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Homogenization Conditions Affect the Oxidative Stability of Fish Oil Enriched Milk Emulsions: Lipid Oxidation

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In this study fish oil was incorporated into commercial homogenized milk using different homogenization temperatures and pressures. The main aim was to understand the significance of homogenization temperature and pressure on the oxidative stability of the resulting milks. Increasing homogenization temperature from 50 to 72 °C decreased droplet size only slightly, whereas a pressure increase from 5 to 22.5 MPa decreased droplet size significantly. Surprisingly, emulsions having small droplets, and therefore large interfacial area, were less oxidized than emulsions having bigger droplets. Emulsions with similar droplet size distributions, but resulting from different homogenization conditions, had significantly different oxidative stabilities, indicating that properties of significance to oxidation other than droplet size itself were affected by the different treatments. In general, homogenization at 72 °C appeared to induce protective effects against oxidation as compared to homogenization at 50 °C. The results thus indicated that the actual composition of the oil−water interface is more important than total surface area itself.

KEYWORDS: Fish oil; lipid oxidation; milk; emulsion; homogenization; droplet size; milk fat globule membrane

INTRODUCTION

Obtainment of an oil-in-water emulsion with sufficient physical stability requires oil droplet sizes as small as possible and stabilization of the oil−water interface. It has been proposed that lipid oxidation in emulsions mainly is initiated at the oil−water interface. For this reason it can be speculated that oxidation may be positively correlated to the interfacial area size and, in turn, that reduction of oil droplet sizes in oil-in-water emulsions may affect oxidative stability negatively (*1*, *2*). However, experimental evidence regarding the effect on oxidation of oil droplet interfacial area in emulsions is inconsistent (*3*−*6*), indicating that interfacial area is not the only factor affecting the initiation and progress of lipid oxidation in emulsions. Previous studies have shown effects on oxidation of attractive and repulsive electrostatic forces between charged surfaces of the dispersed phase and continuous phase metal ions (*7*, *8*). Other studies have shown only minor impact of droplet surface charge, whereas interfacial thickness seemed to be relatively more important with respect to oxidation (*4*). The available knowledge thus suggests that oxidation of a given emulsion may be determined by the area and composition of the interface, including type of emulsifier, surface charge, and thickness of interfacial area, as well as by content of pro- and antioxidants in the oil and aqueous phases. Moreover, there is also a lack of knowledge on how processing conditions affect oxidative stability of fish oil enriched food emulsions. The influence of the processing conditions on the interfacial composition and subsequent oxidation in fish oil enriched milk is further investigated in a separate paper (*9*).

The nutritionally important long-chain omega-3 polyunsaturated fatty acids (PUFA) are very susceptible to lipid oxidation. The main challenge in the incorporation of PUFA-rich fish oils into real food emulsions is therefore to achieve oxidatively stable emulsions with acceptable sensory characteristics with no development of fishy off-flavor during production or storage. As a natural, widely consumed oil-in-water emulsion, regular cow's milk is an interesting medium for fish oil enrichment. Previous studies on this system have shown that especially the fish oil quality, the cold storage temperature employed for keeping the enriched milk, and the addition of antioxidant or rapeseed oil during the manufacture are important factors influencing the oxidative stability of the fish oil enriched milks during storage (*10*, *11*). In addition, previous data indicate that the natural protein material present in cow's milk seems to be sufficient to emulsify the fish oil added. Thus, no extrinsic emulsifier has to be added before or after homogenization. Most available studies on the effect of homogenization temperature and pressure on the protein material in milk have been done on

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Table 1. Experimental Design and Resulting Droplet Size (D[3,2]) and Viscosity of Emulsions

	oil		homogenization				
sample	amount (wt %)	type	temp (°C)	pressure (MPa)	caseinate (g/L)	D[3,2] (μm)	viscosity (mPa·s)
			Experiment 1				
F_T50P5	0.5	fish	50	5		1.26	
F_T72P5	0.5	fish	72	5		1.16	
F_T50P22.5	0.5	fish	50	22.5		0.70	
F_T72P22.5	0.5	fish	72	22.5		0.45	
F_T50P15	0.5	fish	50	15		0.89	
F_T72P15	0.5	fish	72	15		0.81	
			Experiment 2				
F_T50P5	0.5	fish	50	5		1.31	3.9
F_T72P5	0.5	fish	72	5		1.26	4.1
F_T50P22.5	0.5	fish	50	22.5		0.77	4.1
F_T72P22.5	0.5	fish	72	22.5		0.51	4.0
F_cas1	0.5	fish	72	22.5	1	0.50	4.0
F_cas5	0.5	fish	72	22.5	5	0.50	4.7
F_cas10	0.5	fish	72	22.5	10	0.51	5.2
FR_T50P5	0.5	fish + rapeseed	50	5		1.36	4.0
FR_T72P22.5	0.5	fish + rapeseed	72	22.5		0.48	4.0
FR1%_T72P22.5	1.0	fish + rapeseed	72	22.5		0.55	4.0

raw bovine milk. In raw milk, proteins are mainly present in the aqueous phase, and both emulsifying and antioxidative properties of these proteins have been shown. Homogenization of regular cow's milk causes adsorption of both casein and whey proteins to stabilize the fat globule membrane. Overall, the protein load of the interface has been shown to increase with homogenization pressure (*12*−*14*). It is well-established that whey proteins, mainly β-lactoglobulin and α-lactalbumin, are more heat-sensitive than caseins (*12*, *15*−*17*). Heating of raw milk in the range of 65−85 °C leads to denaturation of β-lactoglobulin and exposure of nonpolar residues and sulfhydryl groups, which again enables interaction with membrane proteins of the fat globule and casein molecules. It has been shown that increasing temperature (65−85 °C) increased the ratio of α-lactalbumin and β-lactoglobulin to casein present in the membrane (*13*, *17*).

This study investigated the effect on oxidative stability of different homogenization temperatures and pressures during incorporation of fish oil into commercial pasteurized milk (0.5−1.5 wt % fat) and, thus, examined the overall balance between factors promoting or preventing oxidation when the physical structure of the emulsion was changed by homogenization. Increased temperature during homogenization is necessary to have liquid fat, but is also expected to enhance oxidation, especially of the highly unsaturated n-3 PUFA. In milk, a part of the prooxidant transition metals are bound to protein. Pressure treatment at elevated temperature facilitates protein unfolding, which might increase exposure of these metal ions, which, in turn, may promote oxidation. Finally, the large increase in surface area of the droplets could provide increased contact between oil phase and prooxidants. On the other hand, the above-described changes in milk fat globule membrane during homogenization, including increased or improved coverage of oil droplets and exposure of sulfhydryl groups on β-lactoglobulin, might reduce oxidation of the sensitive polyunsaturated fatty acids in the fish oil (*12*, *13*). On the basis of our previous findings, the temperature and pressure effects were also compared for milk enriched with only fish oil and milk enriched with a stabilized fish and rapeseed oil mixture (**Table 1**) (*18*). Finally, the effect of adding caseinate as an emulsifier prior to homogenization was investigated. Caseinate is an emulsifier, which provides emulsion droplets with both electrostatic and steric stabilization (*19*, *20*). Several experiments have also shown both antioxidative and surface active properties of caseinate in oil-in-water emulsions (*4*, *21*−*23*).

Table 2. Chemical Composition of Fish and Rapeseed Oils

fatty acid[a] (% w/w)	milk fat	fish oil	rapeseed and fish oil mixture
14:0	9.9	3.7	2.0
15:0	1.0		
16:0	34.3	10.8	7.7
17:0	0.5		
18:0	11.7	2.2	2.0
20:0	0.0	1.5	0.3
SAT	**57.4**	**18.2**	**12.0**
14:1	0.4		
16:1(n-7)	1.8	7.3	3.5
18:1(n-9)	22.6	17.0	38.5
18:1(n-7)	2.8	4.4	4.0
20:1(n-9)		11.1	6.1
22:1(n-11)		6.6	3.3
22:1(n-9)		0.8	0.7
MUFA	**27.6**	**47.2**	**56.1**
18:2(n-6)	2.9	1.7	10.5
18:3(n-3)		0.9	4.6
18:4(n-3)		2.8	1.3
20:5(n-3)		9.0	4.2
22:5(n-3)	0.1	0.9	0.5
22:6(n-3)		10.5	5.2
PUFA	**3.0**	**25.8**	**26.3**
PV (± 0.01) (mequiv/kg)			
expt 1		0.09	
expt 2		0.32	0.09
tocopherols (ppm)			
α-		330	273
β-			146

[a] Only fatty acids of C14 or longer chain lengths were determined.

MATERIALS AND METHODS

Materials. Fresh milks with fat contents of 0.5 and 1.5 wt % were purchased locally and mixed in a 1:1 ratio. Refined cod liver oils without added antioxidants and an oil mixture of rapeseed oil and cod liver oil (1:1) with added antioxidants [1840 ppm of citric acid ester (mono- and diglycerides of fatty acids) and 460 ppm of propyl gallate] were provided by Maritex A/S, Sortland, Norway. The oils were described by their fatty acid composition, the peroxide value (PV), and the level of tocopherols of each oil, which are given in **Table 2**. The fatty acid composition was determined by preparation of methyl esters (*24*) that

were subsequently analyzed by gas chromatography (*25*). The levels of tocopherols were determined by HPLC (*26*). Sodium caseinate (Miprodan 30) was provided by Arla Foods, a.m.b.a., Aarhus, Denmark. Chemicals and external standards for identification of volatile oxidation products were all from Sigma Aldrich, Steinheim, Germany, or Merck, Darmstadt, Germany. All solvents were of HPLC grade from Lab-Scan, Dublin, Ireland.

Production of Emulsions and Preparation of Samples for Analyses. Milk (2 L) was pasteurized by heating to 72 °C within 3 min and holding for 15 s, and fish oil (final concentration of 0.5% by weight) was then added. Milk samples were then cooled to 50 °C or homogenized directly according to **Table 1**. Milk samples were homogenized (two-valve Rannie homogenizer, APV, Albertslund, Denmark) at a total pressure of 5, 15, or 22.5 MPa (50, 150, or 225 bar). For emulsions containing sodium caseinate, the caseinate was dissolved in the milk (2 °C) prior to heating and homogenization. The emulsions were stored in sterilized Pyrex bottles at 2 °C in the dark. After storage, emulsions were subjected to sensory evaluation, peroxide value (PV) determination, and dynamic headspace GC-MS analyses. Samples for PV and GC-MS analysis were stored in separate, brown glass bottles, which were immediately flushed with nitrogen and kept at −80 °C until analyses, whereas samples for sensory analyses were evaluated directly at sampling.

Viscosity and Droplet Size of Emulsions. Milk emulsion viscosity was determined on a HAAKE VT 500 rotational viscometer (Thermo Electron Corporation, Karlsruhe, Germany), using the sensor system NV, suitable for low-viscosity liquids. Viscosity was determined at 5 °C by a linear increase in rotation from 0 to 2000 rpm in 1 min followed by a similar linear decrease. The oil droplet size was determined by laser diffraction in a Mastersizer 2000, Malvern Instruments, Worcestershire, U.K. Droplets of milk emulsion were suspended directly in recirculating water (2800 rpm stirring) to an obscuration of 14−17%. The setup used was the Fraunhofer method, which assumes that all sizes of particles scatter with equal efficiencies and that the particles are opaque and transmit no light (*27*). Results are presented graphically as the overall size distribution of the droplets and as the surface area mean diameter, $D[3,2] = \Sigma n_i d_i^3 / \Sigma n_i d_i^2$.

Analyses of Primary Oxidation Products. Lipids from the milk emulsions were extracted by chloroform/methanol (1:1 w/w), using a reduced amount of solvent (*28*, *29*). PV was measured directly on the oils or on the fat extract from the milk emulsions by colorimetric determination of iron thiocyanate (*30*).

Dynamic Headspace Analysis of Volatile Secondary Oxidation Products. Volatile secondary oxidation products from 8 g of milk emulsion were purged and trapped on Tenax GR tubes with nitrogen (150 mL/min) for 30 min at 45 °C using 4-methyl-1-pentanol as internal standard. The volatiles were desorbed (200 °C) from the trap in an automatic thermal desorber (ATD-400, Perkin-Elmer, Norwalk, CT) and cryofocused on a Tenax GR cold trap. Volatiles were separated by gas chromatography (HP 5890 IIA, Hewlett-Packard, Palo Alto, CA) as described previously (*31*) and analyzed by mass spectrometry (HP 5972 mass-selective detector). The oven temperature program was as follows: 45 °C held for 5 min, raised at 1.5 °C/min to 55 °C, at 2.5 °C/min to 90 °C, and at 12 °C/min to 220 °C, and finally held at 220 °C for 4 min. The individual compounds were identified by both MS library searches (Wiley138K, John Wiley and Sons, Hewlett-Packard) and by authentic external standards. The compounds were quantified through individual calibration curves in the range of 2−1500 ng/g of milk. The compounds were dissolved in ethanol, added to a conventional pasteurized milk with 1.5% fat, and headspace analysis was performed in triplicate as described above. The limit of detection and the limit of quantification were determined at signal-to-noise ratios of 2 and 5, respectively, for each of the compounds at the given conditions (8 g of emulsion, 45 °C, 30 min purge with N_2 at 150 mL/min).

Sensory Evaluation. The milk emulsions in the second experiment were evaluated by descriptive analysis by 12 panelists trained in descriptive analysis of fishy off-flavors. ISO standards 6658, 8586, and 6564 were generally followed for training and sensory analysis methods, respectively. The descriptors used for odor and flavor assessment were fishy, rancid, milk, and metallic, and they were evaluated on a continuous intensity scale ranging from 0 intensity to a maximum intensity of 9. Samples (40 mL) were served randomized at 5 °C with crisp bread and cold water in blind trials after 1, 4, and 8 days of storage. Data were collected on PSION mini computers (PSION, London, U.K.).

Statistical Analysis. The data were analyzed by one-way or two-way analysis of variance, and individual samples were compared on a 0.05 level of significance by the Bonferroni multiple comparison.

RESULTS

Effect of Treatments on Droplet Sizes. Changes in temperature and pressure during homogenization affected both the average droplet diameter ($D[3,2]$, **Table 1**) and the droplet size distribution (**Figure 1**) significantly. The size distributions determined in the two experiments were identical for similar samples; thus, only emulsions from experiment 2 are shown in **Figure 1**. At low and medium pressures (5 and 15 MPa), the unimodal distribution of the droplets tended to move only slightly downward as temperature was increased from 50 to 72 °C during homogenization (**Figure 1**). The average droplet diameter was therefore only slightly affected by the temperature during homogenization at low and medium pressures (F_T50P5 vs F_T72P5 and F_T50P15 vs F_T72P15, **Table 1**). Increasing the pressure from 5 to 22.5 MPa at low temperature moved the peak further downward, indicating that high pressure reduced droplet size more than high temperature (F_T50P5 vs F_T50P22.5). The largest difference was observed at both high temperature and pressure (**Table 1**). By studying the droplet size distribution, it was evident that all emulsions homogenized at high temperature and pressure were now bimodal (**Figure 1**). Hence, the apparent decrease in droplet size was not caused by a general decrease in size, but rather a result of the droplets being spilt into two groups: one with unchanged size compared to the emulsion homogenized at only high pressure and one group having very small diameters (**Figure 1**). The emulsion containing 1% added fish oil was homogenized at both high temperature and pressure and was also bimodal and similar to those containing 0.5% added fish oil. However, increase in the oil content to 1% resulted in a small decrease in the portion of the very small droplets (FR_T72P22.5 vs FR1%_T72P22.5) and also led to a slightly increased $D[3,2]$.

Effect of Treatments on Oxidation. The different treatments affected PV, concentrations of volatile oxidation products, and the sensory evaluations of the emulsions during storage. The overall progressions of oxidation were similar in the two storage experiments. Differences in PV were already evident at the beginning of the storage period. Furthermore, a significant and increasing difference between emulsions homogenized at low and high temperature and pressure (F_T50P5 and F_T72P22.5) was observed regarding PV and volatiles. During storage, all samples supplemented with neat fish oil thus had a significant increase in PV and concentrations of 1-penten-3-one, 1-penten-3-ol, 2-penten-1-ol, (*E*)-2-pentenal, (*E*)-2-hexenal, and (*E*,*E*)-2,4-heptadienal (**Figure 2**; **Table 3**). At the end of the storage experiment, the F_T50P5 emulsion had approximately twice the amount of lipid hydroperoxides and volatiles compared to the F_T72P22.5 emulsion (**Figure 2**; **Table 3**). Emulsions homogenized at either high temperature or high pressure had intermediate PVs (**Figure 2**). Overall, the PVs of the emulsions were significantly different after 7 and 11 days in the following order: F_T50P5 > F_T72P5 > F_T50P22.5 > F_T72P22.5. Similar results were obtained regarding the volatiles, as exemplified by development of (*E*)-2-hexenal in the emulsions (**Figure 3**). After 7 days of storage, concentrations of (*E*)-2-hexenal were significantly different in the fish oil enriched milks, in the same order as PV. During the first 7 days, the emulsions

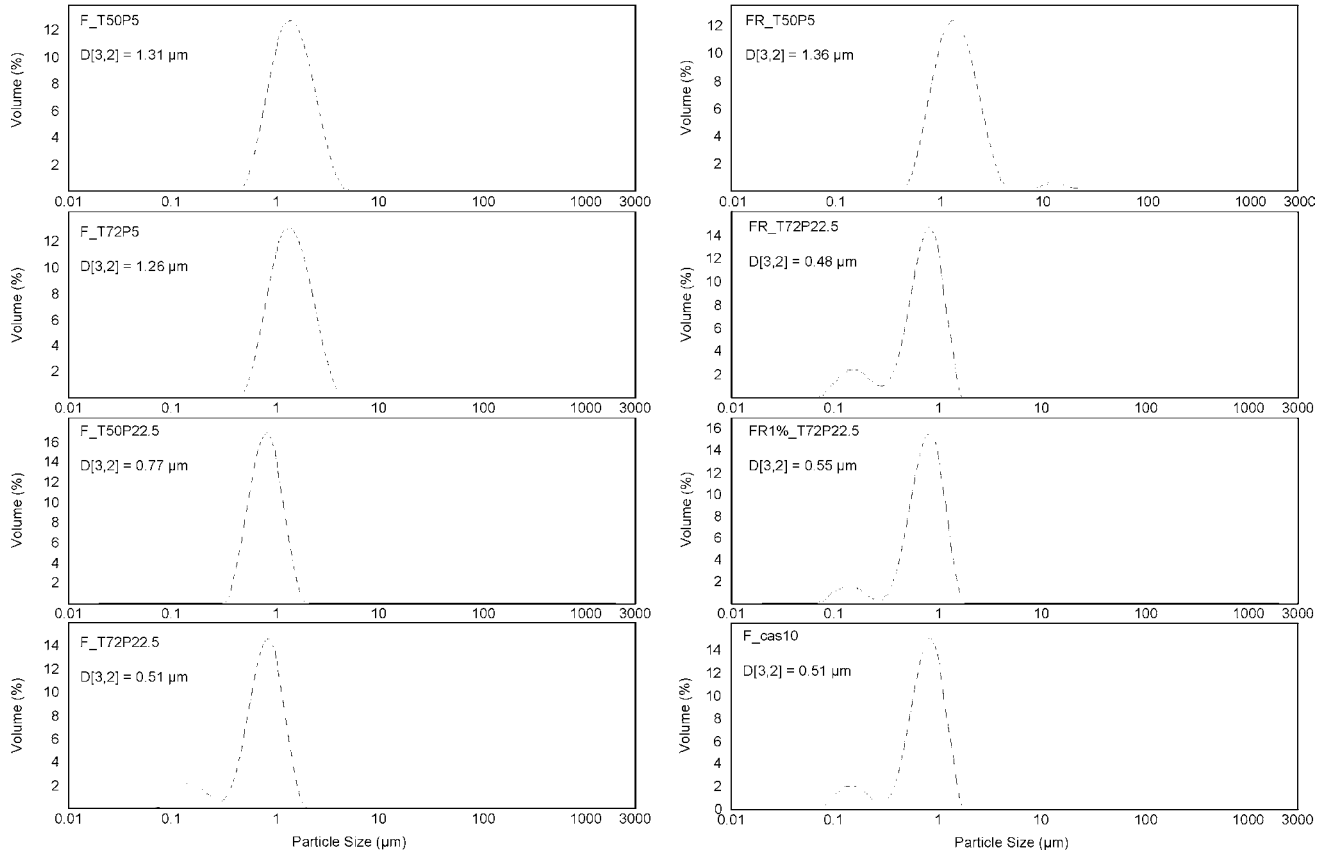

Figure 1. Droplet size distributions in emulsions containing fish oil or fish and rapeseed oil mixture from experiment 2. Sample names refer to **Table 1**.

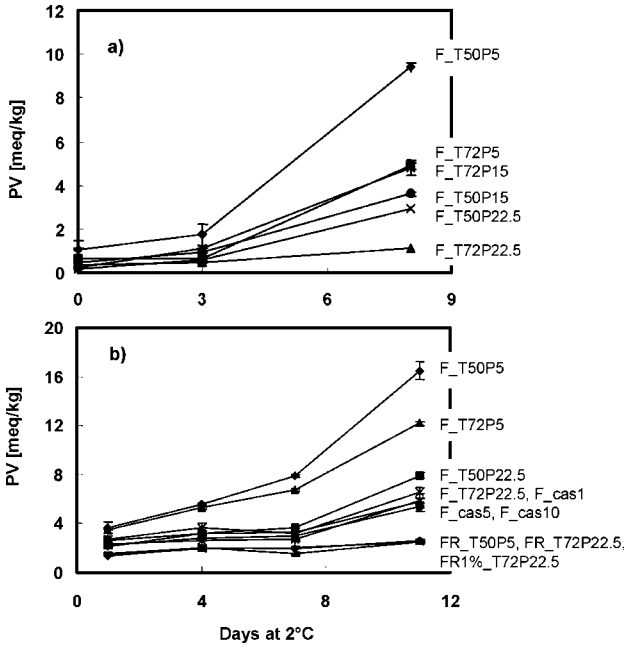

Figure 2. Development in peroxide values (milliequivalents per kilogram) during storage of emulsions in (**a**) experiment 1 and (**b**) experiment 2. Sample names refer to **Table 1**.

homogenized at high temperature and low pressure had significantly higher PV and concentration of volatiles than those homogenized at low temperature and high pressure (F_T72P5 > F_T50P22.5), but after 11 days they had similar and not significantly different concentrations of the quantified volatiles. The emulsions homogenized at intermediate pressure (15 MPa) in the first experiment were located close to the emulsions homogenized at either high temperature (F_T72P5) or high pressure (F_T50P22.5) (**Figure 2**; **Figure 3**). Of these emulsions homogenized at intermediate pressure, the one homogenized at high temperature had significantly higher PV than the one homogenized at low temperature. However, the opposite effect was observed regarding concentrations of volatiles, although these differences were not significant.

Sensory results in **Figure 4** indicated that the emulsion homogenized at high temperature and pressure had less fishy odor and taste than the emulsion homogenized at low temperature and pressure (F_T50P5 vs F_T72P22.5). Moreover, the high temperature−low-pressure emulsion was significantly more fishy than the emulsion homogenized at high temperature and high pressure (F_T72P5 vs F_T72P22.5).

Effects of Extra Caseinate. Emulsions with different levels of added caseinate were homogenized at both high temperature and high pressure and were thus processed similarly to F_T72P22.5 (**Table 1**). The resulting droplet size distributions and average diameters of all four emulsions were almost identical (0.50−0.51 μm). Thus, droplets seemed to remain unchanged with or without caseinate addition. However, caseinate increased the viscosity of the emulsions with increasing caseinate content. All emulsions without caseinate had an average viscosity of 4.0 ± 0.1 mPa·s irrespective of homogenization conditions, whereas emulsions containing 1, 5, and 10 g/L caseinate had viscosities of 4.0, 4.7, and 5.2 mPa·s, respectively (**Table 1**). The differences in viscosity were, however, too small to interfere with odor and taste as perceived by assessors in the sensory panel. Overall, the emulsions homogenized at the high temperature and pressure also had similar fishy off-flavor (i.e., F_T72P22.5 ≈ F_cas1 ≈ F

Table 3. Concentration of Selected Volatile Oxidation Products (Nanograms per Gram of Emulsion) in Emulsions from Experiment 2 after 1 and 11 Days of Storage

	1-penten-3-one[a]		1-penten-3-ol		2-penten-1-ol		(*E*)-2-pentenal		(*E*)-2-hexenal		(*E*,*E*)-2,4-heptadienal	
ng/g of milk emulsion	day 1	day 11	day 1	day 11	day 1	day 11	day 1	day 11	day 1	day 11	day 1	day 11
F_T50P5	3.4	19.4	1.4	23.4	3.5	50.9	0.8	5.9	0.4	11.9	1.8	14.8
F_T72P5	5.0	14.3	2.3	15.6	4.8	37.0	1.4	4.1	1.4	8.7	2.7	10.2
F_T50P22.5	2.9	19.4	1.1	17.6	2.2	40.8	1.3	5.0	0.5	8.5	1.5	11.2
F_T72P22.5	1.3	10.9	0.5	8.7	0.5	20.9	0.4	3.1	0.1	5.0	0.7	7.0
F_cas1	1.2	9.9	0.6	8.0	1.2	18.8	0.3	3.0	0.1	4.6	0.6	6.6
F_cas5	1.4	9.1	0.7	8.4	1.5	18.9	0.6	2.8	0.2	4.5	1.4	6.5
F_cas10	1.9	9.2	0.9	10.6	1.3	23.4	0.4	2.9	0.3	5.1	1.3	6.9
FR_T50P5	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0
FR_T72P22.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0
FR1%_T72P22.5	0.2	0.7	0.0	0.0	0.0	0.0	0.4	0.4	0.0	0.0	0.0	2.1

[a] Average standard deviation of determinations was 0.7 ng/g of emulsion. Sample names refer to **Table 1**.

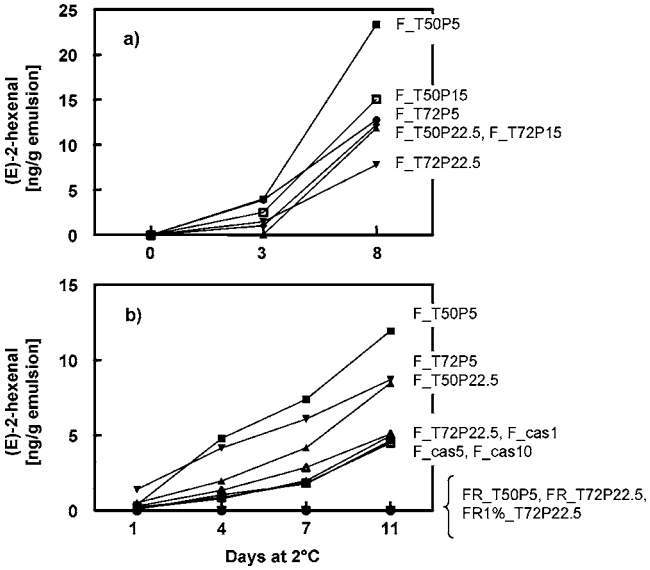

Figure 3. Development of (*E*)-2-hexenal (nanograms per gram of emulsion) during storage of emulsions in (**a**) experiment 1 and (**b**) experiment 2. Sample names refer to **Table 1**.

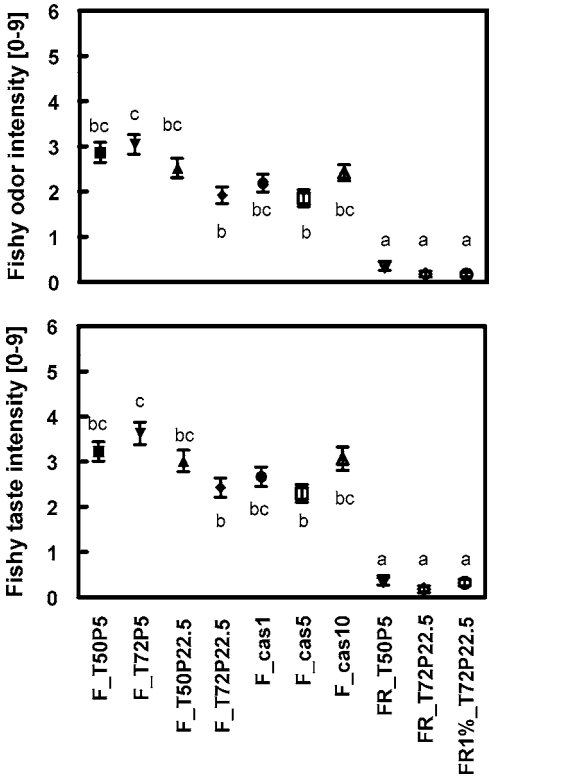

Figure 4. Overall average intensity fishy odor and taste of emulsions enriched with fish oil or fish and rapeseed oil mixture in experiment 2. Sample names refer to **Table 1**.

cas5 ≈ F_cas10). However, the results indicated a slightly higher fishy off-flavor of the emulsion with 10 g/L caseinate. Generally, the concentrations of volatiles in this emulsion were also slightly higher, although the individual volatiles and PV were not significantly different between the four levels of caseinate (0−10 g/L). Sodium caseinate contains traces of transition metal ions, especially iron. Thus, maximum addition of caseinate also results in maximum addition of ferrous ions, which, even in small levels, can promote oxidation by facilitating degradation of lipid hydroperoxides. Hence, rather than protective effects of extra protein emulsifier added in the form of sodium caseinate, a slight prooxidative effect of 10 g/L caseinate was observed.

Milk with Fish Oil versus Fish and Rapeseed Oil Mixture. All three samples with fish and rapeseed oil mixture (FR_T50P5, FR_T72P22.5, FR1%_T72P22.5) had low and stable PVs throughout the storage period of 11 days (1.5−2.0 mequiv/kg) (**Figure 2**). Moreover, the volatiles that are characteristic to lipid oxidation of n-3 PUFA could not be identified in these emulsions, and in particular (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*,*E*)-2,4-hexadienal, (*E*,*E*)-2,4-heptadienal, (*E*,*Z*)-2,6-nonadienal, 1-penten-3-one, 1-penten-3-ol, and 2-penten-1-ol were below the detection limit. The sensory results indicated a slightly increased fishy taste and odor at day 1 of the emulsion containing fish and rapeseed oil mixture homogenized at 50 °C and 5 MPa (FR_T50P5). However, neither PV, volatiles, nor sensory analysis resulted in significant differences between the three emulsions containing fish and rapeseed oil mixture, indicating that the different treatments did not affect oxidation when rapeseed oil including antioxidants was present. Also, doubling the amount of the fish and rapeseed oil mixture did not affect oxidation, and the resulting emulsion with high fish oil content was equally stable (FR_T72P22.5 vs FR1%_T72P22.5). Comparison of the emulsions containing 0.5% fish oil with or without stabilized rapeseed oil (F_T72P22.5 vs FR1%_T72P22.5) clearly showed that the stabilized rapeseed oil reduced PV, concentration of volatiles, and fishy off-flavor of the emulsions. The results therefore indicated that the protective effect of adding the stabilized rapeseed oil exceeded the dilution effect. This is in accordance with several previous studies (*10*, *11*, *18*) showing significant protection of rapeseed oil on fish oil either

with or without additional antioxidants in a fish oil enriched milk emulsion.

DISCUSSION AND CONCLUSIONS

First of all, the results showed only a very slight decrease in droplet size when temperature was increased at low pressure (F_T50P5 vs F_T72P5). On the other hand, a relatively large decrease in droplet sizes was observed when pressure was increased during homogenization at low temperature (F_T50P5 vs F_T50P22.5). Droplet size was further reduced when both temperature and pressure were high. According to the available knowledge pertaining to milk homogenization (*32*), droplets containing solid or partly solid fat are not disrupted sufficiently during homogenization, which is why the homogenization temperature must be above the melting point of the fat to be homogenized and incorporated in the emulsion. At around 37 °C milk fat is completely melted, whereas the more unsaturated fish oil is fluid already at lower temperatures (*32*). In these experiments milk was heated to 50 or 72 °C; thus, no crystalline milk fat or fish oil should be present. Therefore, differences in droplet sizes were less likely to be caused by differences in the physical state of the lipids. Increased temperature facilitates unfolding of whey proteins (*33*, *34*), which might improve adsorption of protein to the lipid globule during the homogenization due to exposure of hydrophobic areas. The decrease in droplet size at high temperature therefore seemed more likely due to conformational changes in the protein material than an effect of lipid fluidity and homogenization. Further investigations will be necessary to determine whether fish oil mixes with milk fat and forms droplets with mixed lipids or if the fish oil is emulsified into droplets separate from milk fat.

An increase in the amount of fish and rapeseed oil mixture from 0.5 to 1.0% resulted in a small decrease in the portion of the very small droplets. This could indicate that some of the protein material used to create an interface around these very small droplets was now needed to cover the increased oil content. On the other hand, additional caseinate did not reduce droplet size further. Competitive adsorption between proteins takes place during homogenization, and aging time affects the adsorption of the individual proteins (*35*). It has also been shown that increasing concentrations of β-lactoglobulin in an emulsion stabilized by sodium caseinate did not result in decreasing droplet sizes. Furthermore, increasing concentrations of caseinate did not result in a continuous decrease in droplet size. Rather, the droplet size reached a plateau, which the droplet size did not go below (*35*). Overall, very complex mechanisms seem to determine the actual droplet size and interfacial composition obtained in this rehomogenized milk enriched with fish oil; thus, more work needs to be done to elucidate this issue.

Overall, the results obtained showed that droplet size data and oxidation data correlated well. High temperature and high pressure resulted in smaller droplets and less oxidation, whereas low temperature and pressure gave larger droplets and faster oxidation. In **Figure 5** the measured oxidation parameters have been plotted against droplet size. From this plot it is clear that a positive linear relationship exists between droplet size and PV. However, as assessed from both the PV and the levels of volatiles, large differences in oxidation tendency were observed between the two milk emulsions having an average droplet size of around 1.30 μm (**Figure 5**). These emulsions were homogenized at 5 MPa and at 50 or 72 °C. It was expected that increased temperature during homogenization would promote oxidation, based on the oxidative susceptibility of long-chain n-3 polyunsaturated fatty acids (*2*). However, even though an increase in temperature during homogenization at low pressure did not alter droplet size, oxidation was significantly reduced with regard to both PV and volatile oxidation products. In contrast to PV, the concentrations of volatiles at the end of the storage did not correlate linearly to droplet size. A large difference in droplet sizes (0.77 vs 1.26 μm) was observed for the emulsions homogenized at either high temperature (F_T72P5) or high pressure (F_T50P22.5), but the concentrations of volatiles were either unchanged or reduced with increasing droplet size.

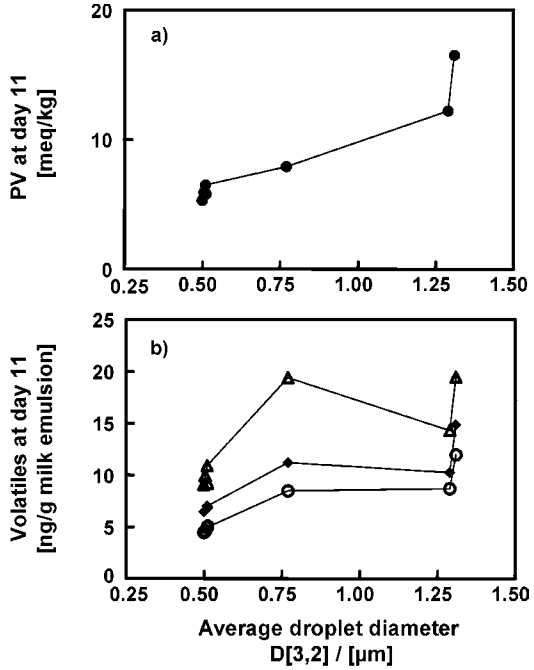

Figure 5. Oxidation measured by (**a**) PV (●) and (**b**) secondary oxidation products [1-penten-3-one (△), (*E*,*E*)-2,4-heptadienal (◆), (*E*)-2-hexenal (○)] versus droplet size in experiment 2.

Previous studies have shown different results concerning the relationship between droplet size and lipid oxidation in emulsions. In fish oil enriched mayonnaise and in an oil-in-water model emulsion based on docosahexaenoic acid (DHA), it was found that decreasing droplet sizes resulted in increased oxidation (*5*, *6*), whereas studies on other oil-in-water model systems have shown no effect of droplet size on initiation or progression of lipid oxidation (*3*, *4*). As mentioned previously, increased homogenization temperature alters the conformation of proteins. Especially, β-lactoglobulin starts to unfold above 65 °C (*13*, *34*) and also contains amino acids with sulfhydryl groups, which has been shown to have antioxidant properties, such as radical scavenging properties (*36*, *37*). We therefore hypothesize that increasing the homogenization temperature from 50 to 72 °C may lead to improved physical coverage of the oil droplets by proteins, most likely β-lactoglobulin, and may expose amino acids, which are able to enhance the oxidative protection of the emulsion. In the present experiment, it therefore seemed that the protective effects caused by homogenization, which were likely to be protein adsorption or protein rearrangement at the interface, significantly prevailed over the effect of heating the relatively unstable fish oil to 72 °C during homogenization of milk containing prooxidant transition metals. This hypothesis regarding possible changes in the protein composition of the interfacial caused by homogenization conditions is further investigated in a separate study (*9*).

As previously described, both heating and homogenization of traditional raw milk cause compositional changes of the fat globule membrane. Homogenization of raw milk introduces whey proteins in the membrane to cover the much larger surface area formed during droplet size reduction. Casein micelles and submicelles also adsorb to the surface of the membrane, providing enhanced protection of the globules against aggregation (*12*, *14*, *38*). Overall, most of the milk fat is covered by new material after homogenization of raw milk (*13*, *14*). In the present study commercial homogenized milk was used as the basis for the fish oil incorporation. Therefore, the milk had already been heated and homogenized once. During incorporation of the fish oil, milk was heated and homogenized again, and additional surface area was created to cover and incorporate the fish oil in the milk emulsion system. In these experiments the effect of dissolved caseinate prior to homogenization was investigated. It was hypothesized that caseinate could adsorb to the newly formed interface and thereby provide enhanced protection by forming a physical barrier. Alternatively, caseinate could also provide additional emulsifying material, which might increase the number of very small droplets. However, addition of caseinate did not reduce droplet size, indicating that the emulsifying material in milk, such as whey proteins and casein, was adequate and that this was not a limiting factor during incorporation of fish oil. Furthermore, the addition of caseinate did not affect lipid oxidation significantly. Caseinate is a surface active molecule, which is able to adsorb to the droplet interface. On the other hand, caseinate is less surface active than β-lactoglobulin. In heated and homogenized oil-in-water emulsions, β-lactoglobulin has been shown to be able to displace casein for the droplet surface, whereas the opposite is not possible (*39*). We speculate that the absence of effect regarding oxidation could be caused by β-lactoglobulin being adsorbed to and covering the droplet interfaces, thus preventing caseinate from providing further physical protection of droplets, as also observed in a separate experiment and reported by Sørensen et al. (*9*).

In summary, these experiments have shown that droplet size and, thus, interfacial area are not the only determining factors of lipid oxidation in the fish oil enriched milk system. Moreover, the results suggest that the actual composition of this interface is more important than the total surface area itself. Finally, these results showed no effect of homogenization conditions on oxidative stability of milk emulsions containing stabilized fish and rapeseed oil mixture. This supports our previous results (*11*, *18*) and highlights the benefits of using this oil mixture in foods enriched with n-3 PUFA.

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