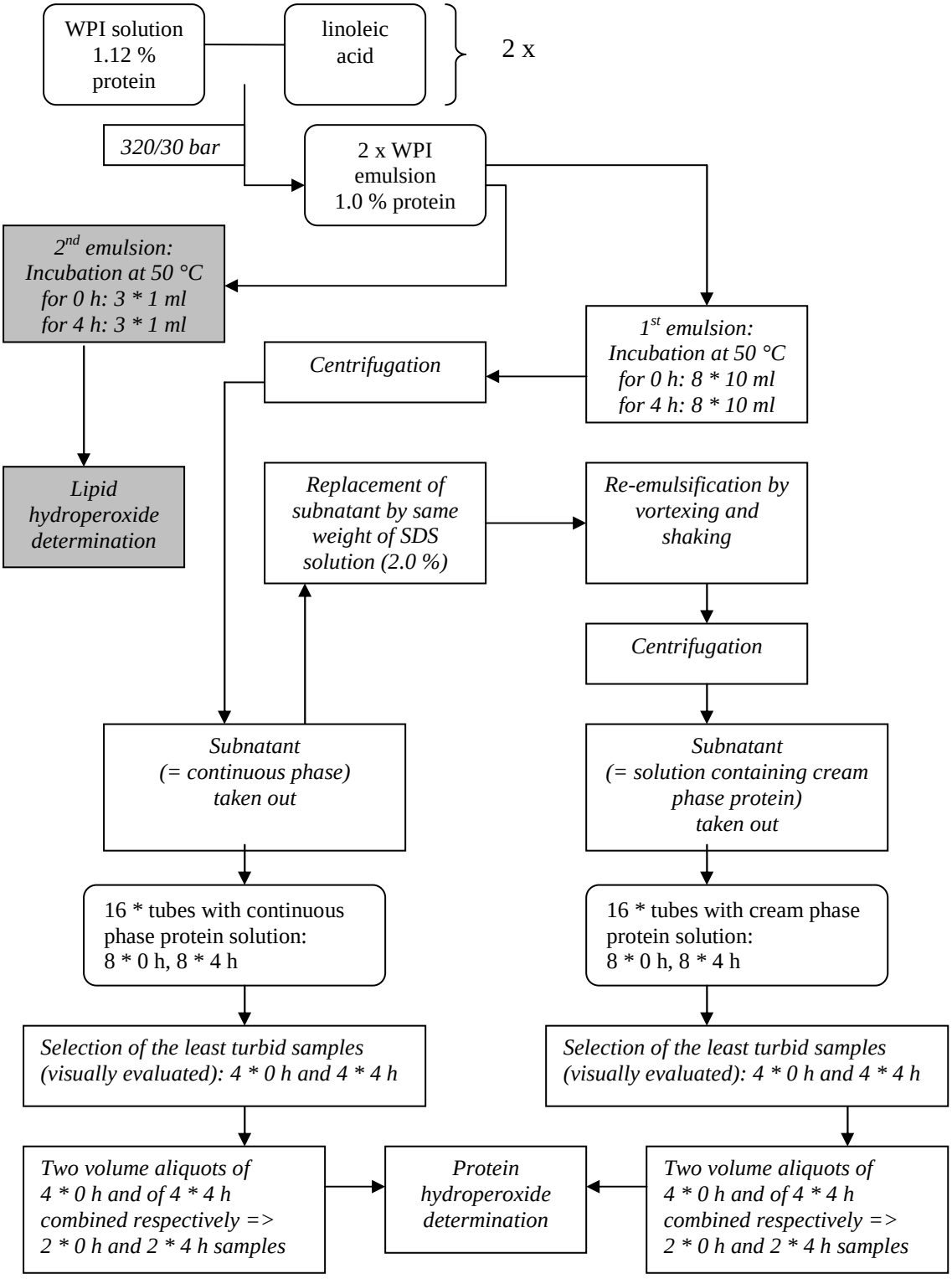


Figure 7.8: Experimental plan: development of protein hydroperoxides in the continuous and cream phase and lipid hydroperoxides in a WPI (1.0 % protein) emulsion

The concentration of a wide range of amino acids and total sulfhydryls in the protein of the continuous and cream phase of a WPI (1.0 % protein) emulsion was followed over time. The emulsion was incubated for up to 20 h at 50 ˚C. Briefly, amino acids were determined by acidic hydrolysis of the protein followed by HPLC analysis according to laboratory procedures (Zou 2005) based on AOAC Official Methods 994.12 and 985.28 and for tryptophan determination alkaline hydrolysis was performed followed by HPLC analysis based on the AOAC Official Method 988.15 (AOAC 2005b; AOAC 2005d). Total sulfhydryls were determined with an adapted method from Taylor and Richardson (1980b). The amino acid and total sulfhydryl concentration were calculated in micromole per g of protein and referred to the protein content of the continuous and cream phase solutions at 0 h of incubation. The protein content was determined by summing up the contents of all amino acids. It decreased from about 0.5 to 0.4 % over 18 h (measurements of 0 h, 4 h and 18 h samples). The decrease was probably due to a structural change of some of the amino acids which could therefore not be detected any more. It was probably not due to a change of the general protein content in the solutions. Therefore the protein contents of only the samples of 0 h incubation time were considered. The protein contents determined by the “Leco, total combustion method” according to AOAC Official Method 968.06 (Dumas Method) gave similar results for the samples of 0 h incubation time (AOAC 2005a). However, the method seemed not suitable to measure the protein content when emulsion samples were incubated for 4 h and longer as the results exhibited a high variability. The experimental plans are depicted in Figure 7.9 and Figure 7.10. More details about the execution can be found in Chapter 3, Materials and methods.

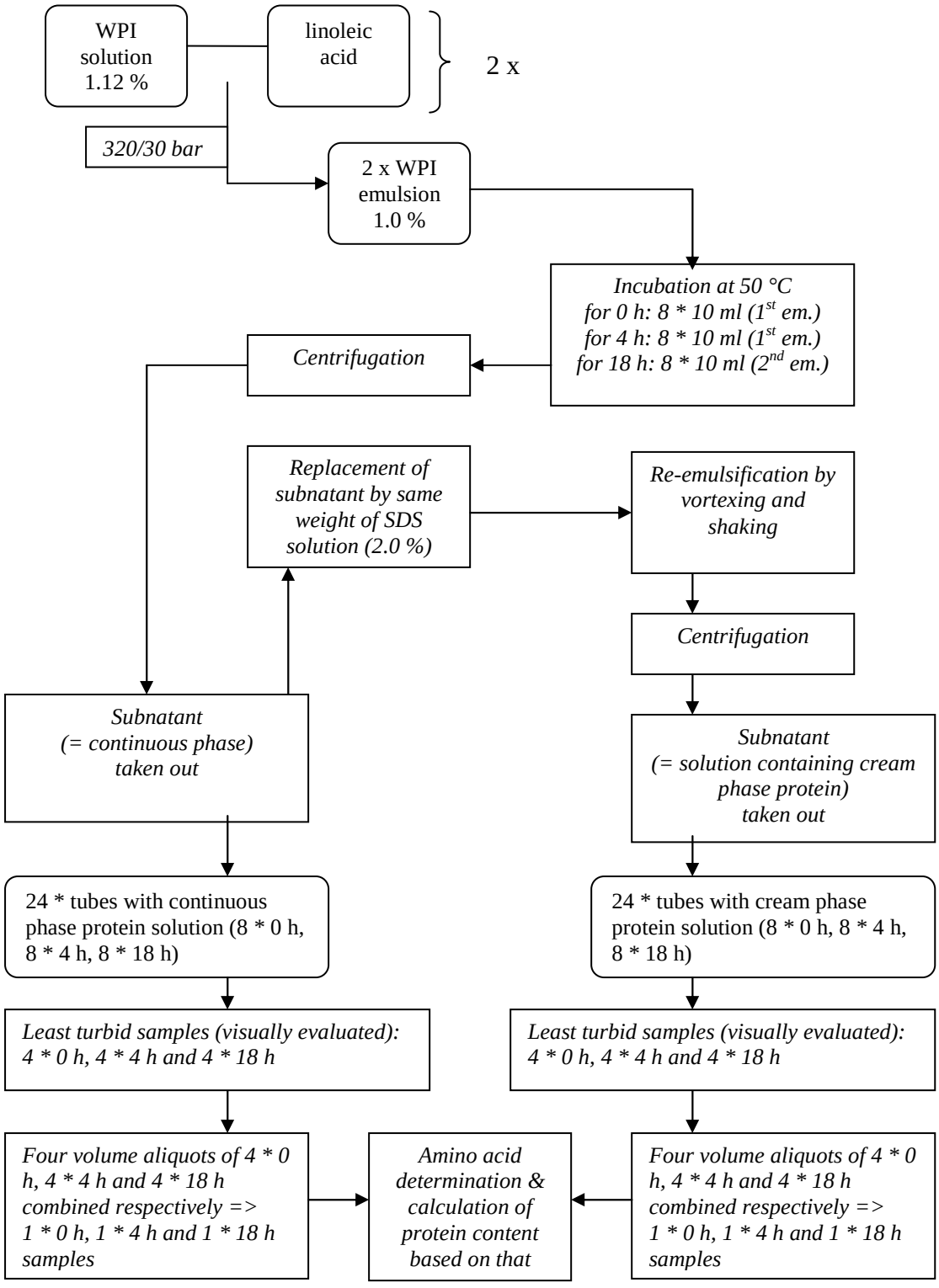


Figure 7.9: Experimental plan: investigation of amino acid concentrations in the protein of continuous and cream phase of a WPI (1.0 %) emulsion incubated at 50 ˚C for 18 h

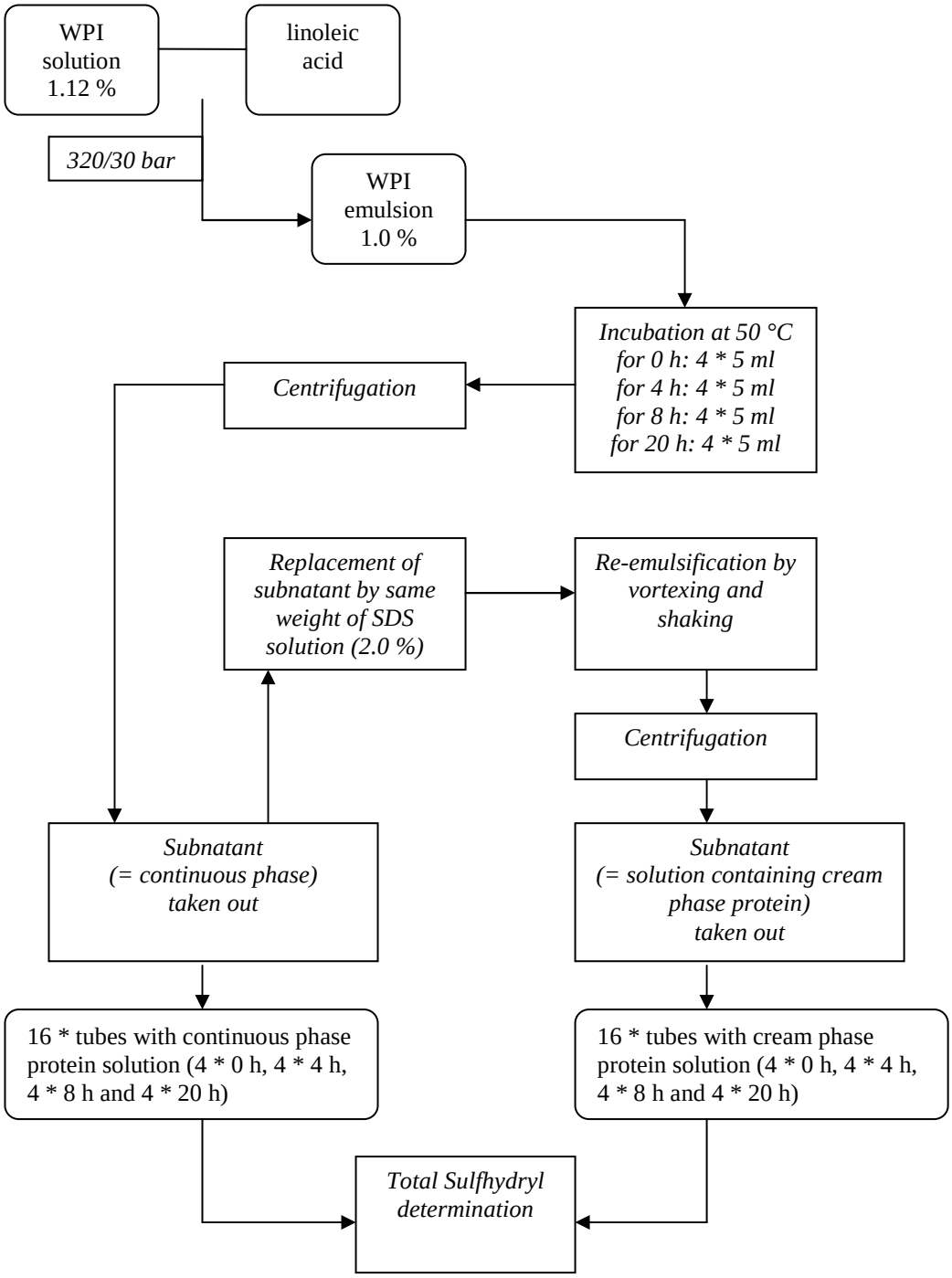


Figure 7.10: Experimental plan: investigation of the total sulfhydryl concentrations in the protein of continuous and cream phase of a WPI (1.0 % protein) emulsion incubated at 50 ˚C for 20 h

WPI (1.12 % protein) solutions with and without the free radical source AAPH (14.7 g/l) were incubated for 18 h at 50 ˚C (four replicates). The protein solutions to which AAPH was added were prepared with phosphate buffer (0.1 M) of pH 6.0 instead of RO water as it was also used for pH stability when AAPH had caused a strong decrease of the oxygen concentration in emulsions. The concentration of a wide range of amino acids and total sulfhydryls in the protein was determined. For the amino acid determination four aliquots were taken from each sample and combined. The amino acids were determined as stated above. For the total sulfhydryl determination, three of the four samples were determined. More details about the execution can be found in Chapter 3, Materials and methods.

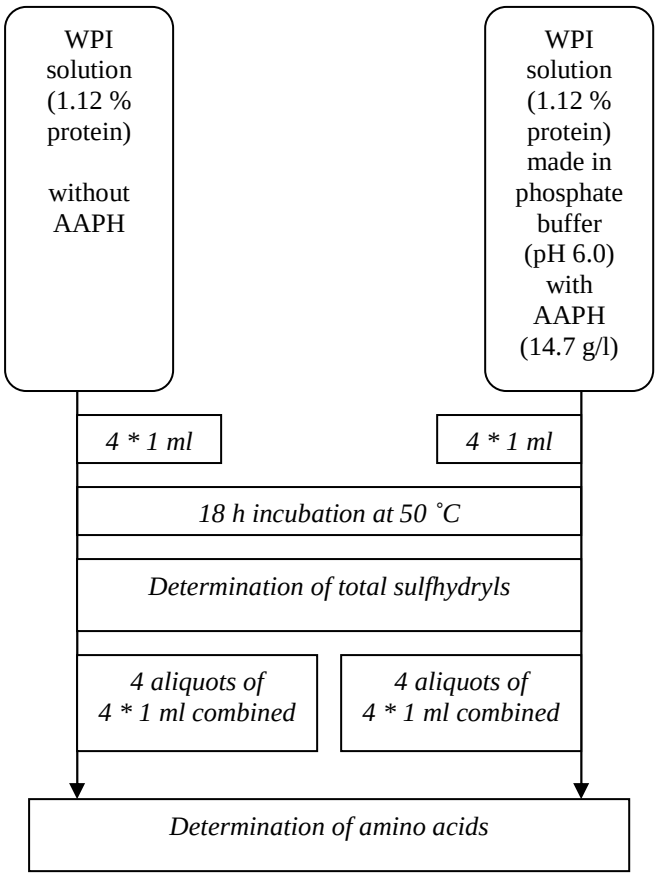


Figure 7.11: Experimental plan: investigation of amino acid and total sulfhydryl concentrations in the protein of a WPI (1.12 % protein) solution with and without AAPH (14.7 g/l) incubated at 50 ˚C for 18 h

The oxygen concentration in WPI and NaCas solutions with AAPH (14.7 g/l) decreased slightly faster than in the control solutions (phosphate buffer of pH 6.0 and RO water). The WPI and NaCas solution of the same protein concentration showed a similar decrease. Also the decrease was similar in solutions with different protein concentration (Figure 7.12). In emulsions with the same level of AAPH the oxygen concentration decreased much faster (see earlier in Section 7.1, Figure 7.2). This indicated a much greater reactivity of linoleic acid rather than the milk proteins with oxygen under pro-oxidative conditions at a high level of free radicals.

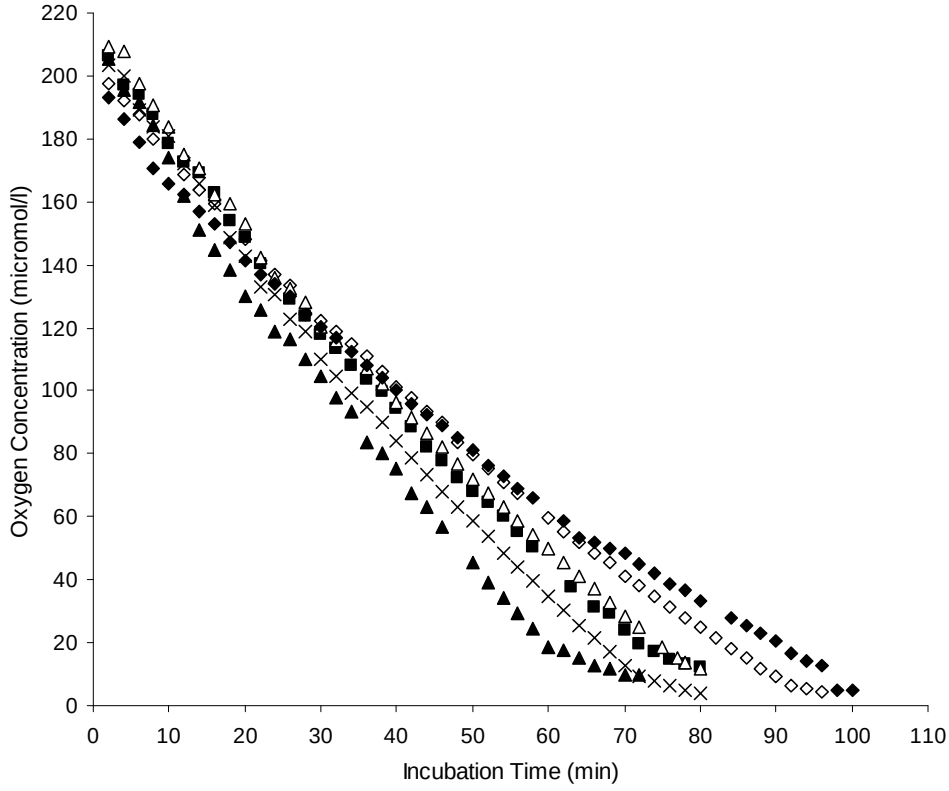


Figure 7.12: Decrease of oxygen concentration in aqueous solutions with AAPH (14.7 g/l): RO water (◆), phosphate buffer of pH 6.0 (◇), WPI 1.12 % (■), WPI 4.48 % (△), WPI 7.84 % (×), NaCas 4.48 % (▲)

The values obtained in the protein hydroperoxide determination were fairly variable (Table 7.3), which was probably due to the extended multi-step procedure in the protein hydroperoxide assay (Chapter 3, Materials and methods, 3.6.4 Methods: Chapter 7.2). The protein hydroperoxide concentration in both, cream phase and continuous phase, appear to be low and stable over time in comparison with the development of lipid hydroperoxides. The lipid hydroperoxide level in the emulsion increased about ten-fold over the same time period (4 h), from 25 ± 2 µmol/l to 247 ± 3 µmol/l (± SEM). This result appears to be in line with the finding that faster oxygen consumption occurred in emulsions than in protein solutions, indicating that oxygen reacted more readily with linoleic acid than with the milk proteins.

Table 7.3: Protein hydroperoxide concentration at 0 h and after 4 h incubation time derived from continuous and cream phase protein in a WPI (1.0 % protein) emulsion

Type of protein source	Incubation time of the emulsion (h)	Mean value of protein hydroperoxides in a WPI (1.0 % protein) emulsion (n = 2) ± SEM* (µmol/l)	Single values of protein hydroperoxides in a WPI (1.0 % protein) emulsion (µmol/l)
Continuous phase protein	0	18.2 ± 9.0	27.3, 9.2
	4	11.6 ± 0.6	11.0, 12.2
Cream phase protein	0	4.2 ± 1.5	2.7, 5.7
	4	11.8 ± 4.5	16.2, 7.3

*SEM = standard error of the mean value

The concentration of total sulfhydryls decreased from 43 to 5 µmol/g protein in the continuous phase and from 14 to 4 µmol/g protein in the cream phase of a WPI (1.0 % protein) emulsion incubated for 20 h (Figure 7.13, see also Table 7.4 and Table 7.5).

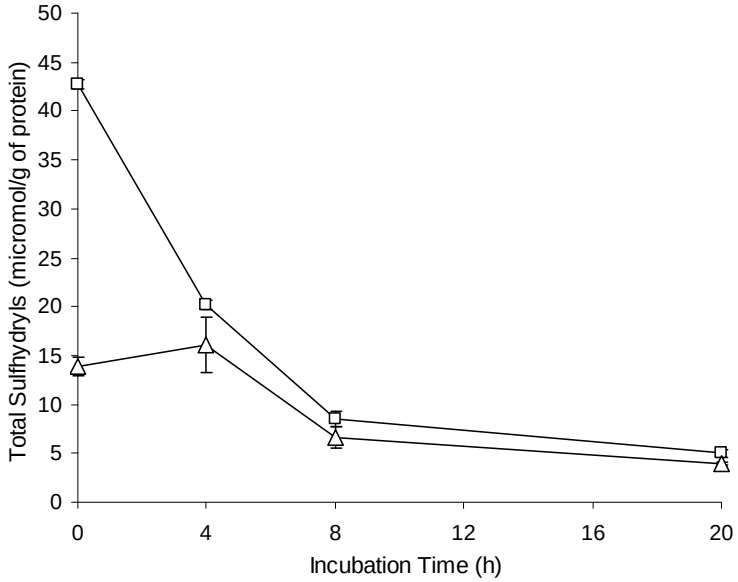
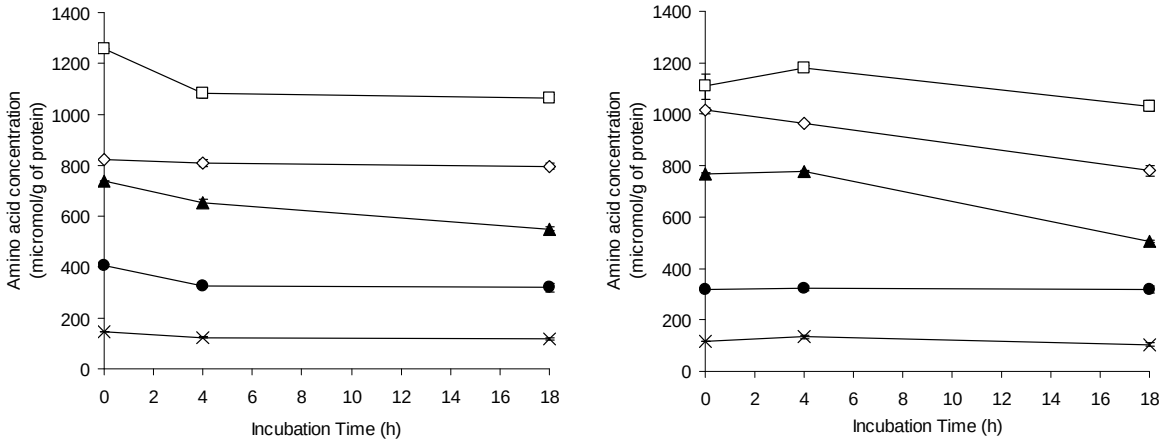


Figure 7.13: Changes in total sulfhydryls in the protein of continuous phase (□) and cream phase (△) of a WPI (1.0 % protein) emulsion

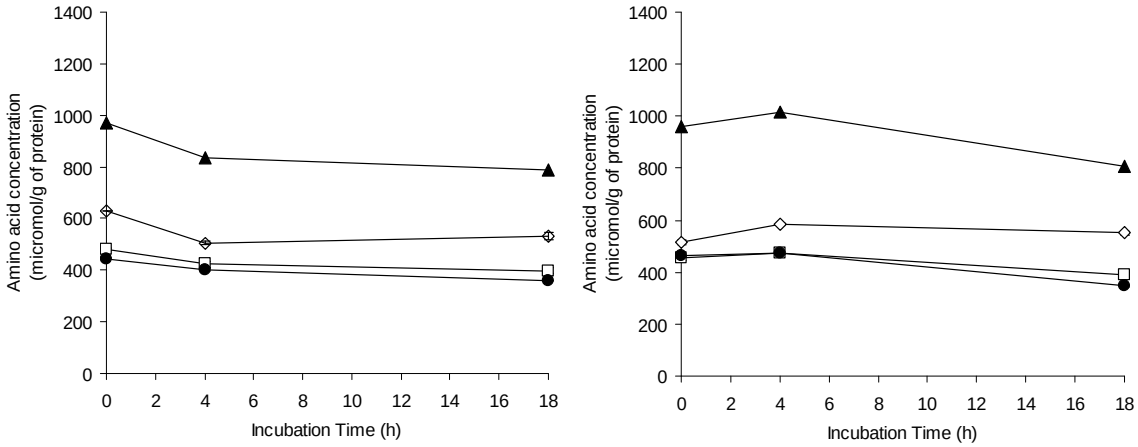
An overview about the concentrations of various amino acids in the continuous and cream phase protein of a WPI (1.0 % protein) emulsion over 18 h incubation time is given in Figure 7.14 and Figure 7.15. More details are given in Table 7.4 and Table 7.5. Overall the decrease in amino acid concentrations was slightly higher in the cream phase protein (1346 µmol/g protein) than in the continuous phase protein (1068 µmol/g protein), excluding total sulfhydryls. The greatest decreases in concentration in the continuous phase protein were found for glutamic acid (including glutamine) (192 µmol/g protein), lysine (186 µmol/g protein) and leucine (182 µmol/g protein). In the cream phase protein, the greatest decreases in concentration were found for lysine (262 µmol/g protein), aspartic acid (including asparagine) (235 µmol/g protein) and leucine (154 µmol/g protein). Although the aspartic acid/asparagine concentration greatly decreased in the cream phase protein only a minor decrease occurred in the continuous phase protein.

For the continuous phase protein, the greatest decreases in percent occurred for total sulfhydryls (cysteine) (88 %), tryptophan (45 %) and methionine (40 %). For the cream phase protein, it was also total sulfhydryls (cysteine) (72 %), tryptophan (58 %) and methionine (37 %) (Table 7.4 and Table 7.5).

For some amino acids an increase in concentration was found, especially from 0 to 4 h. As there was an increase in one phase and at the same time a decrease in the other phase (e.g. for glutamic acid/glutamine, leucine and alanine from 0 to 4 h), proteins and/or peptides could have migrated between the phases.

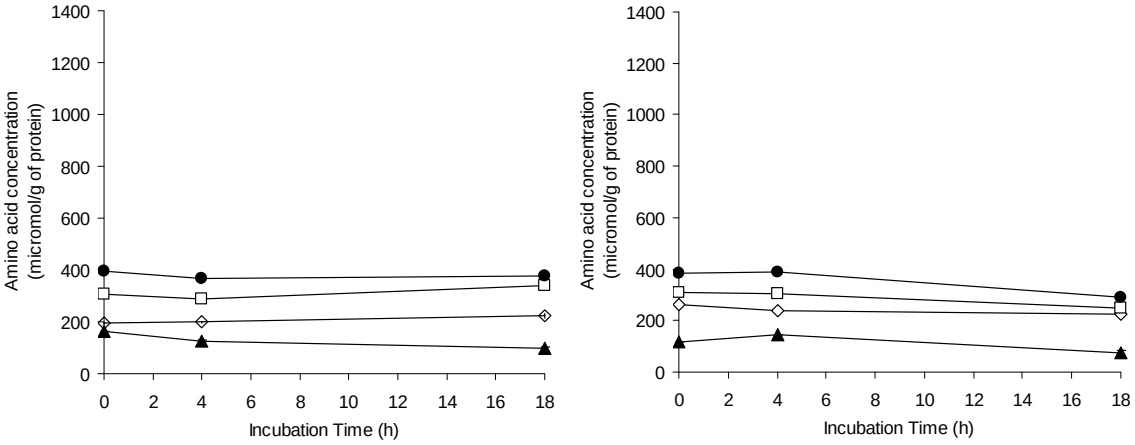


glutamic acid + glutamine (□), aspartic acid + asparagine (◇), lysine (▲), proline (●), arginine (×)

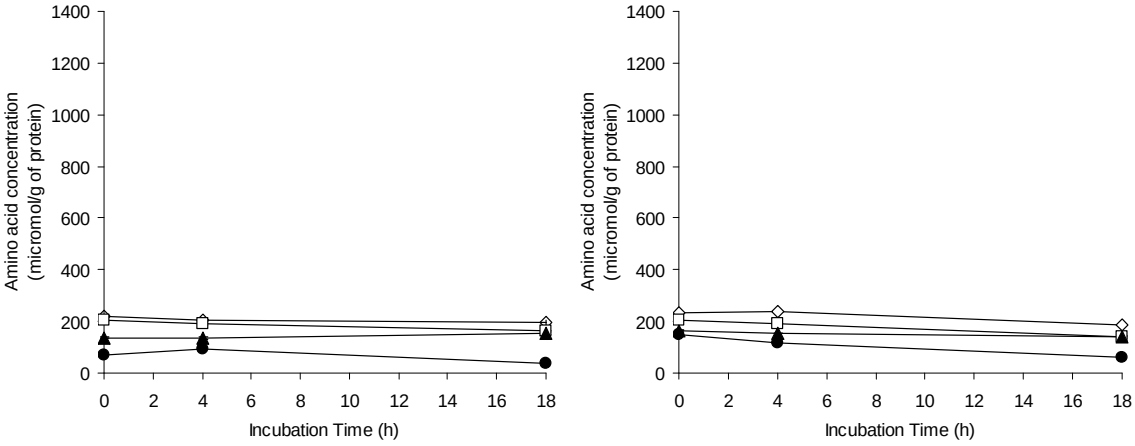


valine (□), alanine (◇), leucine (▲), isoleucine (●)

Figure 7.14: Development of the amino acid concentration in the protein of the continuous phase (left side) and cream phase (right side) of a WPI (1.0 % protein) emulsion incubated at 50 ˚C



serine (□), glycine (◇), methionine (▲), threonine (●)



tyrosine (□), phenylalanine (◇), histidine (▲), tryptophan (●)

Figure 7.15: Development of the amino acid concentration in the protein of the continuous phase (left side) and cream phase (right side) of a WPI (1.0 % protein) emulsion incubated at 50 ˚C

Table 7.4: Decrease of amino acid and total sulfhydryl concentrations in the continuous phase protein in a WPI emulsion (1.0 % protein) incubated for 18 h (20 h for total sulfhydryls) at 50 ˚C

Amino acid or total sulfhydryls	Concentration after 0 h in the continuous phase protein mean value, [1st/2nd replicate] or ± SEM* (µmol/g protein)	Concentration after 18 h (20 h) in the continuous phase protein mean value, [1st/2nd replicate] or ± SEM* (µmol/g protein)	Decrease of the concentration after 18 h (20 h) in the continuous phase protein (µmol/g protein)	Decrease of the concentration after 18 h (20 h) in the continuous phase protein (%)
glutamic acid + glutamine	1258 [1256/1260]	1066 [1076/1057]	192	15
lysine	737 [737/737]	550 [556/544]	186	25
leucine	970 [970/969]	787 [794/781]	182	19
alanine	630 [629/631]	532 [547/518]	97	15
isoleucine	445 [445/446]	361 [361/360]	85	19
valine	480 [483/477]	398 [408/388]	82	17
proline	407 [404/409]	326 [310/341]	81	20
methionine	163 [163/163]	98 [101/94]	65	40
tyrosine	203 [202/204]	162 [163/162]	41	20
tryptophan	71 [71/-]	39 [39/-]	32	45
arginine	147 [145/149]	116 [121/111]	31	21
aspartic acid + asparagine	824 [822/825]	796 [809/784]	27	3
phenylalanine	219 [216/223]	194 [198/190]	25	12
threonine	396 [394/398]	377 [380/374]	19	5
histidine	137 [136/138]	154 [158/149]	-16	-12
glycine	193 [192/194]	222 [231/213]	-29	-15
serine	307 [306/307]	339 [354/324]	-32	-10
total sulfhydryls	43 ± 1	5 ± 1	38	88

* SEM = standard error of the mean value; four replicates

Table 7.5: Decrease of amino acid and total sulfhydryl concentrations in the cream phase protein in a WPI emulsion (1.0 % protein) incubated for 18 h (20 h for total sulfhydryls) at 50 ˚C

Amino acid or total sulfhydryls	Concentration after 0 h in the cream phase protein mean value, [1st/2nd replicate] or ± SEM* (µmol/g protein)	Concentration after 18 h (20 h) in the cream phase protein mean value, [1st/2nd replicate] or ± SEM* (µmol/g protein)	Decrease of the concentration after 18 h (20 h) in the cream phase protein (µmol/g protein)	Decrease of the concentration after 18 h (20 h) in the cream phase protein (%)
lysine	769 [771/766]	506 [512/501]	262	34
aspartic acid + asparagine	1015 [1027/1004]	780 [800/761]	235	23
leucine	960 [964/957]	807 [817/797]	154	16
isoleucine	461 [463/460]	347 [350/345]	114	25
threonine	385 [397/372]	291 [279/303]	94	24
tryptophan	149 [149/-]	62 [62/-]	87	58
glutamic acid + glutamine	1108 [1155/1060]	1031 [1051/1011]	76	7
valine	456 [465/446]	390 [399/381]	65	14
serine	311 [329/293]	246 [231/262]	65	21
tyrosine	203 [205/201]	142 [140/144]	61	30
phenylalanine	233 [234/231]	188 [191/184]	45	19
methionine	117 [117/117]	73 [85/62]	43	37
glycine	261 [266/255]	224 [228/219]	37	14
histidine	164 [161/168]	141 [145/136]	24	14
arginine	117 [115/118]	104 [111/96]	13	11
proline	319 [324/315]	322 [335/309]	-3	-1
alanine	513 [523/504]	550 [557/542]	-36	-7
total sulfhydryls	14 ± 1	4 ± 1	10	72

* SEM = standard error of the mean value; four replicates

The decrease in amino acid and total sulfhydryl concentrations in WPI solution with AAPH (14.7 g/l) in comparison to without AAPH after 18 h is shown in Table 7.6.

Table 7.6: Amino acid and total sulfhydryl concentrations in the protein of a WPI (1.12 % protein) solution, with and without AAPH (14.7 g/l) after 18 h incubation time; decrease in amino acid and total sulfhydryl concentrations in a WPI (1.12 % protein) solution with AAPH (14.7 g/l) in comparison to without AAPH after 18 h incubation time

Amino acid or total sulfhdyryls	*Concentration in a WPI (1.12 %) solution without AAPH incubated for 18 h mean value, [1st/2nd replicate] or ± SEM[x] (µmol/g protein)	**Concentration in a WPI (1.12 %) solution with AAPH (14.7 g/l) incubated for 18 h mean value, [1st/2nd replicate] or ± SEM[x] (µmol/g protein)	Decrease in concentration in a WPI (1.12 %) solution with AAPH (14.7 g/l) in comparison to without AAPH after 18 h incubation (value* - value**) (µmol/g protein)	Decrease in concentration in a WPI (1.12 %) solution with AAPH (14.7 g/l) in comparison to without AAPH after 18 h incubation (%)
tyrosine	203 [203/203]	82 [82/82]	121	60
tryptophan	107 [107/-]	4 [4/-]	103	96
alanine	645 [640/651]	561 [568/553]	84	13
lysine	734 [734/734]	663 [675/651]	71	10
methionine	157 [157/157]	112 [119/105]	45	29
valine	450 [450/451]	409 [415/404]	41	9
proline	383 [384/382]	349 [350/348]	34	9
leucine	940 [937/942]	923 [937/909]	17	2
histidine	144 [147/140]	129 [131/127]	15	10
isoleucine	404 [402/407]	391 [396/386]	13	3
phenylalanine	212 [210/215]	205 [207/203]	7	3
arginine	137 [137/137]	131 [140/121]	6	4
glycine	229 [225/234]	233 [236/230]	-4	-2
threonine	404 [405/403]	411 [419/402]	-7	-2
aspartic acid + asparagine	877 [881/873]	886 [901/871]	-9	-1
glutamic acid + glutamine	1116 [1125/1108]	1138 [1157/1118]	-22	-2
serine	315 [315/315]	364 [372/357]	-49	-16
total sulfhydryls	34 ± 1	6 ± 1	28	82

[x] SEM = standard error of the mean value; three replicates

The strongest decreases in concentration were found for tyrosine (121 µmol/g protein), tryptophan (103 µmol/g protein), alanine (84 µmol/g protein) and lysine (71 µmol/g protein). The strongest decreases in percent were found for tryptophan (96 %), total sulfhydryls (cysteine) (82 %) and tyrosine (60 %). A higher concentration of serine was found in the WPI solution with AAPH. This was an unexpected result as AAPH is a pro-oxidant and could hardly account for the protection of any amino acid. A contamination with serine seems unlikely but may help to explain the finding. For glutamic acid/glutamine also one notably higher value was measured in the protein solution with AAPH, but it was within the variability of the method (± 5 % deviation of the mean value) (Table 7.6).

7.3 General discussion

Oxygen was consumed much faster in emulsions than in protein solutions with the same level of free radical generator (AAPH) (Figure 7.12 and Figure 7.2). The production of protein hydroperoxides had not occurred to a significant extent in a WPI (1.0 % protein) emulsion incubated for 4 h at 50 ˚C (Table 7.3). In contrast, the lipid hydroperoxide concentration had increased by ten-fold of the initial concentration. The consumption of oxygen in the emulsions appeared therefore not to be caused by the formation of protein hydroperoxides, but primarily by the formation of lipid hydroperoxides. It may be stated that milk proteins protected linoleic acid from oxidation and seemed themselves more stable towards oxidative deterioration in terms of reaction with oxygen. In general, it is acknowledged that protein radicals can form when proteins are in contact with oxidising lipids. The protein acts as hydrogen donor to peroxyl or alkoxyl radicals by the creation of a protein radical (Gardner 1979). An example of this reaction is depicted in Figure 7.16 (first reaction step), where a hydrogen atom is abstracted from an α-atom on the peptide backbone.

Once a free radical has formed on a protein, the transfer of this radical from the initial site of generation to another part of the protein can occur, where the radical is more stable. This transfer of radicals can, for example, occur between the residues of

tryptophan, tyrosine and cysteine. Free radical transfer competes with the reaction with oxygen and often takes place when the initial radical has a low reactivity with oxygen (Davies 2003). Free radical transfer on the whey proteins could have been a reason for the low protein hydroperoxide production observed.

In Figure 7.16 (Davies 2003) an example is given for the reaction with oxygen. In this case a radical is generated on an α-carbon atom on the peptide backbone. The radical reacts with oxygen forming a peroxyl radical and after a further reaction step a protein hydroperoxide is produced. In further reactions this may eventually lead to the fragmentation of the peptide backbone and the generation of carbonyl compounds.

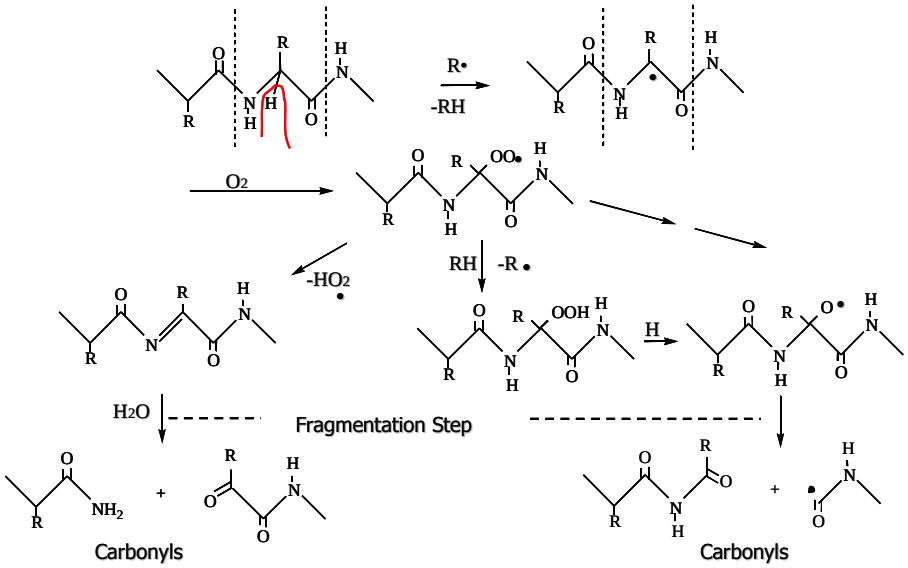


Figure 7.16: Radical-mediated oxidation of proteins: radical generation on α-carbon atom, peroxyl radical and protein hydroperoxide formation, backbone fragmentation and generation of carbonyl compounds

The fragmentation of the peptide backbone has been reported in several sources (Davies and Dean 1997; Elias et al. 2008; Viljanen et al. 2005; Zirlin and Karel 1969). Another oxidative change of protein molecules can be the formation of protein–protein crosslinks (Dean et al. 1997; Elias et al. 2008; Gardner 1979). Also the abstraction of

amino acid side chains can occur (Figure 7.17), which can again lead to the fragmentation of the peptide backbone (Davies 2003).

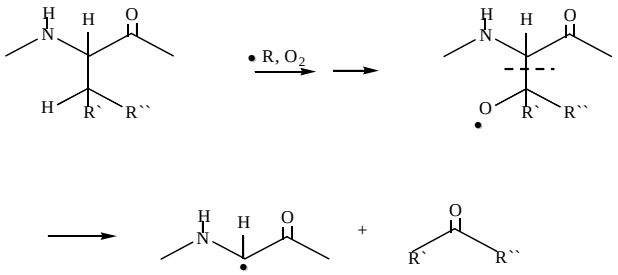


Figure 7.17: Abstraction of amino acid residues in protein oxidation

Another important aspect of protein oxidation is the damage of amino acid residues (Elias et al. 2008; Gardner 1979). In general the oxidation pathways and products are thought to be similar for free amino acids and amino acid residues on proteins or in peptides (Davies and Dean 1997; Elias et al. 2008).

In this study, the concentration of most amino acids and total sulfhydryls decreased in a WPI (1.0 % protein) emulsion over 18 h of incubation at 50 ˚C. For ten out of eighteen amino acids (including total sulfhydryls), the decrease in concentration was greater in the cream phase protein (Table 7.4 and Table 7.5).

Proteins in the cream phase, present on the interface, were close to the oil phase where the concentration of lipid-derived radicals increased over time as lipid oxidation proceeded. From that point of view, the potential for protein damage by free radical reactions would be greater in the cream phase than in the continuous phase. On the other hand, free radicals created in the continuous phase during the emulsification could contribute to protein oxidation. The exchange of proteins between the interface and the continuous phase could contribute to the fairly balanced damage on the protein of both phases. Furthermore, it is possible that free radicals present in the interface or continuous phase could change phases without exchange of the proteins themselves.

Also secondary lipid oxidation products, produced over the 18 h incubation period, may react with protein compounds, thereby contributing to the loss of amino acid residues. In a study of Rampon et al. (2001), the formation of adducts between proteins and lipid oxidation products occurred in bovine-serum-albumin-stabilised sunflower O/W emulsions, indicated by an increase of fluorescence emission at 425 nm.

The greatest deteriorative effect towards amino acids was found for lysine, leucine, glutamic acid + glutamine and aspartic acid + asparagine as part of whey proteins in a WPI-stabilised emulsion. The concentration of these amino acids decreased the most. The greatest loss in percent of the initially present amino acid concentration was found for total sulfhydryls (representing cysteine), tryptophan and methionine, indicating that these amino acids were probably most susceptible towards oxidation (Table 7.4 and Table 7.5).

In the review of Elias et al. (2008) about the antioxidative activity of proteins and peptides, the residues of the amino acids tryptophan, tyrosine, cysteine, methionine, histidine and phenylalanine were pointed out as the most unstable ones towards oxidation. Free radicals are often electrophilic and can easily abstract hydrogen atoms from these side chains as they are nucleophilic (Davies 2003; Elias et al. 2008). These amino acid residues are able to react with free radicals of low energy levels in contrast to less reactive aliphatic amino acid residues (Davies and Dean 1997; Elias et al. 2008).

The concentration of sulfhydryl groups has been shown to decrease in various studies, in which proteins had been exposed to unsaturated lipids in emulsions, e.g. free sulfhydryls as part of a high molecular weight fraction of whey protein added to a salmon oil/Tween 20 emulsion (Tong et al. 2000b) or free sulfhydryls as part of β-lactoglobulin added to a menhaden oil/Brij 35 emulsion (Elias et al. 2005). Subsequent oxidation products of cysteine can be cystine and oxy acids (Elias et al. 2008).

In a study of Rampon et al. (2001), tryptophan oxidation in sunflower O/W emulsions stabilised with bovine serum albumin could be observed within one day of storage. The oxidation was indicated by a decrease of tryptophan fluorescence. Besides Elias et al. (2008), also Gardner (1979) counts tryptophan to the amino acids most susceptible to

oxidation. N-formylkynurenine, kynurenine, 5-hydroxytryptophan and 7-hydroxytryptophan are oxidation products of tryptophan (Elias et al. 2008).

The oxidative lability of methionine on a protein with a high methionine concentration (glutamine synthetase) was investigated in an aqueous solution with hydrogen peroxide (Levine et al. 1996). Surface-exposed methionine residues readily oxidised, whereas the methionine residues buried within the protein structure remained unoxidised. The surface-exposed methionine residues appeared to be arranged in a way that protected the active site of the enzyme, suggesting they would fulfil an antioxidative function. Besides Elias et al. (2008), also Gardner (1979) counts methionine to one of the most labile amino acids towards oxidation. Methionine sulfoxide is a characteristic oxidation product of methionine (Elias et al. 2008).

In the review of Gardner (1979), lysine is mentioned as one of the most sensitive amino acids when exposed to oxidising lipids. The lysine side chain bears an amino group that can take part in Schiff base reactions with aldehydes or can react with α,β-unsaturated aldehydes by Michael addition. Adducts are formed in both cases. It has been reported that β-lactoglobulin and also β-casein underwent such reactions (Leaver et al. 1999). The concentration of lysine in bovine serum albumin was also found to decrease when the protein was exposed to oxidising unsaturated lipids. The higher the degree of unsaturation, the stronger was the decrease (Refsgaard et al. 2000). However, Refsgaard et al. (2000) also pointed out that Schiff base and Michael addition products are not stable under acidic conditions as applied when proteins are hydrolysed before the amino acid analysis. Furthermore a similar loss of lysine was found when potentially present Schiff bases were stabilised towards acidic hydrolysis. Therefore it is questionable to what extent the formation of those products was responsible for the loss of lysine. Although aldehydes and α,β-unsaturated aldehydes were probably generated as secondary lipid oxidation products in the present experiment, the formation of Schiff base and Michael addition products may not fully account for the loss of lysine in WPI. Refsgaard et al. (2000) concluded that the capacity of bovine serum albumin to suppress lipid oxidation was probably due to the reaction of lysine and amino acid residues in general with alkoxyl radicals.

The initial characteristic oxidative modification of leucine is due to the abstraction of hydrogen from its aliphatic residue, as stated by Davies (2003). Further oxidation products of leucine residues can be hydroperoxides, alcohols, α-ketoisocaproic acid, isovaleric acid, isovaleraldehyde, isovaleraldehyde oxime and carbonyl compounds. An explanation for the susceptibility of leucine could be its hydrophobicity. One might expect that more hydrophobic amino acid residues were in contact with the oil phase. Therefore they would be more exposed to lipid-derived radicals. On the other hand, the other amino acid residues whose concentration decreased the most are very hydrophilic. Also valine and isoleucine, which has more hydrophobic side chains than leucine according to the hydropathy index of Kyte and Doolittle (1982), were more stable than leucine. Therefore, the relevance of hydrophobicity for the stability of the amino acid residues is questionable.

For glutamic acid only hydroperoxides and for aspartic acid, glutamine and asparagine no specific side chain oxidation products have been mentioned (Davies 2003; Elias et al. 2008). Glutamic and aspartic acid side chains exhibit the ability to chelate metal ions (Arcan and Yemenicioglu 2007; Elias et al. 2008; Saiga et al. 2003). Therefore it appears possible that they would be particularly prone to free radical attacks if complexed ferrous and ferric ions were still redox active and able to take part in the production of alkoxyl and peroxyl radicals on linoleic acid. These radicals would then be created next to glutamic and aspartic acid side chains, causing deleterious effects. At the same time this would exhibit an antioxidative mechanism of the protein towards the emulsion lipid. Linoleic acid would receive a hydrogen atom from WPI and the radical would be passed on to the protein. As lysyl residues were also reported to exhibit chelating properties (Arcan and Yemenicioglu 2007; Saiga et al. 2003), the same process might also contribute to the observed loss of lysine. It has also been reported that lysine would undergo metal-catalysed oxidation, resulting in a carbonyl compound (amino-adipicsemialdehyde) (Shacter 2008).

When WPI was exposed to free radicals from AAPH in aqueous solution, the following amino acid residues were most labile: tyrosine, tryptophan, alanine and lysine. The greatest loss in percent of the initially present amino acid concentration was found for tryptophan, cysteine/total sulfhydryls and tyrosine (Table 7.6). In general the loss of

amino acids was lesser in the protein of the aqueous solution with AAPH (approximately 500 µmol/g protein) than in the protein of the respective emulsion phase in the previously discussed system (approximately 1100 µmol/g in the continuous phase and 1350 µmol/g in the cream phase).

In the following, an overview about oxidative modifications of the most susceptible amino acid residues (according to Elias et al. (2008)) and an example for aliphatic side chain oxidation is given in Figure 7.18 to Figure 7.22 (Davies 2003; Elias et al. 2008).

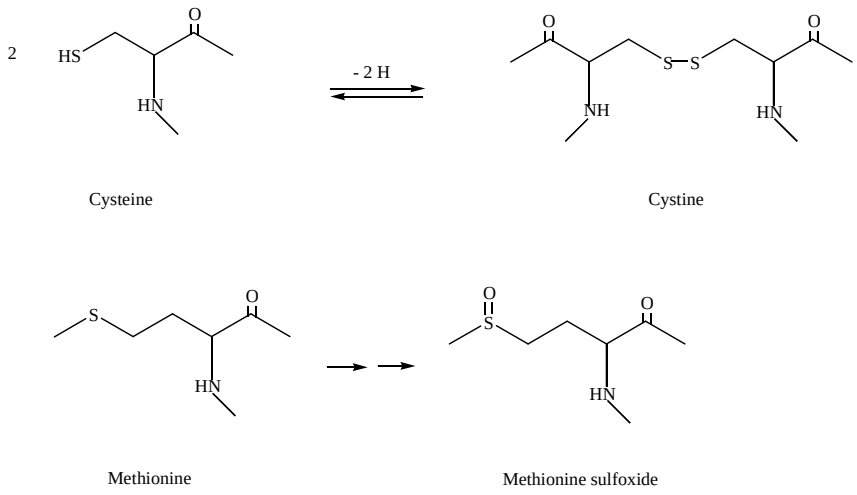


Figure 7.18: Oxidation of sulfur-containing amino acids

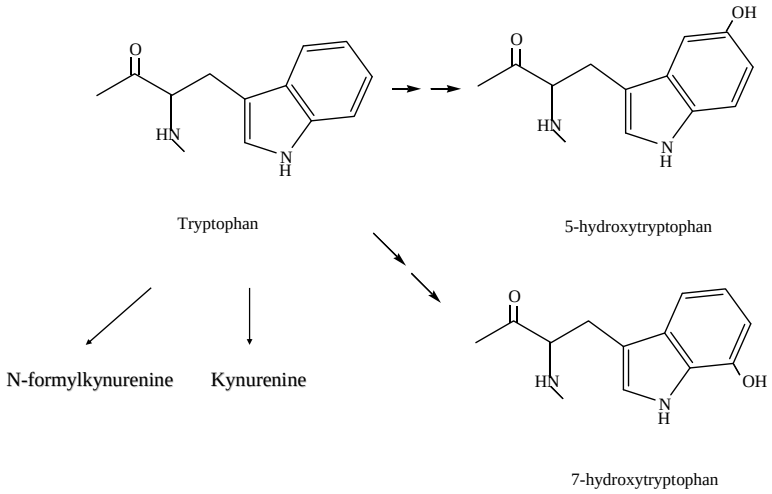


Figure 7.19: Side chain oxidation of aromatic amino acids: tryptophan

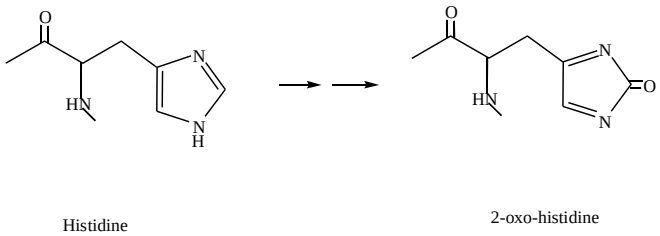


Figure 7.20: Side chain oxidation of histidine

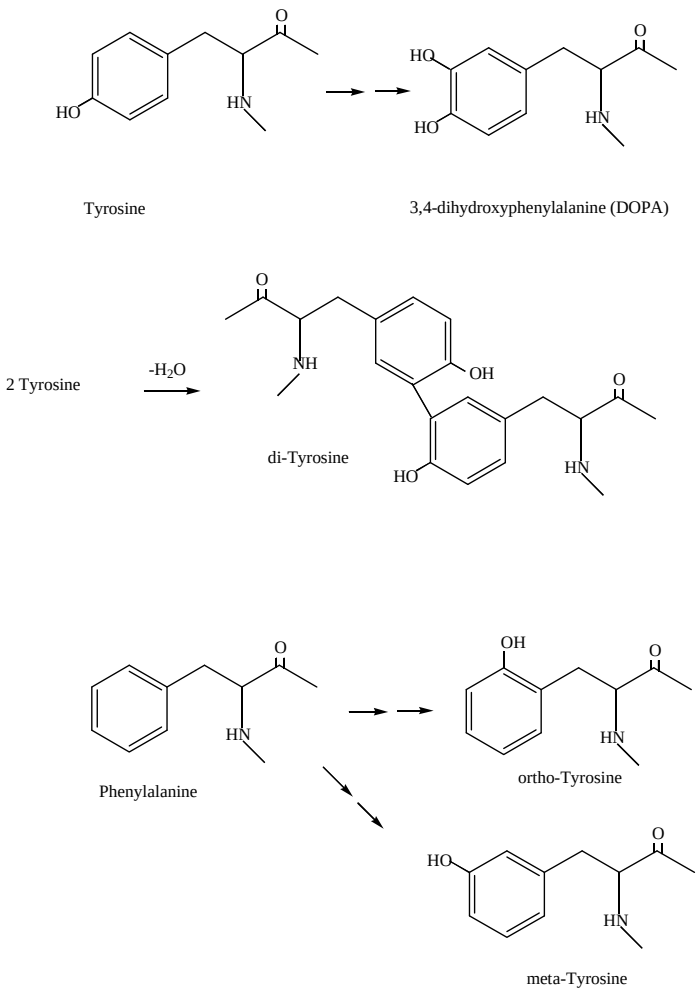


Figure 7.21: Side chain oxidation of aromatic amino acids: tyrosine and phenylalanine

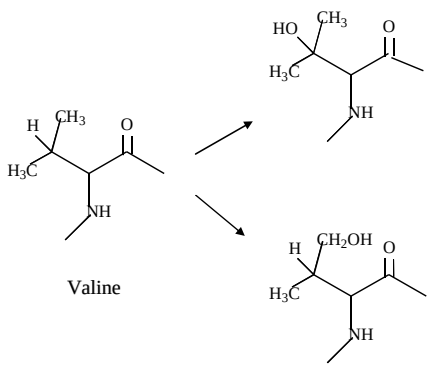


Figure 7.22: Side chain oxidation of aliphatic amino acids: valine

Most of the presumably labile amino acid residues were also amongst the most affected ones in the present study. On the other hand the concentration of histidine and phenylalanine residues did not greatly change, neither in the emulsion nor in the aqueous system with AAPH, in spite of their mentioned susceptibility. Furthermore, alanine was found to be one of the more affected amino acids when WPI was exposed to radicals from AAPH, although alanine has an aliphatic residue and would therefore be less susceptible to oxidation. The same was true for leucine in the emulsion system. Alanine residues together with aspartic acid and asparagine residues had shown a lower reaction constant k ($k \approx 10^7 - 10^8$ l mol^{-1} s^{-1}) in the reaction with hydroxyl radicals compared with the residues of tryptophan, tyrsosine, histidine, methionine, cysteine, phenylalanine and arginine ($k \approx 10^{10}$ l mol^{-1} s^{-1}) (Davies 2003).

The decrease in concentration of certain amino acids also greatly depended on the system or the phase within the system – continuous or cream phase of the emulsion. Alanine was one of the more affected amino acids in the aqueous system with AAPH and also in the continuous phase of the emulsion. In contrast, in the cream phase no decrease in alanine concentration was found. For leucine only a minor loss was observed in the system with AAPH, but a major loss in both phases of the emulsion. No extraordinary loss in concentration was found for tyrosine in the emulsion compared with other amino acids. In contrast, tyrosine was apparently more prone to oxidation in the aqueous solution with AAPH as its concentration greatly decreased. Glutamic acid + glutamine and aspartic acid + asparagine were apparently greatly affected by oxidation in the emulsion system, but no change in concentration was found in the aqueous solution with AAPH. Lysine was overall the amino acid with the greatest losses throughout all investigated protein fractions (whey protein from aqueous solution with AAPH and in the continuous and cream phases of the emulsion).

In a study of Hernandez-Ledesma et al. (2005), the ability of amino acids to scavenge AAPH-derived radicals in aqueous solution (pH 7.4) was investigated. The scavenging activity decreased in the following order (relative activity in brackets), showing the greatest activity for tryptophan and tyrosine: tryptophan (4.65) > tyrosine (1.57) > methionine (1.13) > cysteine (0.15) > histidine (0.07) > phenylalanine (0.003). Free radicals derived from AAPH were also used in the present study, in aqueous solution

with WPI (pH 6.0). The residues of tyrosine and tryptophan oxidised the most according to the decrease in concentration. For aqueous systems with AAPH, the free-radical-scavenging ability and the oxidative modification of tyrosine and tryptophan therefore appear to be closely linked and also seem to be similar for the free amino acids and the amino acid residues on WPI.

The conformation of the whey proteins that occupied the droplet interfaces after homogenisation was different to the conformation of whey proteins in the continuous phase or in aqueous solution before homogenisation. Different molecular regions were therefore exposed to the environment. The environment and the types of free radicals in it did also vary for the studied phases or systems. Whey proteins on the interface were mostly exposed to free radicals derived from linoleic acid and in the outer region to free radicals from the continuous phase. Proteins in the continuous phase were exposed to the latter mentioned. Whey proteins in the aqueous solution with AAPH, were again exposed to a different type of free radicals. Different free radical species also react selectively with amino acid residues, whereas free radicals on a high energy level react less selectively than those on a low energy level (Davies 2003). In a compilation of data from several studies, the loss of certain amino acid residues varied considerably depending on the protein type, when various proteins were exposed to the same oxidising lipid at the same conditions (Gardner 1979).

For the present study and in general, the free-radical-mediated damage of amino acid residues on proteins therefore appears to be influenced by the following factors: susceptibility of amino acid residues to oxidation, exposure of amino acid residues to the environment, free radical species in the environment (which can also be free radicals on the protein itself or on other protein molecules (Davies 2003)), their reactivity and concentration and the possibility of interaction between amino acid residues and free radicals.

Chapter 8

Concluding discussion and recommendations

8.1 Concluding remarks

Proteins have been shown to exhibit antioxidative properties in many studies, including studies on protein-stabilised O/W emulsions. In the present study, these antioxidative effects were confirmed with WPI and NaCas. How the protein type and the protein concentration influenced key parameters of lipid autoxidation (lipid hydroperoxides and a secondary oxidation product, hexanal) over a wide protein concentration range was demonstrated. Lipid oxidation was not inhibited to the same degree over the whole protein concentration range.

In addition, physical characteristics such as the droplet size played a role in oxidative stability. At a given protein concentration, the oxidative stability of emulsions was greater with an average droplet size of 0.31 µm than with an average droplet size of 0.65 µm, because of the larger contact area between proteins and lipids at the smaller average droplet size. The milk proteins were thus able to act more effectively as antioxidants towards the emulsion lipids. The presence of proteins, as opposed to non-protein surfactants, on droplet interfaces had previously been generally shown to result in greater oxidative stability (Donnelly et al. 1998).

As well as the presence of proteins at the droplet surface and the contact area between lipids and proteins, the conformation of the proteins at the interface may also be important in suppressing lipid oxidation. In general, the protein conformation changes in such a way that maximum contact between hydrophobic moieties and the oil phase is achieved as the molecule adsorbs at the interface during the emulsification process (Dalgleish 2004). It is possible that the specific emulsification conditions (e.g. the homogenisation pressure, the temperature and the pH at the point of emulsification) could also influence the conformation of the protein on the interface. Kiokias et al.

(2007) reported increased oxidative stability of WPC-stabilised sunflower O/W emulsions made at high emulsification temperatures. This was attributed to a greater degree of whey protein unfolding at higher temperatures, which resulted in better contact between the emulsion lipids and the whey proteins on the interface. Thus, the emulsification conditions may influence not only the total contact surface between lipids and proteins through changes in the droplet size, but also the types of amino acid residues that come into contact with the emulsion lipids.

The present study also showed that the nature of the continuous phase influenced lipid oxidation after the droplet interfaces had been formed. The oxidative stability was different depending on the protein type (WPI, NaCas or a mixture of WPI/NaCas) and the protein concentration in the continuous phase. The concentrations of lipid hydroperoxides and hexanal showed the same development over the continuous phase protein concentration and the general protein concentration (details in Chapter 4). This indicated the relevance of excess protein (the protein that is not located at the interface after emulsification but remains in the continuous phase) for oxidative stability. Furthermore, a decrease or an increase in the continuous phase protein concentration by diluting emulsions with either RO water or protein solution promoted and inhibited lipid oxidation respectively. Faraji et al. (2004) also observed lower lipid oxidation rates in protein-stabilised emulsions (based on WPI, pH 7) at higher continuous phase protein concentrations. Continuous phase protein also contributed to oxidative stability in some studies with non-protein emulsifiers; however, in those systems, the interfacial properties were different from those of protein-stabilised emulsions (Elias et al. 2005; Elias et al. 2007; Tong et al. 2000a; Tong et al. 2000b).

In the present study, the oxidative stability notably increased when emulsions were diluted with protein solutions of the other protein type, pointing to a synergistic antioxidative effect of WPI and NaCas. The mechanisms for this synergistic effect are still unclear, but interactions of whey proteins and caseins in the continuous phase, or between continuous phase proteins and interfacial proteins, are likely to be involved. A synergistic antioxidative effect may also have occurred in the study of Faraji et al. (2004), when NaCas or soy protein isolate solution was present in the continuous phase

and WPI was on the interface (pH 7). For both, the oxidative stability was greater than for pure WPI emulsions (WPI in the continuous phase and at the interface).

The present study showed that milk proteins on the interface as well as in the continuous phase have the potential to influence the oxidative stability of milk-protein-stabilised O/W emulsions.

In the present study, an enrichment of the emulsions with low molecular weight compounds (molecular weight ≤ 12000–14000) of NaCas and WPI (although not significantly in the case of WPI) increased their oxidative stability. The antioxidative activity of casein and whey protein hydrolysates and peptides thereof has been demonstrated in a number of studies (Diaz and Decker 2004; Diaz et al. 2003; Pena-Ramos and Xiong 2001). Casein-derived peptides are naturally present in milk and are produced during storage by enzymatic activity (e.g. by plasmin, but also by other proteinases). Other low molecular weight compounds in milk have also been associated with antioxidative activity (e.g. urate, organic acids etc.) and may be present in NaCas and/or WPI (see also Chapter 5, Section 5.2).

In the present study, heat treatment at 84 ˚C led to whey protein denaturation (indicated by β-lactoglobulin denaturation), to an increase in the free sulfhydryl concentration and to a decrease in the total sulfhydryl concentration in WPI solutions or mixed solutions of WPI plus NaCas (1:1). However, these effects of heating did not result in changes in the lipid oxidation rates of the subsequently produced emulsions. The lipid oxidation rates were also independent of the heating time of the solutions. Several studies have reported the inhibition of lipid oxidation in O/W emulsion systems in which heat-treated milk protein solutions had been incorporated; however, heat treatments have also had pro-oxidative effects (Elias et al. 2007; Taylor and Richardson 1980b; Tong et al. 2000a). NaCas appeared to increase the physical stability of the emulsions and thus to contribute also to their oxidative stability. As well as this effect, a general synergistic antioxidative effect was observed when the emulsions were produced with mixed solutions of WPI plus NaCas (1:1). Interactions of whey proteins and caseins in the continuous phase, at the interface or between the interface and the continuous phase may account for the increased oxidative stability of the emulsions.

Two major mechanisms inhibit lipid oxidation in general and lipid oxidation in O/W emulsions in particular. One is free-radical-scavenging activity, in the sense that the transfer of free radicals to lipids is inhibited or lipid-based radicals are transferred to other molecules. The other is the prevention of reactions between pro-oxidative metal ions and lipids. At a low concentration of lipid-based radicals, reactions between lipids and oxygen are also limited, even if the oxygen concentration is not a limiting factor for lipid oxidation. Therefore, in investigations of the pro- or antioxidative influence of any factor, e.g. the droplet size, the protein type etc., in O/W emulsions, it makes sense to evaluate it in the light of how it contributes to or antagonises these two antioxidative mechanisms.

Whey proteins, caseins, hydrolysates and peptides thereof have shown free-radical-scavenging activity in numerous studies. To the author's knowledge, this present study was the first to compare the free-radical-scavenging-activities of WPI and NaCas in O/W emulsions, including protein-stabilised O/W emulsions. Both milk protein fractions exhibited free-radical-scavenging activity in emulsions in which the free radical generator AAPH was incorporated. The scavenging activity was greater at higher protein concentrations and in NaCas-stabilised emulsions. This was shown by reduced oxygen consumption and reduced lipid hydroperoxide formation. In protein solutions with similar protein concentration and the same AAPH concentration, the oxygen consumption rate was much lower and similar to that in solutions without protein. This indicated low reactivity of the milk proteins with oxygen compared with linoleic acid. A comparison of the formations of lipid hydroperoxides and protein hydroperoxides in a WPI-stabilised emulsion supported the hypothesis. Free radicals, derived from linoleic acid and AAPH, instead led to oxidative modifications of amino acid residues (shown for WPI). In a study of Rival et al. (2001a), casein, various single caseins and bovine serum albumin exhibited free-radical-scavenging activity (measured by the uptake of oxygen) when they were added to a linoleic acid/Triton X-100 emulsion with AAPH. The oxygen uptake was also found to be linear over time. Whole casein (relative antioxidative activity: 1.56) was found to be more antioxidative than bovine serum albumin (relative antioxidative activity: 1.47). Other caseins had relative antioxidative activities as follows: 1.37 (κ-casein) and 1.16 (β-casein).

For the present study, free radicals derived from hydrophilic AAPH were presumably scavenged by high and low molecular weight compounds of the protein fractions directly in the polar continuous phase. Also, AAPH-derived radicals must have entered areas in which they could react with lipids (the oil phase and/or the interface) – in spite of their hydrophilicity – because they accelerated lipid oxidation compared with emulsions without AAPH. The radicals (two per AAPH molecule) had a molecular weight of 85. Therefore, low molecular weight compounds of the milk protein fractions, present in the continuous phase, may also have protruded into those areas and reacted with various radical types of lipids and AAPH. This is a possible free-radical-scavenging mechanism for emulsions without AAPH. In addition, proteins on the interface would have scavenged both AAPH-derived radicals and lipid-derived radicals (lipid radicals, alkoxyl radicals and peroxyl radicals).

Several factors appear to be relevant for an effective free-radical-scavenging process: the protein concentration in general, the protein type as well as the concentration of low molecular weight compounds with free-radical-scavenging activity that could enter into the interface, the exposure of certain moieties and amino acid residues with free-radical-scavenging activity (e.g. tyrosine, tryptophan, histidine or cysteine), changes in the exposure of those groups during storage of the emulsion, the transfer of radicals to and on proteinaceous materials of high and low molecular weight and a low reactivity of those radicals with lipids. Protein hydrolysates and peptides may be good scavengers of free radicals because of a high content of small and flexible molecules with free-radical-scavenging activity. This may also have contributed to the particular antioxidative effect of the low molecular weight fractions in the present study. Once radicals have formed, they might be transferred easily to other molecules because of a high mobility of the peptides. Davies (2003) mentioned the transfer of radicals on proteins to sites at which they were less reactive and gave an example of amino acid residues (tyrosine, tryptophan and cysteine) on which radical transfers have been found. Ostdal et al. (1999) found stable and long-lived tyrosyl radicals in the interior of bovine serum albumin, whereas radicals on free tyrosine were more reactive and short-lived.

It may be speculated if protein unfolding and increased exposure of some protein moieties could be pro-oxidative under certain conditions the radical-bearing areas would

be exposed to the environment, thus enabling radical transfer to other molecules such as lipids. Baron et al. (2005) mentioned the possibility that proteins may contribute to oxidative damage of lipids, antioxidants or DNA under the influence of heat or light, or in the presence of transition metals (Davies et al. 1995; Luxford et al. 2002; Morgan et al. 2002).

Pro-oxidative metal ions from iron and copper are closely linked to the development of lipid-based radicals.

The ability of milk proteins, milk protein hydrolysates or peptides and low molecular weight compounds of milk proteins to chelate iron and copper ions has been demonstrated in various studies. Milk proteins also naturally contain these metals. In the present study, the treatment of WPI and NaCas solutions with EDTA resulted in greater oxidative stability of the emulsions. Dialysis of protein solutions (molecular weight cut-off 12000–14000) to which no EDTA was added resulted in decreased oxidative stability of the emulsions. This was probably due to a loss of free-radical-scavenging and metal ion chelation activities as a result of the removal of the low molecular weight fractions. The oxidative instability could be balanced out by adding EDTA to the protein solutions before dialysis. This showed that a relevant factor for lipid oxidation in the emulsions was probably the presence of pro-oxidative metal ions in the high molecular weight fractions (molecular weight $\geq$ 12000–14000) of WPI and NaCas. These pro-oxidative metal ions were located in proximity to the emulsion lipids. Nevertheless, both this study and other studies showed that the antioxidative properties of the proteins prevailed.

The ability of iron and copper ions to promote lipid oxidation in O/W emulsions depends on their availability to take part in reactions with lipid hydroperoxides. Free metal ions in the interfacial region, in the lipid phase or in the continuous phase may be the most likely candidates for those reactions. Metal ions that are bound to proteinaceous material may also take part in the lipid oxidation process if they are redox active. This may be the case if they are bound to an exposed molecular moiety and not buried within the protein structure. Furthermore, conditions that lead to a release of protein-bound metal ions are also likely to enhance lipid oxidation. The release of metal

ions probably depends on their location, e.g. if they are bound to buried or exposed moieties and amino acid residues, and the pH, because H^+/H_3O^+ ions compete for the same binding sites as cations. Protein surfaces become more positively charged with decreasing pH, as H^+ binds to COO^- and amino groups. Free cations are therefore more likely to be repelled from proteinaceous material at low pH. Remondetto et al. (2004) stated that a net positive charge causes more cation repulsion than attraction and that a net negative charge causes more cation attraction than repulsion. The oxidative stability of whey-protein-stabilised emulsions, below the isoelectric point of the whey proteins, has been attributed to their net positive surface charge leading to the repulsion of metal ions from the droplet surface into the continuous phase (Donnelly et al. 1998; Hu et al. 2003b). At pH values above the isoelectric point, proteins or low molecular weight compounds, originating from the continuous phase, can be antioxidative through the chelation and removal of pro-oxidative metal ions from the interface (and possibly from the oil phase, as shown by Cho et al. (2003) for other chelators) as these are transferred with the chelating molecules into the continuous phase away from susceptible lipids (Tong et al. 2000b).

In the present study, high and low molecular weight compounds of the milk proteins, present in the continuous phase, could have exhibited such an antioxidative mechanism, because the pH was above their isoelectric points. Low hexanal concentrations were observed at low protein concentrations in NaCas emulsions, whereas higher protein concentrations were needed in WPI emulsions. The reason for this effect may have been a stronger metal chelation ability of the caseins compared with the whey proteins, resulting in a more efficient chelation process. In addition to the metal chelation characteristics, e.g. carboxyl groups or amino groups, of proteins, caseins also possess phosphoseryl groups. Villiere et al. (2005) suggested that the addition of NaCas solution to protein-stabilised emulsions after emulsification may improve their oxidative stability, because caseins could then remove detrimental metal ions away from the interface into the continuous phase.

The access of chelators (such as EDTA or citrate in the present study) to the metal-binding site on a protein and their relative metal binding ability compared with that site will influence the possibility of metal removal. If a metal ion is released from the

protein (e.g. by binding to a chelator), its new location and its redox activity will determine whether or not it has a pro-oxidative influence in the lipid oxidation process.

The interface appears to be a particularly crucial region in terms of chelation ability, redox inactivation and the retention or release of pro-oxidative metal ions, because a high concentration of lipid hydroperoxides is thought to prevail at the interface (Decker et al. 2002; McClements and Decker 2000). The conformation of interfacial proteins may influence the retention/release of bound pro-oxidative metal ions, their ability to chelate free metal ions and their participation in redox reactions. Under some conditions, unfolding of proteins may lead to a greater release of protein-bound metal ions and thus oxidative destabilisation. Elias et al. (2005) pointed out that hydrolysis as well as protein denaturation may increase the exposure of amino acid residues and thus the potential for free radical scavenging, but that metal chelation abilities might also be affected. In this way, different conformations of interfacial proteins, which were partly determined during the emulsification process and which may change during storage, could affect the oxidative stability of an emulsion.

Another relevant aspect for lipid oxidation in O/W emulsions is the emulsion lipid. PUFAs have a tendency to oxidise easily because of a high degree of unsaturation. Also, other factors are likely to play a role in the susceptibility to oxidation. Free fatty acids, as used in the present study (approximately 60 % linoleic acid plus oleic acid and linolenic acids), are partially deprotonised (pK_a of linoleic acid = 7.9 (Belitz et al. 2004)) at neutral or slightly acidic pH and are therefore partly negatively charged, whereas triacylglycerols have no ionic electrical charge. Free fatty acids oxidise faster than their esters (Adachi et al. 1995; Miyashita and Takagi 1986), which could be partly due to their negatively charged carboxyl groups. These can attract pro-oxidative cations.

Furthermore, the hydrophobicity of the lipid, the location of double bonds on the fatty acid molecules and changes in their polarity during lipid oxidation (parts of molecules with lipid hydroperoxides are more hydrophilic and can move to the interface) can influence the oxidative stability of O/W emulsions (McClements and Decker 2000). The oxidative stability of protein-stabilised emulsions could be partly influenced by these factors, as they may play a role in the contact between lipids and proteins at the droplet

interface. Variations in that contact may change the characteristics of free radical scavenging by the proteinaceous material when alkoxyl and peroxyl radicals are created from lipid hydroperoxides.

The following picture (Figure 8.1) offers a concept of protein-stabilised O/W emulsion systems on a molecular level with the depiction of the basic antioxidant processes, metal ion chelation and free radical scavenging. A profile of the interfacial area (in the middle) with the adjacent continuous phase (on the right) and oil phase (on the left) is shown.

The proteins, present in the continuous phase and at the interface, have a net negative surface charge as pH 6 (the pH used in the present study) is above their isoelectric point.

Oxygen (O_2) is present in the continuous phase and in the oil phase. In the oil phase, the reaction with lipid radicals can occur.

Pro-oxidative metal ions such as iron and copper are present in the continuous phase and in the oil phase as free metal ions. Another proportion is bound to proteinaceous material (proteins, peptides and amino acids) in the continuous phase and in the interfacial area. Free metal ions are attracted to net negatively charged proteinaceous material in the continuous phase and at the interface. Some proteinaceous LMW compounds from the continuous phase may be able to enter areas on the inner side of the interface. They may chelate metal ions there and at the interface in general and transfer them into the continuous phase, thus inhibiting lipid oxidation.

The picture shows linoleic acid (protonated and deprotonated), linoleic-acid-based radicals, including alkoxyl radicals and peroxyl radicals, and linoleic acid hydroperoxides on the inner side of the interface. Due to the hydrophilicity of the oxidised forms of linoleic acid, higher levels are present in this area compared with the inner core of the oil phase. Metal ions which are also present in this area can potentially react with lipid hydroperoxides and fuel the autoxidation process.

Other types of free radicals can be present in the continuous phase as they are created during homogenisation. Lipid-based radicals can be scavenged by proteins permanently present on the interface and potentially also by proteinaceous material which is only temporarily in the interfacial area and dynamically exchanges with material from the continuous phase. Proteinaceous material in the continuous phase can also scavenge continuous-phase-born free radicals.

The suggested antioxidant mechanisms which include the idea that proteinaceous material from the continuous phase is in dynamic exchange with material in the interfacial area arises from several findings in the present study and other studies: the continuous phase protein greatly influenced the oxidative stability of lipids inside the droplets, LMW compounds of WPI and NaCas which presumably do not take part in the formation of the interface influenced the oxidative stability, continuous phase protein experienced almost as much damage (indicated by the loss of amino acids) as cream phase protein permanently in contact with the emulsion lipids.

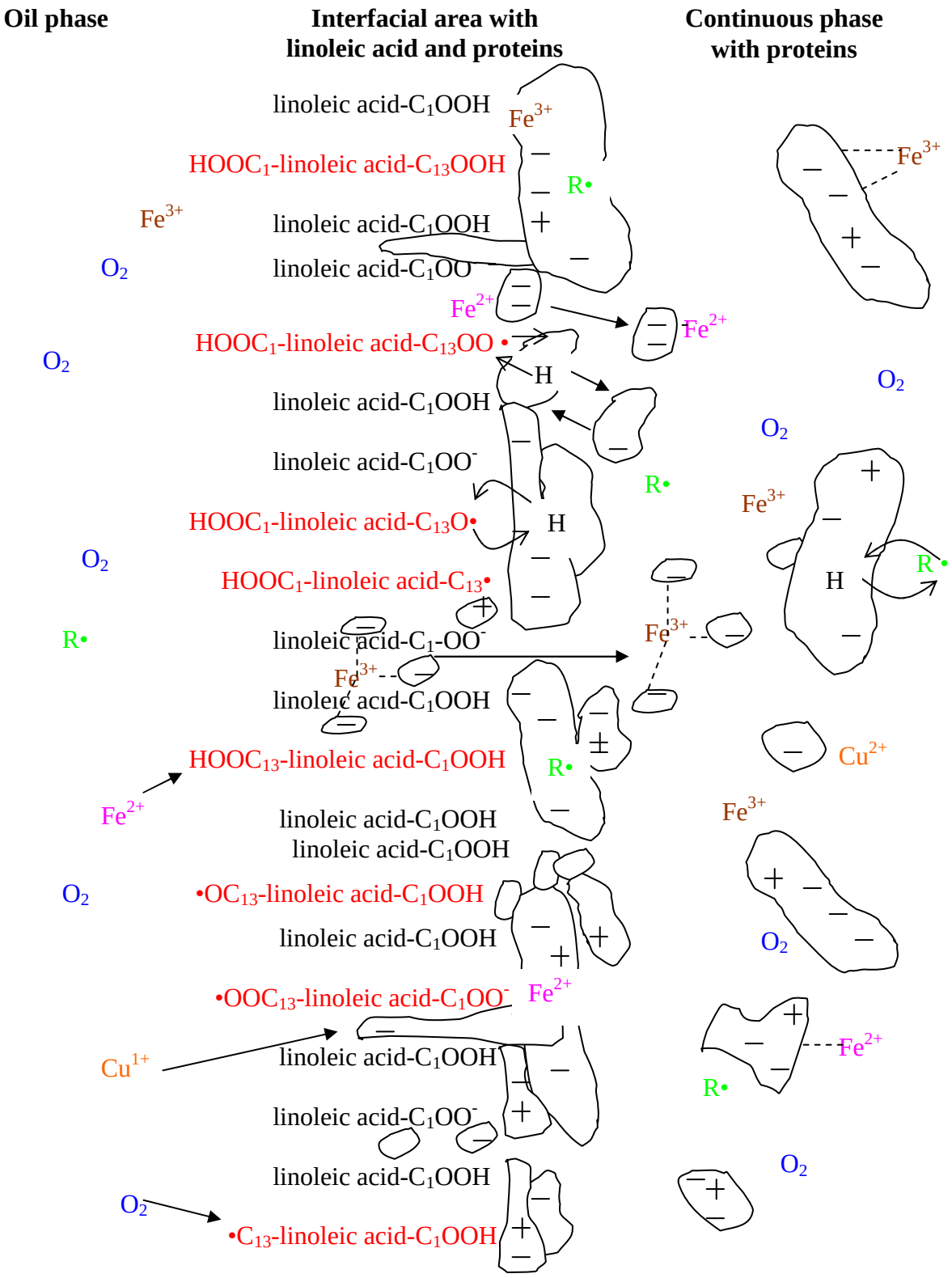


Figure 8.1: Illustration of the emulsion phases in the present system on a molecular level, including the antioxidant mechanisms metal ion chelation and free radical scavenging by proteins; without secondary oxidation products

8.2 Recommendations for future work

- Milk protein products, used in the current study, are complex mixtures of various proteins. For example, NaCas consists of four different caseins: α_{s1}-, α_{s2}-, β- and κ-caseins. It would be of interest to understand more fully the role of individual caseins or whey proteins in providing oxidative stability to O/W emulsions. On a molecular level, more research to identify the peptide sequences that are directly in contact with the oil phase is needed.

- In several studies, the heat treatment of milk protein solutions prior to emulsion preparation led to greater oxidative stability of the emulsions, whereas, in other studies, decreased oxidative stability was observed (see also Chapter 2, Section 2.7.3, and Chapter 6); no effect of heating – except in cases of physical instability – was found in the present study. More research to elucidate under what circumstances, and for which milk proteins, heating contributes to or antagonises oxidative stability is required. Some of the results of such research may be of interest for the food industry, as heat treatments of protein solutions, e.g. pasteurisation, should be applied in a way that also contributes to the oxidative stability of the subsequently produced emulsions. Future studies could also address the question of to what extent the unfolding of the proteins (e.g. whey proteins), and thus improved contact with emulsion lipids in the interfacial region, contributes to improved oxidative stability. Furthermore, the existence of other antioxidative mechanisms that are caused by heat treatment could be investigated, such as the hypothesis that greater oxidative stability of emulsions may be a result of increased rates of metal chelation and free-radical-scavenging reactions when the emulsification is carried out at higher temperatures. For this purpose, solutions of various temperatures, consisting of low molecular weight compounds of the milk proteins (with chelation ability and free-radical-scavenging ability) plus non-protein emulsifiers, could be used to produce emulsions. Thus, if antioxidative effects were observed at higher heating temperatures, they could clearly be attributed to the low molecular weight compounds and not to protein unfolding (as proteins would not be present).

- Low molecular weight compounds appeared to be particularly effective antioxidants in the present study. More research is needed to determine the composition of the low molecular weight fractions of WPI and NaCas, as well as the antioxidative functionality, or possibly partially pro-oxidative functionality, of the constituent compounds. Furthermore, factors that influence the composition of these fractions and that might possibly lead to a controlled improvement of their antioxidative properties could be investigated.

- Enzymatic hydrolysates of WPI and NaCas have exhibited marked antioxidative properties. Expertise that is already present in the dairy industry could be used to produce mixtures of antioxidative milk protein peptides. Emulsions stabilised with WPI or NaCas could also be fortified with such hydrolysates to improve their oxidative stability.

- An antioxidative synergistic effect was found when WPI and NaCas were used in combination. More details of the nature of this effect and optimum ratios for maximum oxidative stability should be investigated. The experimental designs could consider measurement of the oxidative stability of some of the following emulsions: WPI only on the interface and caseins only in the continuous phase and vice versa, combinations of the proteins at various ratios on the interface and WPI or NaCas only in the continuous phase, and pure proteins on the interface and mixed proteins at various ratios in the continuous phase. The results of such studies may also be of interest for commercial food applications in which maximum protection of PUFAs may be achieved by adjusting the WPI/NaCas ratios.

- Metal ions, contained in the high molecular weight fractions of WPI and NaCas, appeared to be pro-oxidative in the present study, because their removal contributed to greater oxidative stability of the emulsions. The chelation capacities of WPI and NaCas and their natural contents of iron and copper, in terms of their influence on oxidative stabilisation and destabilisation respectively, could be investigated further. Although whey proteins and caseins have been shown to exhibit chelation capacity in several studies, the actual redox inactivation of chelated iron and copper ions

needs further examination. Such studies should also include variations in the pH value as it influences the retention of metal ions.

- Furthermore, the role of the location of the high molecular weight fractions of WPI and NaCas, in which the natural metal content is still present or has been lowered by EDTA treatment, in the oxidative stability of emulsions could be further elucidated. A possible experimental design may include the following: preparation of emulsions using the high molecular weight fraction of WPI or NaCas, which has been treated with EDTA, and replacement of the continuous phase with WPI or NaCas solution respectively, which has not been treated with EDTA (and vice versa). A logical hypothesis would be that the respective high molecular weight fraction with the original metal content would be more pro-oxidative if present on the interface and would be less pro-oxidative if present in the continuous phase. Whether high molecular weight fractions with lowered metal content, present in the continuous phase, have chelation capacity for metal ions present in interfacial protein that consists of the high molecular weight fractions with the original metal content could also be investigated.

- Finally, further research is required to elucidate the transfer of material across the interface in milk-protein-stabilised emulsions and how this is related to their oxidative stability under different conditions (e.g. various oxygen and metal concentrations in the continuous phase, interface and oil phase).

Appendix

Appendix Chapter 4

Table 4A: Probability values from ANOVA for the difference in lipid oxidation of differently droplet sized NaCas emulsions (1.0 % protein) over the whole incubation time (droplet size*incubation time)

Lipid hydroperoxides		Hexanal	
Droplet Size* Incubation Time	*Probability p	Droplet Size* Incubation Time	*Probability p
0.31 versa 0.43	0.436	0.31 versa 0.43	0.966
0.31 versa 0.59	0.000	0.31 versa 0.59	0.000
0.43 versa 0.59	0.000	0.43 versa 0.59	0.009

*Samples that were not significantly different ($p > 0.05$) are marked red.

Table 4B: Probability values from two sample t-tests for the difference in lipid oxidation between WPI and NaCas emulsions at certain protein concentrations

Droplet size (µm)	Protein concentration (%)	Comparison WPI – NaCas *Probability p	
		Lipid hydroperoxides after 4 h	Hexanal after 24 h
0.3	0.5	0.401	0.063
	1.0	0.000	0.461
	2.0	0.138	0.618
	3.0	0.033	0.614
	4.0	0.016	0.003
	7.0	0.000	0.764
	10.0	0.021	0.008
0.6	0.5	0.113	0.024
	1.0	0.009	0.105
	2.0	0.008	0.000
	3.0	0.013	0.237
	4.0	0.000	0.000
	7.0	0.002	0.001
	10.0	0.014	0.009

*Samples that were not significantly different ($p > 0.05$) are marked red.

Table 4C: Probability values from two sample t-tests for the difference in hexanal concentration (24 h) of small droplet sized emulsions with protein concentrations from 0.5 to 4.0 %

Protein concentrations compared (%)	*Probability p	
	WPI emulsion, Hexanal after 24 h	NaCas emulsion, Hexanal after 24 h
0.5 – 1.0	0.356	0.590
0.5 – 2.0	0.058	0.451
0.5 – 3.0	0.056	0.289
0.5 – 4.0	0.025	0.583
1.0 – 2.0	0.184	0.481
1.0 – 3.0	0.165	0.407
1.0 – 4.0	0.058	0.721
2.0 – 3.0	0.558	0.682
2.0 – 4.0	0.000	0.309
3.0 – 4.0	0.028	0.134

*Samples that were not significantly different ($p > 0.05$) are marked red.

Table 4D: Probability values from two sample t-tests for the difference in lipid oxidation for emulsions (4.0 % protein) where the continuous phase was replaced with protein solution (4.48 % protein) or RO water

[*]Samples compared	[**]Probability p	
	Lipid hydroperoxides after 4 h	Hexanal after 24 h
(WPI control) – (WPI + RO water)	0.000	0.000
(WPI control) – (WPI + WPI)	0.000	0.089
(WPI + ROwater) – (WPI + WPI)	0.000	0.000
(NaCas control) – (NaCas + RO water)	0.003	0.001
(NaCas control) – (NaCas + NaCas)	0.001	0.012
(NaCas + RO water) – (NaCas + NaCas)	0.000	0.041

[*] WPI control = WPI emulsion; WPI + RO water = WPI emulsion, continuous phase replaced with RO water; WPI + WPI = WPI emulsion, continuous phase replaced with WPI solution; NaCas control = NaCas emulsion; NaCas + RO water = NaCas emulsion, continuous phase replaced with RO water; NaCas + NaCas = NaCas emulsion, continuous phase replaced with NaCas solution. [**]Samples that were not significantly different ($p > 0.05$) are marked red.

Table 4E: Probability values from two sample t-tests for the difference in lipid oxidation of emulsions with varying concentrations of continuous phase protein (comparisons within one protein type)

Comparison of emulsions with various continuous phase protein concentration	[*]Probability p	
	Lipid hydroperoxides after 4 h	Hexanal after 24 h
WPI 0 % - WPI 0.5 %	0.005	0.002
WPI 0 % - WPI 2.0 %	0.000	0.002
WPI 0 % - WPI 4.0 %	0.000	0.002
WPI 0.5 % - WPI 2.0 %	0.000	0.008
WPI 0.5 % - WPI 4.0 %	0.001	0.001
WPI 2.0 % - WPI 4.0 %	0.004	0.007
NaCas 0 % - NaCas 0.5 %	0.039	0.047
NaCas 0 % - NaCas 2.0 %	0.000	0.155
NaCas 0 % - NaCas 4.0 %	0.000	0.838
NaCas 0.5 % - NaCas 2.0 %	0.003	0.555
NaCas 0.5 % - NaCas 4.0 %	0.002	0.059
NaCas 2.0 % - NaCas 4.0 %	0.004	0.158

[*]Samples that were not significantly different ($p > 0.05$) are marked red.

Table 4F: Probability values from two sample t-tests for the difference in lipid oxidation of emulsions with the same concentration of continuous phase protein and of different protein type

Comparison of emulsions with various continuous phase protein concentration	*Probability p	
	Lipid hydroperoxides after 4 h	Hexanal after 24 h
WPI 0 % - NaCas 0 %	0.000	0.004
WPI 0.5 % - NaCas 0.5 %	0.008	0.008
WPI 2.0 % - NaCas 2.0 %	0.002	0.032
WPI 4.0 % - NaCas 4.0 %	0.000	0.001

*Samples that were not significantly different ($p > 0.05$) are marked red.

Table 4G: Probability values from two sample t-tests for the difference in lipid oxidation of emulsions that were not diluted or diluted with protein solution or RO water

[*]Samples compared (1 – 8)	[**]Probability p	
	Lipid hydroperoxides after 4 h	Hexanal after 24 h
1 – 2	0.009	0.489
1 – 3	0.034	0.014
1 – 4	0.006	0.005
1 – 5	0.055	0.150
1 – 6	0.004	0.162
1 – 7	0.048	0.159
1 – 8	0.017	0.001
2 – 3	0.001	0.022
2 – 4	0.000	0.009
2 – 5	0.022	0.106
2 – 6	0.003	0.114
2 – 7	0.000	0.158
2 – 8	0.000	0.009
3 – 4	0.000	0.002
3 – 5	0.011	0.060
3 – 6	0.000	0.007
3 – 7	0.110	0.004
3 – 8	0.008	0.004
4 – 5	0.004	0.014
4 – 6	0.000	0.001
4 – 7	0.000	0.001
4 – 8	0.001	0.806
5 – 6	0.007	0.648
5 – 7	0.014	0.578
5 – 8	0.008	0.016
6 – 7	0.000	0.877
6 – 8	0.000	0.001
7 – 8	0.005	0.001

[*] 1 = WPI emulsion; 2 = WPI emulsion + RO water; 3 = WPI emulsion + WPI solution; 4 = WPI emulsion + NaCas solution; 5 = NaCas emulsion; 6 = NaCas emulsion + RO water; 7 = NaCas emulsion + NaCas solution; 8 = NaCas emulsion + WPI solution. [**]Samples that were not significantly different ($p > 0.05$) are marked red.

Appendix Chapter 5

Table 5A: Probability values from two sample t-tests for the difference in lipid oxidation of emulsions with varying protein type and dialysis application to protein solutions used to prepare the emulsions at certain chelator levels

Sample types compared	*Probability p		Chelator concentration
	Lipid hydroperoxides after 4 h	Hexanal after 24 h	
WPI dialysed - WPI not-dialysed	0.066	0.000	
NaCas dialysed - NaCas not-dialysed	0.003	0.015	
WPI dialysed - NaCas dialysed	0.695	0.942	Control
WPI not-dialysed - NaCas not-dialysed	0.018	0.003	no chelator
WPI dialysed - NaCas not-dialysed	0.017	0.000	
NaCas dialysed - WPI not-dialysed	0.014	0.007	
WPI dialysed - WPI not-dialysed	0.263	0.095	
NaCas dialysed - NaCas not-dialysed	0.003	0.007	
WPI dialysed - NaCas dialysed	0.085	0.013	Citrate
WPI not-dialysed - NaCas not-dialysed	0.001	0.000	low
WPI dialysed - NaCas not-dialysed	0.004	0.009	
NaCas dialysed - WPI not-dialysed	0.253	0.013	
WPI dialysed - WPI not-dialysed	0.000	0.002	
NaCas dialysed - NaCas not-dialysed	0.011	0.085	
WPI dialysed - NaCas dialysed	0.001	0.001	Citrate
WPI not-dialysed - NaCas not-dialysed	0.000	0.967	high
WPI dialysed - NaCas not-dialysed	0.000	0.003	
NaCas dialysed - WPI not-dialysed	0.074	0.149	
WPI dialysed - WPI not-dialysed	0.000	0.035	
NaCas dialysed - NaCas not-dialysed	0.025	0.075	
WPI dialysed - NaCas dialysed	0.096	0.114	EDTA
WPI not-dialysed - NaCas not-dialysed	0.000	0.379	low
WPI dialysed - NaCas not-dialysed	0.003	0.017	
NaCas dialysed - WPI not-dialysed	0.012	0.074	
WPI dialysed - WPI not-dialysed	0.365	0.971	
NaCas dialysed - NaCas not-dialysed	0.275	0.893	
WPI dialysed - NaCas dialysed	0.534	0.748	EDTA
WPI not-dialysed - NaCas not-dialysed	0.034	0.670	high
WPI dialysed - NaCas not-dialysed	0.001	0.798	
NaCas dialysed - WPI not-dialysed	0.650	0.649	

*Samples that were not significantly different ($p > 0.05$) are marked red.

Table 5B: Probability values from two sample t-tests for the difference in lipid oxidation of emulsions with different chelator treatments

Chelator levels compared	*Probability p		Protein type and application of dialysis to protein solution
	Lipid hydroperoxides after 4 h	Hexanal after 24 h	
Control - Citrate low	0.335	0.003	
Control - Citrate high	0.001	0.000	
Control - EDTA low	0.000	0.000	
Control - EDTA high	0.000	0.000	
Citrate low - Citrate high	0.015	0.000	WPI, not-dialysed
Citrate low - EDTA low	0.001	0.000	
Citrate low - EDTA high	0.001	0.000	
Citrate high - EDTA low	0.000	0.075	
Citrate high - EDTA high	0.000	0.040	
EDTA low - EDTA high	0.080	0.361	
Control - Citrate low	0.155	0.000	
Control - Citrate high	0.087	0.000	
Control - EDTA low	0.004	0.000	
Control - EDTA high	0.002	0.000	
Citrate low - Citrate high	0.750	0.496	WPI, dialysed
Citrate low - EDTA low	0.006	0.022	
Citrate low - EDTA high	0.003	0.003	
Citrate high - EDTA low	0.000	0.024	
Citrate high - EDTA high	0.000	0.001	
EDTA low - EDTA high	0.001	0.016	
Control - Citrate low	0.000	0.000	
Control - Citrate high	0.000	0.000	
Control - EDTA low	0.000	0.000	
Control - EDTA high	0.000	0.000	
Citrate low - Citrate high	0.082	0.109	NaCas, not-dialysed
Citrate low - EDTA low	0.182	0.045	
Citrate low - EDTA high	0.000	0.003	
Citrate high - EDTA low	0.104	0.069	
Citrate high - EDTA high	0.053	0.003	
EDTA low - EDTA high	0.000	0.852	
Control - Citrate low	0.004	0.022	
Control - Citrate high	0.003	0.002	
Control - EDTA low	0.001	0.011	
Control - EDTA high	0.001	0.002	
Citrate low - Citrate high	0.140	0.001	NaCas, dialysed
Citrate low - EDTA low	0.003	0.155	
Citrate low - EDTA high	0.002	0.005	
Citrate high - EDTA low	0.020	0.080	
Citrate high - EDTA high	0.002	0.478	
EDTA low - EDTA high	0.033	0.072	

*Samples that were not significantly different ($p > 0.05$) are marked red.

Table 5C: To results of experimental plan (A): Probability values from two sample t-tests for the difference in lipid oxidation of various emulsion samples

*Samples compared	**Probability p	
	Lipid hydro-peroxides after 4 h	Hexanal after 24 h
WPI – (WPI + 0.030 % LMW-WPI)	0.085	0.080
WPI – NaCas	0.003	0.014
NaCas – (NaCas + 0.025 % LMW-NaCas)	0.001	0.017
(WPI + 0.030 % LMW-WPI) – (NaCas + 0.025 % LMW-NaCas)	0.001	0.136
NaCas – (WPI + 0.030 % LMW-WPI)	0.004	0.002
WPI – (NaCas + 0.025 % LMW-NaCas)	0.001	0.825

*WPI = WPI emulsion, WPI + 0.030 % LMW-WPI = WPI emulsion with additional 0.030 % LMW-WPI compounds, NaCas = NaCas emulsion, NaCas + 0.025 % LMW-NaCas = NaCas emulsion with additional 0.025 % LMW-NaCas compounds. **Samples that were not significantly different ($p > 0.05$) are marked red.

Table 5D: To results of experimental plan (B): Probability values from two sample t-tests for the difference in lipid oxidation of various emulsion samples

*Samples compared	**Probability p		
	Lipid hydroperoxides		Hexanal
	after 8 h	after 16 h	after 24 h
NaCas – (NaCas + 100 ppm EDTA)	0.001	0.000	0.000
NaCas – (NaCas + 0.022 % LMW-NaCas)	0.003	0.064	0.141
NaCas – (NaCas + 0.045 % LMW-NaCas)	0.002	0.022	0.000
(NaCas + 100 ppm EDTA) – (NaCas + 0.022 % LMW-NaCas)	0.001	0.001	0.004
(NaCas + 100 ppm EDTA) – (NaCas + 0.045 % LMW-NaCas)	0.003	0.001	0.001
(NaCas + 0.022 % LMW-NaCas) – (NaCas + 0.045 % LMW-NaCas)	0.001	0.388	0.010

*NaCas = NaCas emulsion, NaCas + 100 ppm EDTA = NaCas emulsion with 100 ppm EDTA, NaCas + 0.022 % LMW-NaCas = NaCas emulsion with additional 0.022 % LMW-NaCas compounds, NaCas + 0.045 % LMW-NaCas = NaCas emulsion with additional 0.045 % LMW-NaCas compounds. **Samples that were not significantly different ($p > 0.05$) are marked red.

References

Ackman, R. G. (2004). The ocean supplies more EPA and DHA than we can use. *OCL - Oleagineux, Corps Gras, Lipides*, 11(2), 112-115.

Adachi, S., Ishiguro, T., and Matsuno, R. (1995). Autoxidation kinetics for fatty acids and their esters. *Journal of the American Oil Chemists Society*, 72(5), 547-551.

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry*, 27(6), 1256-1262.

Agboola, S. O., Singh, H., Munro, P. A., Dalgleish, D. G., and Singh, A. M. (1998). Stability of emulsions formed using whey protein hydrolysate: effects of lecithin addition and retorting. *Journal of Agricultural and Food Chemistry*, 46(5), 1814-1819.

Ahn, D. U., and Kim, S. M. (1998). Prooxidant effects of ferrous iron, hemoglobin, and ferritin in oil emulsion and cooked-meat homogenates are different from those in raw-meat homogenates. *Poultry Science*, 77(2), 348-355.

Allen, J. C., and Wrieden, W. L. (1982). Influence of milk proteins on lipid oxidation in aqueous emulsion. 1. Casein, whey protein and α-lactalbumin. *Journal of Dairy Research*, 49(2), 239-248.

Anema, S. G. (2007). Role of κ-casein in the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. *Journal of Agricultural and Food Chemistry*, 55(9), 3635-3642.

Anema, S. G., and McKenna, A. B. (1996). Reaction kinetics of thermal denaturation of whey proteins in heated reconstituted whole milk. *Journal of Agricultural and Food Chemistry*, 44(2), 422-428.

AOAC. (2005a). AOAC official method 968.06, protein (crude) in animal feed. *Official Methods of Analysis of AOAC International*, 18th Edition, W. Horwitz, and G. W. Latimer, eds., AOAC International, Gaithersburg, MD, Chapter 4, p. 25.

AOAC. (2005b). AOAC official method 988.15, tryptophan in foods and food and feed ingredients. *Official Methods of Analysis of AOAC International*, 18th Edition, W. Horwitz, and G. W. Latimer, eds., AOAC International, Gaithersburg, MD, Chapter 45, p. 83.

AOAC. (2005c). AOAC official method 991.20, nitrogen (total) in milk. *Official Methods of Analysis of AOAC International,* 18th Edition, W. Horwitz, and G. W. Latimer, eds., AOAC International, Gaithersburg, MD, Chapter 33, p. 10.

AOAC. (2005d). AOAC official method 994.12, amino acids in feeds. *Official Methods of Analysis of AOAC International*, 18th Edition, W. Horwitz, and G. W. Latimer, eds., AOAC International, Gaithersburg, MD, Chapter 4, p. 8.

Arcan, I., and Yemenicioglu, A. (2007). Antioxidant activity of protein extracts from heat-treated or thermally processed chickpeas and white beans. *Food Chemistry*, 103(2), 301-312.

Bachran, K., and Bernhard, R. A. (1980). Interaction of iron(II) with lactose. *Journal of Agricultural and Food Chemistry*, 28(3), 536-540.

Baron, C. P., Berner, L., Skibsted, L. H., and Refsgaard, H. H. F. (2005). Evaluation of activity of selected antioxidants on proteins in solution and in emulsions. *Free Radical Research*, 39(7), 777-785.

Baumy, J. J., and Brule, G. (1988). Effect of pH and ionic strength on the binding of bivalent cations to β-casein. *Lait*, 68(4), 409-417.

Bayram, T., Pekmez, M., Arda, N., and Yalcin, A. S. (2008). Antioxidant activity of whey protein fractions isolated by gel exclusion chromatography and protease treatment. *Talanta*, 75(3), 705-709.

Belitz, H. D., Grosch, W., and Schieberle, P. (2004). Lipids. *Food Chemistry*, 3rd revised edition, Springer, NY, pp. 157-244.

Beltran, G., Aguilera, M. P., and Gordon, M. H. (2005). Solid phase microextraction of volatile oxidation compounds in oil-in-water emulsions. *Food Chemistry*, 92(3), 401-406.

Benjelloun, B., Talou, T., Delmas, M., and Gaset, A. (1991). Oxidation of rapeseed oil - effect of metal traces. *Journal of the American Oil Chemists Society*, 68(3), 210-211.

Bennett, T., Desmond, A., Harrington, M., McDonagh, D., FitzGerald, R., Flynn, A., and Cashman, K. D. (2000). The effect of high intakes of casein and casein phosphopeptide on calcium absorption in the rat. *British Journal of Nutrition*, 83(6), 673-680.

Berge, J. P., and Barnathan, G. (2005). Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects. *Marine Biotechnology I*, Y. Le Gal, and R. Ulber, Eds., Springer, NY, pp. 49-125.

Bhattacharyya, J., and Das, K. P. (1999). Molecular chaperone-like properties of an unfolded protein, α_s-casein. *Journal of Biological Chemistry*, 274(22), 15505-15509.

Bouhallab, S., Leonil, J., and Maubois, J. L. (1991). Study of β-casein phosphopeptide (1-25)-iron complex: action of alcalase and acid-phosphatase. *Lait*, 71(4), 435-443.

Brisson, G., Britten, M., and Pouliot, Y. (2007). Heat-induced aggregation of bovine lactoferrin at neutral pH: effect of iron saturation. *International Dairy Journal*, 17(6), 617-624.

http://www.caymanchem.com/app/template/Product.vm/catalog/82235/promo/searchresult.82235/a/z (2008). 82235 AAPH. Cayman Chemical Company, Ann Arbor, MI, USA.

Cervato, G., Cazzola, R., and Cestaro, B. (1999). Studies on the antioxidant activity of milk caseins. *International Journal of Food Sciences and Nutrition*, 50(4), 291-296.

Chan, K. M., and Decker, E. A. (1994). Endogenous skeletal muscle antioxidants. *Critical Reviews in Food Science and Nutrition*, 34(4), 403-426.

Chanamai, R., and McClements, D. J. (2002). Comparison of gum arabic, modified starch, and whey protein isolate as emulsifiers: influence of pH, $CaCl_2$ and temperature. *Journal of Food Science*, 67(1), 120-125.

Chandan, R. (1997). Production and specifications of milk concentrates. *Dairy-based Ingredients*, Eagan Press Handbook Series, Eagan Press, St. Paul, MN, pp. 23-39.

Chaud, M. V., Izumi, C., Nahaal, Z., Shuhama, T., Bianchi, M. D. P., and de Freitas, O. (2002). Iron derivatives from casein hydrolysates as a potential source in the treatment of iron deficiency. *Journal of Agricultural and Food Chemistry*, 50(4), 871-877.

Chen, H. M., Muramoto, K., Yamauchi, F., Fujimoto, K., and Nokihara, K. (1998). Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *Journal of Agricultural and Food Chemistry*, 46(1), 49-53.

Chen, J. S., Dickinson, E., and Edwards, M. (1999). Rheology of acid-induced sodium caseinate stabilized emulsion gels. *Journal of Texture Studies*, 30(4), 377-396.

Chin, S. C. K., and Kitts, D. D. (2004). Antioxidant characterization of caseinophosphopeptides from bovine milk. *Nutraceutical Beverages: Chemistry, Nutrition, and Health Effects*, F. Shahidi, and D. K. Weerasinghe, eds., ACS Symposium Series, 871, pp. 279-289.

Chiue, H., Kusano, T., and Iwami, K. (1997). Deamidation-induced fragmentation of maize zein, and its linked reduction in fatty acid-binding capacity as well as antioxidative effect. *Food Chemistry*, 58(1-2), 111-117.

Cho, Y. J., Alamed, J., McClements, D. J., and Decker, E. A. (2003). Ability of chelators to alter the physical location and prooxidant activity of iron in oil-in-water emulsions. *Journal of Food Science*, 68(6), 1952-1957.

Clark, L. C., Wolf, R., Granger, D., and Taylor, Z. (1953). Continuous recording of blood oxygen tensions by polarography. *Journal of Applied Physiology*, 6(3), 189-193.

Colbert, L. B., and Decker, E. A. (1991). Antioxidant activity of an ultrafiltration permeate from acid whey. *Journal of Food Science*, 56(5), 1248-1250.

Coupland, J. N., Zhu, Z., Wan, H., McClements, D. J., Nawar, W. W., and Chinachoti, P. (1996). Droplet composition affects the rate of oxidation of emulsified ethyl linoleate. *Journal of the American Oil Chemists Society*, 73(6), 795-801.

Cunnane, S. C. (2003). Problems with essential fatty acids: time for a new paradigm? *Progress in Lipid Research*, 42(6), 544-568.

Dalgleish, D. G. (2004). Food emulsions: their structures and properties. *Food Emulsions*, S. E. Friberg, K. Larsson, and J. Sjoeblom, Eds., Marcel Dekker, NY, pp. 1-44.

Dalgleish, D. G. (1997). Adsorption of protein and the stability of emulsions. *Trends in Food Science & Technology*, 8(1), 1-6.

Dalgleish, D. G., Srinivasan, M., and Singh, H. (1995). Surface properties of oil-in-water emulsion droplets containing casein and Tween 60. *Journal of Agricultural and Food Chemistry*, 43(9), 2351-2355.

Dalgleish, D. G. (1993). The sizes and conformations of the proteins in adsorbed layers of individual caseins on latices and in oil-in-water emulsions. *Colloids and Surfaces B-Biointerfaces*, 1(1), 1-8.

Davies, M. J. (2003). Protein oxidation: concepts, mechanisms and new insights. *http://www.healthcare.uiowa.edu/research/sfrbm/Papers/Sunrise%20Papers/2003/Davies%202003.ppt#22*. The Heart Research Institute, Sydney, Australia.

Davies, M. J., and Dean, R. T. (1997). *Radical-Mediated Protein Oxidation: From Chemistry to Medicine*, Oxford University Press, Oxford.

Davies, M. J., Fu, S. L., and Dean, R. T. (1995). Protein hydroperoxides can give rise to reactive free radicals. *Biochemical Journal*, 305(Part 2), 643-649.

Dean, R. T., Fu, S. L., Stocker, R., and Davies, M. J. (1997). Biochemistry and pathology of radical-mediated protein oxidation. *Biochemical Journal*, 324(Part 1), 1-18.

Dean, R. T., Hunt, J. V., Grant, A. J., Yamamoto, Y., and Niki, E. (1991). Free radical damage to proteins: the influence of the relative localization of radical generation, antioxidants, and target proteins. *Free Radical Biology and Medicine*, 11(2), 161-168.

Decker, E. A., McClements, D. J., Chaiyasit, W., Nuchi, C. D., Silvestre, M. P. C., Mancuso, J. R., Tong, L. M., and Mei, L. Y. (2002). Factors influencing free radical generation in food emulsions. *Free Radicals in Food: Chemistry, Nutrition and Health Effects*, M. J. Morello, F. Shahidi, C-T Ho, Eds., ACS Symposium Series 807, American Chemical Society, Washington, DC, pp. 83-97.

Decker, E. A., Ivanov, V., Zhu, B. Z., and Frei, B. (2001). Inhibition of low-density lipoprotein oxidation by carnosine and histidine. *Journal of Agricultural and Food Chemistry*, 49(1), 511-516.

Delcourt, C. (2007). Application of nutrigenomics in eye health. *Nutrigenomics - Opportunities in Asia*, E. S. Tai, and P. J. Gillies, eds., Karger, NY, pp. 168-175.

Diaz, M., and Decker, E. A. (2004). Antioxidant mechanisms of caseinophosphopeptides and casein hydrolysates and their application in ground beef. *Journal of Agricultural and Food Chemistry*, 52(26), 8208-8213.

Diaz, M., Dunn, C. M., McClements, D. J., and Decker, E. A. (2003). Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 51(8), 2365-2370.

Dickinson, E. (1992). *An Introduction to Food Colloids*, Oxford University Press, Oxford.

Dickinson, E., and Stainsby, G. (1982). *Colloids in Food,* Applied Science Publishers, London.

Djordjevic, D., McClements, D. J., and Decker, E. A. (2004). Oxidative stability of whey protein-stabilized oil-in-water emulsions at pH 3: potential omega-3 fatty acid delivery systems (part B). *Journal of Food Science*, 69(5), C356-C362.

Donnelly, J. L., Decker, E. A., and McClements, D. J. (1998). Iron-catalyzed oxidation of menhaden oil as affected by emulsifiers. *Journal of Food Science*, 63(6), 997-1000.

Doucet, D., Otter, D. E., Gauthier, S. F., and Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by alcalase: peptide identification and determination of enzyme specificity. *Journal of Agricultural and Food Chemistry*, 51(21), 6300-6308.

Elias, R. J., Kellerby, S. S., and Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition*, 48(5), 430-441.

Elias, R. J., McClements, D. J., and Decker, E. A. (2007). Impact of thermal processing on the antioxidant mechanisms of continuous phase β-lactoglobulin in oil-in-water emulsions. *Food Chemistry*, 104(4), 1402-1409.

Elias, R. J., Bridgewater, J. D., Vachet, R. W., Waraho, T., McClements, D. J., and Decker, E. A. (2006). Antioxidant mechanisms of enzymatic hydrolysates of β-lactoglobulin in food lipid dispersions. *Journal of Agricultural and Food Chemistry*, 54(25), 9565-9572.

Elias, R. J., McClements, D. J., and Decker, E. A. (2005). Antioxidant activity of cysteine, tryptophan, and methionine residues in continuous phase β-lactoglobulin in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 53(26), 10248-10253.

Emery, T. (1992). Iron oxidation by casein. *Biochemical and Biophysical Research Communications*, 182(3), 1047-1052.

Fang, Y., and Dalgleish, D. G. (1993). Dimensions of the adsorbed layers in oil-in-water emulsions stabilized by caseins. *Journal of Colloid and Interface Science*, 156(2), 329-334.

Faraji, H., McClements, D. J., and Decker, E. A. (2004). Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 52(14), 4558-4564.

FDA, U. (2007). Paragraph 172.135, Disodium EDTA. *Code of Federal Regulations*, Food and Drug Administration, U.S. Department of Health and Human Services, Silver Spring, MD.

Feng, M., VanderDoes, L., and Bantjes, A. (1995). Preparation of apolactoferrin with a very low iron saturation. *Journal of Dairy Science*, 78(11), 2352-2357.

Ferguson, J. D. (2008). Milk components. *http://cahpwww.vet.upenn.edu/dairy/mc.index.htm*. University of Pennsylvania, PA, USA.

Fox, P. F. (2003). The major constituents of milk. *Dairy Processing: Improving Quality*, G. Smit, ed., Woodhead Publishing Limited, Cambridge, pp. 5-41.

Fox, P. F., and McSweeney, P. L. H. (1998a). Milk proteins. *Dairy Chemistry and Biochemistry*, Blackie Academic & Professional, London, pp. 146-238.

Fox, P. F., and McSweeney, P. L. H. (1998b). Heat-induced changes in milk. *Dairy Chemistry and Biochemistry*, Blackie Academic & Professional, London, pp. 347-378.

Fox, P. F., and McSweeney, P. L. H. (1998c). Physical properties of milk. *Dairy Chemistry and Biochemistry*, Blackie Academic & Professional, London, pp. 437-461.

Frankel, E. N., Satue-Gracia, T., Meyer, A. S., and German, J. B. (2002). Oxidative stability of fish and algae oils containing long-chain polyunsaturated fatty acids in bulk and in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 50(7), 2094-2099.

Frankel, E. N. (1998a). Hydroperoxide formation, *Lipid Oxidation*, The Oily Press, Dundee, Scotland, pp. 23-41.

Frankel, E. N. (1998b). Hydroperoxide decomposition, *Lipid Oxidation*, The Oily Press, Dundee, Scotland, pp. 55-77.

Frankel, E. N. (1998c). Methods to determine extent of oxidation. *Lipid Oxidation*, The Oily Press, Dundee, Scotland, pp. 79-98.

Frankel, E. N. (1993). In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends in Food Science & Technology*, 4(7), 220-225.

Gallaher, J. J., Hollender, R., Peterson, D. G., Roberts, R. F., and Coupland, J. N. (2005). Effect of composition and antioxidants on the oxidative stability of fluid milk supplemented with an algae oil emulsion. *International Dairy Journal*, 15(4), 333-341.

Gardner, H. W. (1979). Lipid hydroperoxide reactivity with proteins and amino acids: a review. *Journal of Agricultural and Food Chemistry*, 27(2), 220-229.

Gaucheron, F., LeGraet, Y., Boyaval, E., and Piot, M. (1997a). Binding of cations to casein molecules: importance of physicochemical conditions. *Milchwissenschaft - Milk Science International*, 52(6), 322-327.

Gaucheron, F., LeGraet, Y., Raulot, K., and Piot, M. (1997b). Physicochemical characterization of iron-supplemented skim milk. *International Dairy Journal*, 7(2-3), 141-148.

Gaucheron, F., Famelart, M. H., and LeGraet, Y. (1996). Iron-supplemented caseins: preparation, physicochemical characterization and stability. *Journal of Dairy Research*, 63(2), 233-243.

Gaucheron, F., Molle, D., Leonil, J., and Maubois, J. L. (1995). Selective determination of phosphopeptide β-cn(1-25) in a β-casein digest by adding iron: characterization by liquid-chromatography with online electrospray ionization mass spectrometric detection. *Journal of Chromatography B - Biomedical Applications*, 664(1), 193-200.

Gay, C., Collins, J., and Gebicki, J. M. (1999). Hydroperoxide assay with the ferric–xylenol orange complex. *Analytical Biochemistry*, 273(2), 149-155.

Genot, C., Meynier, A., and Riaublanc, A. (2003). Lipid oxidation in emulsions. *Lipid Oxidation Pathways*, A. Kamal-Edin, ed., AOCS Press, Champaign, IL, pp. 190-244.

Gieseg, S., Duggan, S., and Gebicki, J. M. (2000). Peroxidation of proteins before lipids in U937 cells exposed to peroxyl radicals. *Biochemical Journal*, 350, 215-218.

Gohtani, S., Sirendi, M., Yamamoto, N., Kajikawa, K., and Yamano, Y. (1999). Effect of droplet size on oxidation of docosahexaenoic acid in emulsion system. *Journal of Dispersion Science and Technology*, 20(5), 1319-1325.

Gordon, M. H. (2001). The development of oxidative rancidity in foods. *Antioxidants in Food: Practical Applications*, J. Pokorny, N. Yanlieva, and M. H. Gordon, eds., Woodhead Publishing Limited, Cambridge, p. 7-21.

Graf, E., Mahoney, J. R., Bryant, R. G., and Eaton, J. W. (1984). Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. *Journal of Biological Chemistry*, 259(6), 3620-3624.

Guingamp, M. F., Humbert, G., and Linden, G. (1993). Determination of sulfhydryl groups in milk using Ellman procedure and clarifying reagent®. *Journal of Dairy Science*, 76(8), 2152-2155.

Hamm, D. L., Hammond, E. G., and Hotchkis.Dk. (1968). Effect of temperature on rate of autoxidation of milk fat. *Journal of Dairy Science*, 51(4), 483-491.

Harris, W. S. (2007). International recommendations for consumption of long-chain omega-3 fatty acids. *Journal of Cardiovascular Medicine*, 8(Supplement 1), S50-S52.

Havea, P., Singh, H., Creamer, L. K., and Campanella, O. H. (1998). Electrophoretic characterization of the protein products formed during heat treatment of whey protein concentrate solutions. *Journal of Dairy Research*, 65(1), 79-91.

Hawkins, C. L., and Davies, M. J. (2001). Generation and propagation of radical reactions on proteins. *Biochimica Et Biophysica Acta - Bioenergetics*, 1504(2-3), 196-219.

Hegenauer, J., Saltman, P., Ludwig, D., Ripley, L., and Bajo, P. (1979a). Effects of supplemental iron and copper on lipid oxidation in milk. 1. Comparison of metal-complexes in emulsified and homogenized milk. *Journal of Agricultural and Food Chemistry*, 27(4), 860-867.

Hegenauer, J., Saltman, P., Ludwig, D., Ripley, L., and Ley, A. (1979b). Iron-supplemented cow milk. Identification and spectral properties of iron bound to casein micelles. *Journal of Agricultural and Food Chemistry*, 27(6), 1294-1301.

Hegenauer, J., Saltman, P., and Nace, G. (1979c). Iron(III)-phosphoprotein chelates: stoichiometric equilibrium constant for interaction of iron(III) and phosphorylserine residues of phosvitin and casein. *Biochemistry*, 18(18), 3865-3879.

Hekmat, S., and McMahon, D. J. (1998). Distribution of iron between caseins and whey proteins in acidified milk. *Food Science and Technology - Lebensmittel-Wissenschaft & Technologie*, 31(7-8), 632-638.

Hernandez-Ledesma, B., Davalos, A., Bartolome, B., and Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, 53(3), 588-593.

Hirai, Y., Permyakov, E. A., and Berliner, L. J. (1992). Proteolytic digestion of α-lactalbumin: physiological implications. *Journal of Protein Chemistry*, 11(1), 51-57.

http://www.foodsci.uoguelph.ca/dairyedu/chem.html (2008). Dairy chemistry and physics. Department of Food Science, University of Guelph, Guelph, Canada.

Hu, M., McClements, D. J., and Decker, E. A. (2004). Impact of chelators on the oxidative stability of whey protein isolate-stabilized oil-in-water emulsions containing omega-3 fatty acids. *Food Chemistry*, 88(1), 57-62.

Hu, M., McClements, D. J., and Decker, E. A. (2003a). Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 51(5), 1435-1439.

Hu, M., McClements, D. J., and Decker, E. A. (2003b). Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. *Journal of Agricultural and Food Chemistry*, 51(6), 1696-1700.

Huang, S. W., Satue-Gracia, M. T., Frankel, E. N., and German, J. B. (1999). Effect of lactoferrin on oxidative stability of corn oil emulsions and liposomes. *Journal of Agricultural and Food Chemistry*, 47(4), 1356-1361.

Huang, X. L. L., Catignani, G. L., and Swaisgood, H. E. (1996). Improved emulsifying properties of β-barrel domain peptides obtained by membrane-fractionation of a limited tryptic hydrolysate of β-lactoglobulin. *Journal of Agricultural and Food Chemistry*, 44(11), 3437-3443.

Hunt, J. A., and Dalgleish, D. G. (1994). Adsorption behavior of whey protein isolate and caseinate in soya oil-in-water emulsions. *Food Hydrocolloids*, 8(2), 175-187.

Imai, H., Maeda, T., Shima, M., and Adachi, S. (2008). Oxidation of methyl linoleate in oil-in-water micro- and nanoemulsion systems. *Journal of the American Oil Chemists Society*, 85(9), 809-815.

IUPAC. (1987). Method 2.504. Determination of the p-anisidine value (p-A.V.). *IUPAC Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th Edition, C. Paquot, and A. Hautfenne, Eds., Blackwell Scientific Publications, Oxford.

Jacobsen, C., Let, M. B., Andersen, G., and Meyer, A. S. (2006). Oxidative stability of fish oil enriched yoghurts. *Seafood Research from Fish to Dish: Quality, Safety and Processing of Wild and Farmed Seafood*, J. B. Luten, C. Jacobsen, K. Bakaert, A. Saebø, and J. Oehlenschläger, Eds., Wageningen Academic Publishers, Wageningen.

Jacobsen, C., Hartvigsen, K., Thomsen, M. K., Hansen, L. F., Lund, P., Skibsted, L. H., Holmer, G., Adler-Nissen, J., and Meyer, A. S. (2001). Lipid oxidation in fish oil enriched mayonnaise: calcium disodium ethylenediaminetetraacetate, but not gallic acid, strongly inhibited oxidative deterioration. *Journal of Agricultural and Food Chemistry*, 49(2), 1009-1019.

Jacobsen, C., Hartvigsen, K., Lund, P., Thomsen, M. K., Skibsted, L. H., Adler-Nissen, J., Holmer, G., and Meyer, A. S. (2000). Oxidation in fish oil-enriched mayonnaise 3. Assessment of the influence of the emulsion structure on oxidation by discriminant partial least squares regression analysis. *European Food Research and Technology*, 211(2), 86-98.

Jacobsen, C., Hartvigsen, K., Lund, P., Meyer, A. S., Adler-Nissen, J., Holstborg, J., and Holmer, G. (1999). Oxidation in fish-oil-enriched mayonnaise 1. Assessment of propyl gallate as an antioxidant by discriminant partial least squares regression analysis. *European Food Research and Technology*, 210(1), 13-30.

Je, J. Y., Park, P. J., and Kim, S. K. (2005). Antioxidant activity of a peptide isolated from Alaska pollack (Theragra chalcogramma) frame protein hydrolysate. *Food Research International*, 38(1), 45-50.

Jonsdottir, R., Bragadottir, M., and Arnarson, G. O. (2005). Oxidatively derived volatile compounds in microencapsulated fish oil monitored by solid-phase microextraction (SPME). *Journal of Food Science*, 70(7), C433-C440.

Ju, Z. Y., and Kilara, A. (1998). Gelation of pH-aggregated whey protein isolate solution induced by heat, protease, calcium salt, and acidulant. *Journal of Agricultural and Food Chemistry*, 46(5), 1830-1835.

Kamal-Edin, A., Mäkinen M., and Lampi, A-M (2003). The challenging contribution of hydroperoxides to the lipid oxidation mechanism. *Lipid Oxidation Pathways*, A. Kamal-Edin, ed., AOCS Press, Champain, IL, pp. 1-36.

Katz, S., Shinaberry, R. G., Heck, E. L., and Squire, W. (1980). Structure–volume relationships: singular volume effects produced by cupric ion–globular protein interaction. *Biochemistry*, 19(16), 3805-3813.

Ke, P. J., and Ackman, R. G. (1973). Bunsen coefficient for oxygen in marine oils at various temperatures determined by an exponential dilution method with a polarographic oxygen electrode. *Journal of the American Oil Chemists Society*, 50(11), 429-435.

Kellerby, S. S., Gu, Y. S., McClements, D. J., and Decker, E. A. (2006). Lipid oxidation in a menhaden oil-in-water emulsion stabilized by sodium caseinate cross-linked with transglutaminase. *Journal of Agricultural and Food Chemistry*, 54(26), 10222-10227.

Kim, G. N., Jang, H. D., and Kim, C. I. (2007a). Antioxidant capacity of caseinophosphopeptides prepared from sodium caseinate using alcalase. *Food Chemistry*, 104(4), 1359-1365.

Kim, S. B., Seo, I. S., Khan, M. A., Ki, K. S., Nam, M. S., and Kim, H. S. (2007b). Separation of iron-binding protein from whey through enzymatic hydrolysis. *International Dairy Journal*, 17(6), 625-631.

Kiokias, S., Dimakou, C., and Oreopoulou, V. (2007). Effect of heat treatment and droplet size on the oxidative stability of whey protein emulsions. *Food Chemistry*, 105(1), 94-100.

Kiokias, S., and Bot, A. (2006). Temperature cycling stability of pre-heated acidified whey protein-stabilised o/w emulsion gels in relation to the internal surface area of the emulsion. *Food Hydrocolloids*, 20(2-3), 245-252.

Kiokias, S. N., Dimakou, C. P., Tsaprouni, I. V., and Oreopoulou, V. (2006). Effect of compositional factors against the thermal oxidative deterioration of novel food emulsions. *Food Biophysics*, 1(3), 115-123.

Kitts, D. D. (2005). Antioxidant properties of casein-phosphopeptides. *Trends in Food Science & Technology*, 16(12), 549-554.

Kritchevsky, D. (2002). Fats and oils in human health. *Food Lipids - Chemistry, Nutrition, and Biotechnology*, 2nd edition, C. C. Akoh, and D. B. Min, Eds., Marcel Dekker, NY, pp. 543-558.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology*, 157(1), 105-132.

Lagarde, M. (2008). Docosahexaenoic acid: nutrient and precursor of bioactive lipids. *European Journal of Lipid Science and Technology*, 110(8), 673-678.

Leaver, J., Law, A. J. R., and Brechany, E. Y. (1999). Covalent modification of emulsified β-casein resulting from lipid peroxidation. *Journal of Colloid and Interface Science*, 210(1), 207-214.

Lee, H., Kizito, S. A., Weese, S. J., Craig-Schmidt, M. C., Lee, Y., Wei, C. I., and An, H. (2003). Analysis of headspace volatile and oxidized volatile compounds in DHA-enriched fish oil on accelerated oxidative storage. *Journal of Food Science*, 68(7), 2169-2177.

Leonil, J., Molle, D., Gaucheron, F., Arpino, P., Guenot, P., and Maubois, J. L. (1995). Analysis of major bovine milk proteins by online high-performance liquid chromatography and electrospray ionization mass spectrometry. *Lait*, 75(3), 193-210.

Let, M. B., Jacobsen, C., and Meyer, A. S. (2007a). Lipid oxidation in milk, yoghurt, and salad dressing enriched with neat fish oil or pre-emulsified fish oil. *Journal of Agricultural and Food Chemistry*, 55(19), 7802-7809.

Let, M. B., Jacobsen, C., Sorensen, A. D. M., and Meyer, A. S. (2007b). Homogenization conditions affect the oxidative stability of fish oil enriched milk emulsions: lipid oxidation. *Journal of Agricultural and Food Chemistry*, 55(5), 1773-1780.

Lethuaut, L., Metro, F., and Genot, C. (2002). Effect of droplet size on lipid oxidation rates of oil-in-water emulsions stabilized by protein. *Journal of the American Oil Chemists Society*, 79(5), 425-430.

Levine, R. L., Mosoni, L., Berlett, B. S., and Stadtman, E. R. (1996). Methionine residues as endogenous antioxidants in proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 93(26), 15036-15040.

Liu, H. C., Chen, W. L., and Mao, S. J. T. (2007). Antioxidant nature of bovine milk β-lactoglobulin. *Journal of Dairy Science*, 90(2), 547-555.

Liu, Y. W., Han, C. H., Lee, M. H., Hsu, F. L., and Hou, W. C. (2003). Patatin, the tuber storage protein of potato (Solanum tuberosum L.), exhibits antioxidant activity in vitro. *Journal of Agricultural and Food Chemistry*, 51(15), 4389-4393.

Luxford, C., Dean, R. T., and Davies, M. J. (2002). Induction of DNA damage by oxidised amino acids and proteins. *Biogerontology*, 3(1-2), 95-102.

Mackie, A. R., Mingins, J., and North, A. N. (1991). Characterization of adsorbed layers of a disordered coil protein on polystyrene latex. *Journal of the Chemical Society - Faraday Transactions*, 87(18), 3043-3049.

Mancuso, J. R., McClements, D. J., and Decker, E. A. (1999a). Ability of iron to promote surfactant peroxide decomposition and oxidize α-tocopherol. *Journal of Agricultural and Food Chemistry*, 47(10), 4146-4149.

Mancuso, J. R., McClements, D. J., and Decker, E. A. (1999b). The effects of surfactant type, pH, and chelators on the oxidation of salmon oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 47(10), 4112-4116.

Manson, W., and Cannon, J. (1978). Reaction of α_{s1} and β-casein with ferrous ions in presence of oxygen. *Journal of Dairy Research*, 45(1), 59-67.

Marcuse, R. (1962). Effect of some amino acids on oxidation of linoleic acid and its methyl ester. *Journal of the American Oil Chemists Society*, 39(2), 97-103.

Marcuse, R. (1960). Antioxidative effect of amino acids. *Nature*, 186(4728), 886-887.

McClements, D. J., and Decker, E. A. (2000). Lipid oxidation in oil-in-water emulsions: impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science*, 65(8), 1270-1282.

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

Mei, L. Y., McClements, D. J., and Decker, E. A. (1999). Lipid oxidation in emulsions as affected by charge status of antioxidants and emulsion droplets. *Journal of Agricultural and Food Chemistry*, 47(6), 2267-2273.

Mei, L. Y., Decker, E. A., and McClements, D. J. (1998a). Evidence of iron association with emulsion droplets and its impact on lipid oxidation. *Journal of Agricultural and Food Chemistry*, 46(12), 5072-5077.

Mei, L. Y., McClements, D. J., Wu, J. N., and Decker, E. A. (1998b). Iron-catalyzed lipid oxidation in emulsion as affected by surfactant, pH and NaCl. *Food Chemistry*, 61(3), 307-312.

http://wordnetweb.princeton.edu/perl/webwn?s=free%20radical (2009). Word to search for: "free radical". WordNet®, Cognitive Science Laboratory, Princeton University, Princeton, NJ, USA.

Miyashita, K., and Takagi, T. (1986). Study on the oxidative rate and prooxidant activity of free fatty acids. *Journal of the American Oil Chemists Society*, 63(10), 1380-1384.

Morgan, P. E., Treweek, T. M., Lindner, R. A., Price, W. E., and Carver, J. A. (2005). Casein proteins as molecular chaperones. *Journal of Agricultural and Food Chemistry*, 53(7), 2670-2683.

Morgan, P. E., Dean, R. T., and Davies, M. J. (2002). Inactivation of cellular enzymes by carbonyls and protein-bound glycation/glycoxidation products. *Archives of Biochemistry and Biophysics*, 403(2), 259-269.

Mossini, V., and Feather, M. S. (1998). Metal-binding properties of glycated proteins and amino acids. *The Maillard Reaction in Foods and Medicine*, J. O'Brien, H. E. Nursten, M. J. C. Crabbe, and J. Ames, Eds., The Royal Society of Chemistry, London, pp. 429.

Moure, A., Dominguez, H., and Parajo, J. C. (2006). Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochemistry*, 41(2), 447-456.

Murase, H., Nagao, A., and Terao, J. (1993). Antioxidant and emulsifying activity of N-(long-chain-acyl)histidine and N-(long-chain-acyl)carnosine. *Journal of Agricultural and Food Chemistry*, 41(10), 1601-1604.

Nagasako, Y., Saito, H., Tamura, Y., Shimamura, S., and Tomita, M. (1993). Iron-binding properties of bovine lactoferrin in iron-rich solution. *Journal of Dairy Science*, 76(7), 1876-1881.

Nakaya, K., Ushio, H., Matsukawa, S., Shimizu, M., and Ohshima, T. (2005). Effects of droplet size on the oxidative stability of oil-in-water emulsions. *Lipids*, 40(5), 501-507.

Nielsen, N. S., Debnath, D., and Jacobsen, C. (2007). Oxidative stability of fish oil enriched drinking yoghurt. *International Dairy Journal*, 17(12), 1478-1485.

Nielsen, N. S., Petersen, A., Meyer, A. S., Timm-Heinrich, M., and Jacobsen, C. (2004). Effects of lactoferrin, phytic acid, and EDTA on oxidation in two food emulsions enriched with long-chain polyunsaturated fatty acids. *Journal of Agricultural and Food Chemistry*, 52(25), 7690-7699.

Nuchi, C. D., Hernandez, P., McClements, D. J., and Decker, E. A. (2002). Ability of lipid hydroperoxides to partition into surfactant micelles and alter lipid oxidation rates in emulsions. *Journal of Agricultural and Food Chemistry*, 50(19), 5445-5449.

Nuchi, C. D., McClements, D. J., and Decker, E. A. (2001). Impact of Tween 20 hydroperoxides and iron on the oxidation of methyl linoleate and salmon oil dispersions. *Journal of Agricultural and Food Chemistry*, 49(10), 4912-4916.

O'Kennedy, B. T., and Mounsey, J. S. (2006). Control of heat-induced aggregation of whey proteins using casein. *Journal of Agricultural and Food Chemistry*, 54(15), 5637-5642.

Osborn, H. T., and Akoh, C. C. (2004). Effect of emulsifier type, droplet size, and oil concentration on lipid oxidation in structured lipid-based oil-in-water emulsions. *Food Chemistry*, 84(3), 451-456.

Ostdal, H., Andersen, H. J., and Nielsen, J. H. (2000). Antioxidative activity of urate in bovine milk. *Journal of Agricultural and Food Chemistry*, 48(11), 5588-5592.

Ostdal, H., Andersen, H. J., and Davies, M. J. (1999). Formation of long-lived radicals on proteins by radical transfer from heme enzymes - a common process? *Archives of Biochemistry and Biophysics*, 362(1), 105-112.

Ostdal, H., Daneshvar, B., and Skibsted, L. H. (1996). Reduction of ferrylmyoglobin by β-lactoglobulin. *Free Radical Research*, 24(6), 429-438.

Paraskevopoulou, D., Boskou, D., and Paraskevopoulou, A. (2007). Oxidative stability of olive oil–lemon juice salad dressings stabilized with polysaccharides. *Food Chemistry*, 101(3), 1197-1204.

Patel, H. A. (2007a). Milk proteins. *Studies On Heat- And Pressure-Induced Interactions Of Milk Proteins*. Ph.D. Thesis, Massey University, Palmerston North, New Zealand, pp. 5-16.

Patel, H. A. (2007b). Materials and methods. *Studies On Heat- And Pressure-Induced Interactions Of Milk Proteins*. Ph.D. Thesis, Massey University, Palmerston North, New Zealand, pp. 49-66.

Pazos, M., Andersen, M. L., and Skibsted, L. H. (2006). Amino acid and protein scavenging of radicals generated by iron/hydroperoxide system: an electron spin resonance spin trapping study. *Journal of Agricultural and Food Chemistry*, 54(26), 10215-10221.

Pena-Ramos, E. A., and Xiong, Y. L. (2001). Antioxidative activity of whey protein hydrolysates in a liposomal system. *Journal of Dairy Science*, 84(12), 2577-2583.

Philippe, M., Le Graet, Y., and Gaucheron, F. (2005). The effects of different cations on the physicochemical characteristics of casein micelles. *Food Chemistry*, 90(4), 673-683.

Ponginebbi, L., Nawar, W. W., and Chinachoti, P. (1999). Oxidation of linoleic acid in emulsions: effect of substrate, emulsifier, and sugar concentration. *Journal of the American Oil Chemists Society*, 76(1), 131-138.

Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y., and Kim, S. K. (2005). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Research International*, 38(2), 175-182.

Rampon, V., Lethuaut, L., Mouhous-Riou, N., and Genot, C. (2001). Interface characterization and aging of bovine serum albumin stabilized oil-in-water emulsions as revealed by front-surface fluorescence. *Journal of Agricultural and Food Chemistry*, 49(8), 4046-4051.

Reddy, M. I., and Mahoney, A. W. (1991a). Binding of Fe(III) to bovine α_{s1}-casein. *Journal of Dairy Science*, 74(Supplement 1), D58, 100.

Reddy, M. I., and Mahoney, A. W. (1991b). A study of the interaction of Fe(III) with bovine α_{s1}-casein using ultraviolet and fluorescence spectroscopy. *Journal of Dairy Science*, 74(Supplement 1), D59, 100.

Refsgaard, H. H. F., Tsai, L., and Stadtman, E. R. (2000). Modifications of proteins by polyunsaturated fatty acid peroxidation products. *Proceedings of the National Academy of Sciences of the United States of America*, 97(2), 611-616.

Remondetto, G. E., Beyssac, E., and Subirade, M. (2004). Iron availability from whey protein hydrogels: an in vitro study. *Journal of Agricultural and Food Chemistry*, 52(26), 8137-8143.

Renye, J. A., and Somkuti, G. A. (2008). Cloning of milk-derived bioactive peptides in *Streptococcus thermophilus*. *Biotechnology Letters*, 30(4), 723-730.

Rival, S. G., Boeriu, C. G., and Wichers, H. J. (2001a). Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *Journal of Agricultural and Food Chemistry*, 49(1), 295-302.

Rival, S. G., Fornaroli, S., Boeriu, C. G., and Wichers, H. J. (2001b). Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. *Journal of Agricultural and Food Chemistry*, 49(1), 287-294.

Roozen, J. P., Frankel, E. N., and Kinsella, J. E. (1994). Enzymatic and autoxidation of lipids in low-fat foods: model of linoleic acid in emulsified hexadecane. *Food Chemistry*, 50(1), 33-38.

Ruxton, C. H. S., Reed, S. C., Simpson, M. J. A., and Millington, K. J. (2004). The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *Journal of Human Nutrition and Dietetics*, 17(5), 449-459.

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

Satue-Gracia, M. T., Frankel, E. N., Rangavajhyala, N., and German, J. B. (2000). Lactoferrin in infant formulas: effect on oxidation. *Journal of Agricultural and Food Chemistry*, 48(10), 4984-4990.

Schnitzer, E., Pinchuk, I., Bor, A., Fainaru, M., and Lichtenberg, D. (1997). The effect of albumin on copper-induced LDL oxidation. *Biochimica Et Biophysica Acta - Lipids and Lipid Metabolism*, 1344(3), 300-311.

Segall, K. I., and Goff, H. D. (2002). Secondary adsorption of milk proteins from the continuous phase to the oil–water interface in dairy emulsions. *International Dairy Journal*, 12(11), 889-897.

Shacter, E. (2008). Protein oxidation: a primer on characterization, detection, and consequences. *http://www.healthcare.uiowa.edu/research/sfrbm/Papers/Virtual%20School%20Papers/Shacter-ProtOx.ppt#1*. University of Iowa, Iowa City, IA, USA.

Shantha, N. C., and Decker, E. A. (1994). Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *Journal of AOAC International*, 77(2), 421-424.

Sharp, J. S., Becker, J. M., and Hettich, R. L. (2004). Analysis of protein solvent accessible surfaces by photochemical oxidation and mass spectrometry. *Analytical Chemistry*, 76(3), 672-683.

Shears, G. E., Ledward, D. A., and Neale, R. J. (1987). Iron complexation to carboxyl groups in a bovine serum albumin digest. *International Journal of Food Science and Technology*, 22(3), 265-272.

Shen, Z. P., Rusli, J. K., Sanguansri, L., and Augustin, M. A. (2007). Retention of propanal in protein-stabilised tuna oil-in-water emulsions. *Food Chemistry*, 101(2), 746-752.

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

Shimada, K., Muta, H., Nakamura, Y., Okada, H., Matsuo, K., Yoshioka, S., Matsudaira, T., and Nakamura, T. (1994). Iron-binding property and antioxidative activity of xanthan on the autoxidation of soybean oil in emulsion. *Journal of Agricultural and Food Chemistry*, 42(8), 1607-1611.

Shimada, K., Fujikawa, K., Yahara, K., and Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry*, 40(6), 945-948.

Silvestre, M. P. C., Chaiyasit, W., Brannan, R. G., McClements, D. J., and Decker, E. A. (2000). Ability of surfactant headgroup size to alter lipid and antioxidant oxidation in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 48(6), 2057-2061.

Simopoulos, A. P. (2001). n-3 fatty acids and human health: defining strategies for public policy. *Lipids*, 36, S83-S89.

Simopoulos, A. P. (1999). Essential fatty acids in health and chronic disease. *American Journal of Clinical Nutrition*, 70(3), 560S-569S.

Sims, R. J., Fioriti, J. A., and Trumbetas, J. (1979). Effect of sugars and sugar alcohols on autoxidation of safflower oil in emulsions. *Journal of the American Oil Chemists Society*, 56(8), 742-745.

Smith, A. K., and Campbell, B. E. (2007). Microstructure of milk components. *Structure of Dairy Products*, A. Y. Tamime, ed., Blackwell Publishing, Oxford, pp. 59-71.

Smith, C. A., Anderson, B. F., Baker, H. M., and Baker, E. N. (1994). Structure of copper-substituted and oxalate-substituted human lactoferrin at 2.0 Angstrom resolution. *Acta Crystallographica Section D - Biological Crystallography*, 50(Part 3), 302-316.

Sorensen, A. D. M., Baron, C. P., Let, M. B., Bruggemann, D. A., Pedersen, L. R. L., and Jacobsen, C. (2007). Homogenization conditions affect the oxidative stability of fish oil enriched milk emulsions: oxidation linked to changes in protein composition at the oil–water interface. *Journal of Agricultural and Food Chemistry*, 55(5), 1781-1789.

Srinivasan, M., Singh, H., and Munro, P. A. (1999). Adsorption behaviour of sodium and calcium caseinates in oil-in-water emulsions. *International Dairy Journal*, 9(3-6), 337-341.

Srinivasan, M., Singh, H., and Munro, P. A. (1996). Sodium caseinate-stabilized emulsions: factors affecting coverage and composition of surface proteins. *Journal of Agricultural and Food Chemistry*, 44(12), 3807-3811.

Steijns, J. M., and van Hooijdonk, A. C. M. (2000). Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *British Journal of Nutrition*, 84(Supplement 1), S11-S17.

Suetsuna, K., Ukeda, H., and Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *Journal of Nutritional Biochemistry*, 11(3), 128-131.

Sun, C. H., and Gunasekaran, S. (2009). Effects of protein concentration and oil-phase volume fraction on the stability and rheology of menhaden oil-in-water emulsions stabilized by whey protein isolate with xanthan gum. *Food Hydrocolloids*, 23(1), 165-174.

Svenning, C., and Vegarud, G. E. (1998). *Proceeding 'Dairy Foods in Health'*. IDF Nutrition Week, New Zealand, p. 12.

Tamime, A. Y., Robinson, R. K., and Michel, M. (2007). Microstructure of concentrated and dried milk products. *Structure of Dairy Products*, A. Y. Tamime, ed., Blackwell Publishing, Oxford, pp. 104-133.

Taylor, M. J., and Richardson, T. (1980a). Antioxidant activity of cysteine and protein sulfhydryls in a linoleate emulsion oxidized by hemoglobin. *Journal of Food Science*, 45(5), 1223-1227.

Taylor, M. J., and Richardson, T. (1980b). Antioxidant activity of skim milk: effect of heat and resultant sulfhydryl groups. *Journal of Dairy Science*, 63(11), 1783-1795.

Thomsen, M. K., Jacobsen, C., and Skibsted, L. H. (2000). Mechanism of initiation of oxidation in mayonnaise enriched with fish oil as studied by electron spin resonance spectroscopy. *European Food Research and Technology*, 211(6), 381-386.

Tong, L. M., Sasaki, S., McClements, D. J., and Decker, E. A. (2000a). Antioxidant activity of whey in a salmon oil emulsion. *Journal of Food Science*, 65(8), 1325-1329.

Tong, L. M., Sasaki, S., McClements, D. J., and Decker, E. A. (2000b). Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *Journal of Agricultural and Food Chemistry*, 48(5), 1473-1478.

Turini, M. E., Crozier, G. L., Donnet-Hughes, A., and Richelle, M. A. (2001). Short-term fish oil supplementation improved innate immunity, but increased ex vivo oxidation of LDL in man – a pilot study. *European Journal of Nutrition*, 40(2), 56-65.

Tzvetkova, I., Dalgalarrondo, M., Danova, S., Iliev, I., Ivanova, I., Chobert, J. M., and Haertle, T. (2007). Hydrolysis of major dairy proteins by lactic acid bacteria from Bulgarian yogurts. *Journal of Food Biochemistry*, 31(5), 680-702.

van Duynhoven, J. P. M., Goudappel, G. J. W., van Dalen, G., van Bruggen, P. C., Blonk, J. C. G., and Eijkelenboom, A. (2002). Scope of droplet size measurements in food emulsions by pulsed field gradient NMR at low field. *Magnetic Resonance in Chemistry*, 40(13), S51-S59.

Vegarud, G. E., Langsrud, T., and Svenning, C. (2000). Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. *British Journal of Nutrition*, 84(Supplement 1), S91-S98.

Viljanen, K., Kylli, P., Hubbermann, E. M., Schwarz, K., and Heinonen, M. (2005). Anthocyanin antioxidant activity and partition behavior in whey protein emulsion. *Journal of Agricultural and Food Chemistry*, 53(6), 2022-2027.

Villiere, A., Viau, M., Bronnec, I., Moreau, N., and Genot, C. (2005). Oxidative stability of bovine serum albumin- and sodium caseinate-stabilized emulsions depends on metal availability. *Journal of Agricultural and Food Chemistry*, 53(5), 1514-1520.

Watkins, S. M., and German, J. B. (2002). Unsaturated fatty acids. *Food Lipids - Chemistry, Nutrition, and Biotechnology*, C. C. Akoh, and D. B. Min, Eds., Marcel Dekker, NY, pp. 559-561.

Weel, K. G. C., Boelrijk, A. E. M., Burger, J. J., Claassen, N. E., Gruppen, H., Voragen, A. G. J., and Smit, G. (2003). Effect of whey protein on the in vivo release of aldehydes. *Journal of Agricultural and Food Chemistry*, 51(16), 4746-4752.

Wijendran, V., and Hayes, K. C. (2004). Dietary n-6 and n-3 fatty acid balance and cardiovascular health. *Annual Review of Nutrition*, 24, 597-615.

Wong, P. Y. Y., and Kitts, D. D. (2003). Chemistry of buttermilk solid antioxidant activity. *Journal of Dairy Science*, 86(5), 1541-1547.

Yanishlieva-Maslarova, N. V. (2001). Inhibiting oxidation. *Antioxidants in food: Practical Applications*, J. Pokorny, N. Yanishlieva, and M. H. Gordon, eds., Woodhead Publishing, Cambridge, pp. 22-70.

Ye, A., and Singh, H. (2000). Influence of calcium chloride addition on the properties of emulsions stabilized by whey protein concentrate. *Food Hydrocolloids*, 14(4), 337-346.

Yong, Y. H., and Foegeding, E. A. (2008). Effects of caseins on thermal stability of bovine β-lactoglobulin. *Journal of Agricultural and Food Chemistry*, 56(21), 10352-10358.

Zirlin, A., and Karel, M. (1969). Oxidation effects in a freeze-dried gelatin-methyl linoleate system. *Journal of Food Science*, 34(2), 160-165.

Zou, M. (2005). Laboratory procedure: determination of amino acids profile in food and feedstuffs by means of ion-exchange chromatography, AOAC methods 994.12 & 985.28. Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand.