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Protection against Oxidation of Fish-Oil-Enriched Milk Emulsions through Addition of Rapeseed Oil or Antioxidants

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The ability of rapeseed oil and/or different antioxidants (α- and γ-tocopherol mixture, ascorbyl palmitate, and EDTA) to protect fish-oil-enriched milk emulsions against oxidation was investigated. Tocopherol isomers in concentrations similar to those found in natural rapeseed oil were added to rapeseed oil stripped of natural tocopherols. The rapeseed oil with added tocopherols significantly inhibited oxidation in the fish-oil-enriched milk emulsions. In contrast, the emulsions with only fish oil and added α- and γ-tocopherol were less stable than the emulsions with fish oil alone. When added individually, the γ-tocopherol seemed to inhibit oxidation more efficiently than α-tocopherol. Ascorbyl palmitate (AP) almost completely retarded oxidation in the fish-oil-enriched milk emulsions, as determined by PV, volatile oxidation products, and sensory evaluation. AP also prevented the otherwise prooxidant effect of tocopherols added to fish oil before emulsification. No interactions between AP, tocopherols, and EDTA were observed, and EDTA added alone to fish oil did not show antioxidant properties in the milk emulsions. Overall, the results showed that addition of AP or rapeseed oil containing natural tocopherols to fish oil was equally efficient in inhibiting oxidation in the fish-oil-enriched milk emulsions.

KEYWORDS: Fish oil; lipid oxidation; milk; emulsion; rapeseed oil; ascorbyl palmitate; tocopherol; EDTA

INTRODUCTION

The use of fish oil in food lipid emulsions is complicated by the high oxidative susceptibility of these polyunsaturated oils, which leads to unacceptable fishy off-flavors. Different means of protecting emulsions against oxidation have been attempted, mainly focusing on adding antioxidants and designing antioxidant mixtures suitable for the specific type of food emulsion (*1*). Oxidation in oil-in-water emulsions is suggested to occur at the interface between the lipid and the aqueous phases, and the composition of the interfacial area is therefore highly important regarding oxidative stability (*2*). Several factors influence the overall effectiveness of the different antioxidants added, primarily the solubility of the antioxidant, the antioxidative mechanism, and interactions with other compounds. Nonpolar antioxidants have been shown to be particularly effective in oil-in-water emulsions. This phenomenon is assumed to be a result of these antioxidants being located in the lipid phase closer to the interface where oxidation is likely to occur (*3*, *4*).

Tocopherols are nonpolar antioxidants and are recognized as very potent chain-breaking antioxidants based on their ability to scavenge free radicals. It has been suggested that fish oil can be protected against oxidation via other means than by adding antioxidants. Thus, industrial claims have been made that addition of vegetable oil can protect fish oil against oxidation (*5*, *6*). In agreement with these claims, we previously showed a significant protective effect of rapeseed oil against oxidation, when a rapeseed and fish oil mixture was emulsified in milk and subsequently stored cold (*7*, *8*). Fish oil itself contains the α-tocopherol isomer, while rapeseed oil contains both the α-tocopherol and in addition relatively high amounts of the γ-tocopherol isomer. We therefore hypothesized that the specific tocopherol composition naturally present in the rapeseed oil and especially in the presence of γ-tocopherol might play an important role in protecting fish oil from oxidation in the milk emulsions.

However, the water soluble EDTA (calcium disodium ethylenediaminetetraacetate) has also shown antioxidative effects in both mayonnaise and milk oil-in-water emulsions containing fish oil (*9*, *10*). The antioxidative effect of EDTA presumably rests on metal chelation, indicating that trace metals present in the water phase or at the oil−water interface are able to mediate degradation of lipid hydroperoxides to form secondary lipid oxidation products as well as radicals that may contribute to further oxidation reactions (*3*, *4*).

The palmitic acid ester of ascorbic acid, ascorbyl palmitate (AP), is a more hydrophobic antioxidant than the water-soluble ascorbic acid, but antioxidative mechanisms are presumably

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Table 1. Experimental Designs

a: experiment 1	oil addition		antioxidant addition (ppm/oil)[a]				total concentration of tocs. (ppm/oil)	
	0.25% fish	0.25% rapeseed	α	β	γ	AP	α	γ
RC		chrom.						
F + RC	+	chrom.					165	
F + RC + T	+	chrom.	260	40	360		295	180
F + RC + T + AP	+	chrom.	260	40	360	300	295	180
F + RN(1)	+	natural[b]					275	165
F	+						330	
F + tocs	+		260	40	360		590	360

b: experiment 2	oil addition		antioxidant addition[c]			total concentration of tocs. (ppm/oil)	
	0.5% fish	0.5% rapeseed	AP (ppm/oil)	α + γ (ppm/oil)	EDTA (ppm/milk)	α	γ
F	+					330	
F + AP	+		300			330	
F + T	+			260 + 360		590	360
F + EDTA	+				5	330	
F + AP + T	+		300	260 + 360		590	360
F + AP + EDTA	+		300		5	330	
F + T + EDTA	+			260 + 360	5	590	360
F + AP + T + EDTA	+		300	260 + 360	5	590	360
F + RN(2)	+	natural[d]				275	150

c: experiment 3	oil addition		antioxidant addition (ppm/oil)		total concentration of tocs. (ppm/oil)	
	0.5% fish	0.5% rapeseed	α	γ	α	γ
F	+				330	
F + α	+		220		550	
F + $^1/_2\gamma$	+			165	330	165
F + 1γ	+			330	330	330
F + 2γ	+			660	330	660
F + RN(3)	+	natural[b]			275	165

[a] On the basis of the complete emulsion, the molar concentrations of the antioxidants were 1.5 μM α-tocopherol, 0.2 μM β-tocohperol, 2.2 μM γ-tocopherol, and 1.8 μM AP. [b] The natural content of tocopherols in the rapeseed oil was 220 ppm α-tocopherol and 330 ppm γ-tocopherol. [c] On the basis of the complete emulsion, the molar concentrations of the antioxidants were 3.6 μM AP, 3.0 and 4.3 μM α- and γ-tocopherol, respectively, and 13.4 μM EDTA. F = fish oil, RC = chromatographed rapeseed oil, RN = natural rapeseed oil, AP = ascorbyl palmitate, T = tocopherol, EDTA = calcium disodium ethylenediaminetetraacetate. [d] In total was added 1% of a fish and rapeseed oil mixture containing 275 and 150 ppm of natural α- and γ-tocopherol, respectively.

based on the ascorbic acid group. Several mechanisms have been proposed for the antioxidant activity of ascorbic acid and ascorbyl palmitate, including singlet oxygen quenching and metal chelation, and more importantly, also a free-radical-scavenging potential of AP has been shown (*11*−*14*). In bulk oils, several storage experiments have shown that AP works synergistically with tocopherols and that tocopherols are spared at the expense of AP during oxidation. The general view has been that AP is used to regenerate tocopherols (*15*, *16*). AP donates a hydrogen to the tocopheroxyl radical formed by tocopherol donating a hydrogen to the lipid radical. However, some studies have suggested that, on the basis of the free-radical-scavenging potential of AP, AP itself is capable of donating hydrogen directly to the lipid radical (*12*, *17*). In both cases, however, any tocopherols present would be spared compared to AP. Because of the potential synergistic effect with tocopherols, we included AP in this work.

In the present work, three separate storage experiments were performed to investigate the protection of fish-oil-enriched milk emulsions by different antioxidant mechanisms and means of antioxidant addition (**Table 1**). Experiment 1 was designed to investigate the previously observed protective effect of rapeseed oil tocopherols on fish oil through the addition of tocopherols having the same α and γ composition as the natural rapeseed oil. It was also investigated whether an additional protective effect of AP could be observed. Rapeseed oil stripped from tocopherol by chromatography was used for some of the emulsions to avoid confounding effects of naturally present tocopherol. The second experiment aimed at investigating the individual and possible synergistic effects of α- and γ-tocopherol, AP, and EDTA. Oxidation in the emulsions was also compared to the oxidation of an emulsion with natural rapeseed oil and fish oil. In a follow-up study (experiment 3), we investigated the effects on oxidation of the individual α- and γ-tocopherol isomers.

MATERIALS AND METHODS

Materials. Fresh milk with fat contents of 0.5 and 1.5 wt % were purchased locally and mixed in a 1:1 ratio, to obtain milk with 1% milk fat. Two 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 citric acid ester (mono- and diglycerides of fatty acids) and 460 ppm propyl gallate] were provided by Maritex A/S, Århus, Denmark. Also a refined, deodorized rapeseed oil was supplied by Maritex A/S (natural rapeseed oil). A part of this oil was then chromatographed to remove the tocopherols naturally present in the rapeseed oil, using the method by Lampi et al. (*18*). The oils were described by their fatty acid composition, the peroxide value (PV), the amount of free fatty acids, and the level of tocopherols in each of the oils (**Table 2**). The fatty acid composition was determined by preparation of methyl esters that were in turn analyzed by gas chromatography (*19*, *20*). The levels of tocopherols were determined by HPLC (*21*). Tocopherol standards were purchased from Calbiochem, San Diego, CA, and ascorbyl palmitate were provided by Danisco, Brabrand, Denmark. Calcium disodium ethylenediaminetetraacetate

Table 2. Chemical Data of the Fish Oils and the Fish Oil and Rapeseed Oil Mixture

	experiment 1 + 3				experiment 2	
fatty acid (% w/w)	milk	fish oil	rapeseed oil, natural	rapeseed oil, chromatographed	fish oil	fish and rapeseed oil mixture
14:0	10.8	3.5			4.1	2.2
15:0	1.1	0.3				
16:0	37.6	10.3	4.0	3.8	11.9	8.2
17:0	0.6	0.1			0.0	0.0
18:0	13.3	2.3	1.6	1.6	2.4	2.1
20:0	0.2	0.1	1.6	1.8	1.7	0.3
SAT	**63.6**	**16.6**	**7.2**	**7.2**	**20.1**	**12.8**
14:1(*n*–5)	1.0	0.1				
16:1(*n*–7)	1.9	6.7	0.2		8.1	3.7
18:1(*n*–9)	25.3	18.9	60.0	64.5	18.8	41.0
18:1(*n*–7)	3.2	4.4	2.9	0.1	4.9	4.3
20:1(*n*–9)	0.2	14.3	0.1	0.1	12.3	6.6
22:1(*n*–9)		8.7			7.3	3.5
MUFA	**31.6**	**53.1**	**63.2**	**64.7**	**51.3**	**59.1**
18:2(*n*–6)	3.3	1.9	19.8	19.7	1.9	11.2
18:3(*n*–3)	0.6	1.1	8.9	7.6	1.0	4.9
18:4(*n*–3)	0.7	3.0			3.1	1.4
19:2			0.7	0.6		
20:5(*n*–3)	0.1	9.7			10.0	4.5
22:5(*n*–3)	0.1	1.2			1.0	0.5
22:6(*n*–3)		13.4	0.2	0.2	11.6	5.6
PUFA	**4.8**	**30.3**	**29.6**	**28.1**	**28.6**	**28.1**
total	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
PV (±0.02) (meq/kg)	0.76	0.19	0.29	0.65	0.66	0.04
FFA (%)	2.0	0.03	0.03	0.02	0.08	0.14
tocopherols (ppm)						
α	17	330	220	nd[a]	330	275
γ			330	nd		150

[a] nd = not detected.

(EDTA), chemicals, and external standards for identification of volatile oxidation products were all purchased from Sigma Aldrich, Steinheim, Germany. All solvents were of HPLC grade from Lab-Scan, Dublin, Ireland.

Production of Emulsions and Preparation of Samples for Analyses. In experiment 1, the tocopherols and fish oil were added to chromatographed rapeseed oil before emulsification and storage. The added tocopherol profiles were attempted to mimic the compositional profile of the native tocopherols as closely as possible. Milk (3 L) was pasteurized by heating to 72 °C within 3 min and holding for 15 s. Fish oil and/or rapeseed oil (0.5 wt %) and antioxidants were added to the milk according to the designs (**Table 1**). Because of the slightly different objectives of experiment 1 compared to experiments 2 and 3, the reference sample in the latter two experiments (F + RN) contained higher levels of rapeseed oil (0.5%) compared to experiment 1 (0.25%). Milk samples were then cooled to 50 °C and homogenized immediately (total pressure of 50 bar, two-valve Rannie homogenizer, APV, Albertslund, Denmark). The emulsions were stored in sterilized Pyrex bottles at 2 °C in the dark. The emulsions were subjected to sensory evaluation, peroxide value (PV) determination, and dynamic headspace GC−MS analyses. The samples for chemical analysis were transferred to separate, brown glass bottles, flushed with nitrogen, and stored at −80 °C until analyses, while samples for sensory analyses were evaluated directly at sampling.

Analyses of Primary Oxidation Products. Lipids from the emulsions were extracted by chloroform/methanol (1:1, w/w) using a reduced amount of solvent (*22*, *23*). PV was measured directly on the oils or on the fat extract from the milk emulsions by colorimetric determination of iron-thiocyanate (*24*).

Dynamic Headspace Analysis of Volatile Secondary Oxidation Products. Volatile secondary oxidation products from 8 g of 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 the 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) and analyzed by mass spectrometry (HP 5972 mass-selective detector). Oven temperature program: 45 °C held for 5 min, 1.5 °C/min to 55 °C, 2.5 °C/min to 90 °C, 12 °C/min to 220 °C, and finally held at 220 °C for 4 min. The individual compounds were identified by both MS-library searches (Wiley138K, John Wiley and Sons, Hewlett−Packard) and by authentic external standards. The individual compounds were quantified through calibration curves.

Sensory Evaluation. The milk emulsions were evaluated by descriptive analysis by 9−13 panelists trained in descriptive analysis of milk samples with 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 these were evaluated on a continuous intensity scale ranging from zero 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 7 days of storage. Data were collected on PSION mini computers (PSION, London, U.K.).

Statistical Analysis. The data were analyzed by two-way analysis of variance, and individual samples were compared on a 0.05-level of significance by the Bonferroni multiple comparison test using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA).

To illustrate the overall results of the two main storage experiments, three-dimensional multivariate methods of data analysis were also employed. We have previously used this type of data analysis in the evaluation of storage experiments (*7*). The three-dimensional structure of the data is called a three-way array (**Figure 1**). The 3 directions in the array are called modes (*25*). With *i*, *j*, and *k* variables in the 3 modes, the array consists of $i \times j \times k$ elements. The analytical data for PV and volatiles and the sensory data were investigated by the parallel factor analysis (PARAFAC) using the algorithms in the N-way toolbox for Matlab (*25*, *26*). PARAFAC is a three-dimensional extension of the principal component analysis (PCA), which is used in two-dimensional data analysis. As in PCA, the PARAFAC model aims at describing the experimental data by computing latent variables. These latent variables, here referred to as components, are computed in all 3 modes in a PARAFAC model. Prior to the modeling, the data from these experiments were centered across the first mode to remove off-

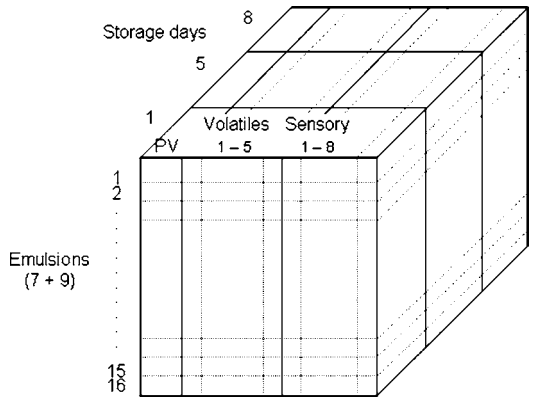

Figure 1. Arrangement of data in a three-way array. First mode: the 16 different emulsions from the two storage experiments. Second mode: six analytical variables of PV, 2-pentene-1-ol, 1-pentene-3-ol, 2-pentenal, 2-hexenal, and 2,4-heptadienal and eight sensory descriptors of fishy smell, rancid smell, milky smell, metallic smell, fishy taste, rancid taste, milky taste, and metallic taste. Third mode: storage times for day 1, 5, and 8.

sets of the different emulsions and scaled within the second mode to normalize the chemical and sensory variables (*26*, *27*). The optimum number of components was determined by segmented cross-validation (*28*). The sensory results were preprocessed prior to the PARAFAC analysis. When the average values of the assessors at each sensory session as described previously are projected away (*10*, *29*), 3 pseudoreplicates of the sensory data were constructed and subsequently used in the PARAFAC analysis.

RESULTS AND DISCUSSION

Experiment 1: Effects of Adding Tocopherols and Ascorbyl Palmitate to Chromatographed Rapeseed Oil and Fish Oil before Emulsification into Milk. Seven milk emulsions were prepared (**Table 1**). The design aimed at investigating the protective effect of rapeseed oil against oxidation of fish oil, when emulsified into milk. Rapeseed oil was stripped for tocopherols, and the effect of adding back the tocopherols to the stripped rapeseed oil was evaluated. The fatty acid composition of the rapeseed oil after the chromatographic stripping was generally in accordance with the fatty acid composition determined in the natural, unchromatographed oil, and the chromatographed oil did not show any traces of tocopherols in the HPLC analysis performed (**Table 2**). The PV of chromatographed rapeseed oil was slightly elevated as compared to the natural rapeseed oil (0.65 versus 0.29 meq/kg). This was likely due to the chromatographic purification step applied and to the fact that the removal of tocopherols invariably renders the polyunsaturated rapeseed oil very susceptible to oxidation.

PV and Volatiles. Already after 1 day of storage, two emulsions deviated clearly from the remaining five emulsions with respect to PV (**Table 3**). These two emulsions containing the chromatographed rapeseed oil (RC) and chromatographed rapeseed oil together with the fish oil (F + RC) had significantly higher PVs than the other emulsion already from day 1 and throughout the storage period. Moreover, the emulsion with only the chromatographed rapeseed oil (RC) had a significantly higher PV than the emulsion with the chromatographed rapeseed oil and fish oil (F + RC). This indicated that the fish oil, with its natural content of α-tocopherol, now protected the rapeseed oil stripped from tocopherols during storage of the emulsions (**Table 3**). We assume that the combination of a very oxidatively unstable rapeseed oil without any tocopherols and the slightly elevated PV of this oil (0.65 meq/kg) might be responsible for the very high degree of oxidation in these milk emulsions observed during the storage period.

The remaining five emulsions had low PVs and were not significantly different at the beginning of the storage experiment. The emulsion with natural rapeseed oil and fish oil [F + RN(1)] was not significantly different from the emulsion with the chromatographed rapeseed oil, fish oil, and added tocopherols (F + RC + T) during the entire storage period. In comparison to the emulsion with only the chromatographed rapeseed oil and fish oil (F + RC), these results indicated a significant protective effect of the tocopherols when added back to the rapeseed oil before emulsification into the milk. During the entire storage period, the emulsion containing the chromatographed rapeseed oil, fish oil, tocopherols, and AP (F + RC + T + AP) had the lowest PV. After 8 and 12 days, this difference was significant.

In contrast, the PV of the emulsion with only fish oil and added tocopherols (F + T) increased during the storage period, and after 8 and 12 days of storage, the PVs were significantly higher than the PV of the emulsion containing only fish oil (F). This indicated a prooxidative effect of the tocopherols, when added to fish oil alone before emulsification. This finding was investigated further in experiment 2.

The emulsion with only fish oil (F) had a slightly higher PV than the emulsion with fish oil and natural rapeseed oil [F + RN(1)]. The difference was significant after 12 days of storage, indicating a slightly protective effect of the rapeseed oil on the fish oil when emulsified into milk and stored a 2 °C. The development in the concentrations of volatile compounds during storage was generally very similar to the development of the PVs (data not shown).

Sensory Analysis. Sensory evaluations were performed after 1, 5, and 8 days of storage. The results of the taste and odor evaluations were very similar, and the intensities of rancid and fishy taste of the emulsions are given in **Figure 2**. Regarding both rancid and fishy taste of the emulsions, different groups of emulsions were observed. The four emulsions containing the chromatographed rapeseed oil had a significant and increasing rancid taste during storage, except the emulsion with added AP

Table 3. Peroxide Values [meq/kg] of the Milk Emulsions Containing the Different Oils and Antioxidants during Storage (Emulsions Listed According to Their PV Level)[a]

	day 1	day 5	day 8	day 12
F + RC + T + AP	0.6 a, u ± 0.2	1.1 a, u ± 0.0	0.7 a, u ± 0.1	1.1 a, u ± 0.0
F + RN(1)	0.5 a, u ± 0.1	1.7 ab, u ± 0.1	2.0 b, u ± 0.3	2.0 b, u ± 0.4
F + RC + T	0.4 a, u ± 0.0	1.9 b, uv ± 0.1	2.6 b, v ± 0.0	2.3 bc, v ± 0.5
F	0.6 a, u ± 0.0	2.3 b, uv ± 0.3	2.6 b, v ± 0.3	2.8 c, v ± 0.4
F + T	0.4 a, u ± 0.0	2.2 b, v ± 0.1	3.4 c, vx ± 0.3	4.0 d, x ± 0.2
F + RC	3.1 b, u ± 0.2	22.7 c, x ± 0.4	27.4 d, y ± 0.4	26.6 e, y ± 0.5
RC	11.7 c, u ± 3.2	33.6 d, v ± 0.2	36.4 e, xy ± 1.1	37.8 f, y ± 0.3

[a] Emulsion names refer to Table 1. F = fish oil, RC = chromatographed rapeseed oil, RN = natural rapeseed oil, T = tocopherol, AP = ascorbyl palmitate.

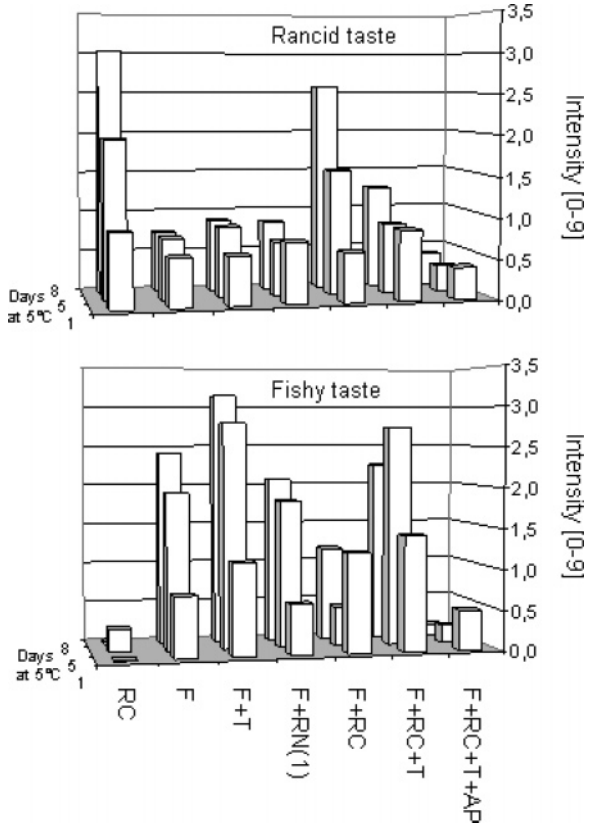

Figure 2. Results of the sensory evaluation of fishy and rancid taste [0–9] of the emulsions. Emulsion names refer to **Table 1**.

(F + RC + T + AP). This emulsion remained stable during storage with respect to both the fishy and rancid taste. The rancid taste of the emulsion with the chromatographed rapeseed oil, fish oil and added tocopherols (F + RC + T) increased less than the other two emulsions (RC and F + RC), and after 8 days, they were significantly different. This supports the observed protective effect of the added tocopherols on the oxidative stability, as suggested by the PV results. The rancid taste of the remaining four emulsions was equally low and did not change during storage.

Except for the emulsion with only the chromatographed rapeseed oil, all other emulsions had a fishy taste. The emulsions with only fish oil, fish oil and tocopherols, and fish oil and natural rapeseed oil [F, F + T, and F + RN(1)] all had an increasing fishy taste during storage. However, the fishy taste did not increase throughout storage for the emulsions containing the chromatographed rapeseed oil and fish oil with or without added tocopherols (F + RC and F + RC + T). Thus, in contrast to the observations for PV, volatiles, and rancid off-flavor, the protective effect of adding tocopherols to the emulsion containing the chromatographed rapeseed oil and fish oil was not observed regarding the fishy off-flavor (**Figure 2**). The increase in rancid taste during storage also indicated that the tocopherols did not block oxidation in these emulsions. The fishy taste of the F + RC and F + RC + T was similar at day 1, but afterward, the emulsion with tocopherols (F + RC + T) was more fishy than the emulsion without tocopherols (F + RC, **Figure 2**). This increase in fishy taste could be caused by the decreased masking of fishy odor by the rancid odor in the emulsion with tocopherols.

Experiment 2: Effects of Adding the Different Antioxidants to Fish Oil before Emulsification into Milk. On basis of the results of the first storage experiment, where a prooxidative effect of the tocopherols was indicated when tocopherols were added to the fish oil alone before emulsification, a second storage experiment was designed. This experiment concerned the individual and possible interactive effects of adding three different types of antioxidants (tocopherols, AP, and EDTA) to fish oil before emulsification into milk.

PV Analysis. The emulsions could be categorized into three groups according to their PV (**Table 4**). The emulsion with fish oil and added tocopherols (F + T) had significantly higher PV than the emulsion with only fish oil, with fish oil and EDTA, and with fish oil, tocopherols, and EDTA (F, F + EDTA, and F + T + EDTA) after 4, 7, and 11 days of storage. The latter three emulsions had similar PVs throughout the storage period. This supports our findings of prooxidant activity of the tocopherols when added to the fish oil alone before emulsification into milk. The group of least oxidized emulsions included the emulsion containing fish and rapeseed oil mixture [F + RN(2)] and all of the four emulsions containing fish oil and added AP regardless of the presence of other antioxidants (EDTA and/or tocopherols). PVs of these five emulsions were stable throughout the storage period.

Volatiles. A total range of 14 compounds was quantified. These were 1-pentanol, 2-penten-1-ol, 1-penten-3-ol, 1-penten-3-one, pentanal, hexanal, nonanal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*,*Z*)-2,4-hexadienal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-nonadienal, (*E*,*Z*)-2,6-nonadienal, and (*E*,*E*)-2,4-decadienal. The volatiles 1-pentanol, pentanal, hexanal, (*E*)-2-heptenal, (*E*,*Z*)-2,4-hexadienal, and (*E*,*E*)-2,4-decadienal were not detected or detected in very low concentrations and did not show any changes during storage. The general development in the concentrations of the remaining compounds is exemplified by (*E*,*E*)-2,4-heptadienal in **Figure 3**. The volatiles 2-penten-1-ol, 1-penten-3-ol, 1-penten-3-one, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*,*E*)-2,4-nonadienal, and (*E*,*Z*)-2,6-nonadienal showed very similar behavior during storage. The development profile of these volatiles was also very similar to the development in PVs during storage. Thus, the same groups could be identified as the emulsion with fish oil and tocopherols (F + T) had the highest concentration of all of the quantified volatiles, and the emulsion with fish and rapeseed oil mixture [F + RN(2)] as well as those with AP (F + AP, F + AP + T, F + AP + EDTA, and F + AP + T + EDTA) had the lowest concentrations.

Sensory Analysis. Although a weak tendency to increased fishy taste could be discerned during storage for F + T + EDTA milk, there was generally no worsening of the fishy taste during storage. Hence, in all milks, the fishy taste seemed to mainly be formed during the production (**Table 5**). This finding was in disagreement with the findings in experiment 1, where fishy off-flavor developed during storage in milks without rapeseed oil. The results of the sensory evaluations also showed that the emulsions with and without AP could clearly be distinguished regarding fishy odor and taste of the emulsions, which developed similarly during storage (**Table 5**). After 8 days of storage, the emulsions containing fish oil, fish oil and EDTA, fish oil and tocopherols, and fish oil, EDTA, and tocopherols (F, F + T, F + EDTA, and F + T + EDTA) had significantly more fishy odor and taste than the emulsions containing fish oil and AP in any combination. After 1 day of storage, these four emulsions with AP had significantly higher intensity of fishy taste than the emulsion with fish and rapeseed oil, but after 8 days of storage, all five emulsions had an equal fishy taste.

There was a significant difference between the emulsions containing fish oil and natural rapeseed oil in the two experi-

Table 4. Peroxide Values [meq/kg] of the Milk Emulsions Containing the Different Combinations of Antioxidants during Storage (Emulsions Listed According to Their PV Level)[a]

	day 1	day 4	day 7	day 11
F + RN(2)	2.3 ab, x ± 0.1	0.4 a, u ± 0.0	1.7 a, v ± 0.1	1.5 a, v ± 0.4
F + AP	2.4 ab, x ± 0.2	0.5 a, u ± 0.1	2.0 a, vx ± 0.2	1.7 a, v ± 0.3
F + AP + T	2.1 a, v ± 0.4	0.5 a, u ± 0.0	1.5 a, v ± 0.1	1.5 a, v ± 0.2
F + AP + EDTA	1.7 a, v ± 0.1	0.9 a, u ± 0.1	1.5 a, v ± 0.0	1.4 a, v ± 0.1
F + AP + T + EDTA	1.9 a, v ± 0.3	0.5 a, u ± 0.3	1.9 a, v ± 0.0	1.4 a, v ± 0.2
F + EDTA	2.9 b, u ± 0.5	2.4 b, u ± 0.0	5.2 b, v ± 0.2	7.3 b, x ± 0.0
F	3.8 c, v ± 0.7	2.4 b, u ± 0.3	5.8 bc, x ± 0.2	7.6 b, y ± 0.3
F + T + EDTA	2.3 ab, u ± 0.2	2.5 b, u ± 0.0	6.2 c, v ± 0.0	8.4 c, x ± 0.2
F + T	4.2 c, u ± 0.4	3.8 c, u ± 0.1	8.1 d, v ± 0.1	12.3 d, x ± 0.1

[a] Emulsion names refer to **Table 1**. F = fish oil, RN = natural rapeseed oil, AP = ascorbyl palmitate, T = tocopherol, EDTA = calcium disodium ethylenediaminetetraacetate.

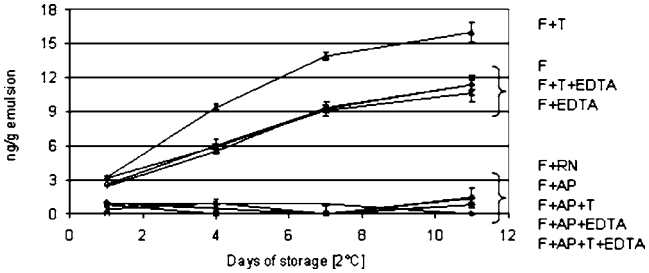

Figure 3. Concentration of (*E*,*E*)-2,4-heptadienal [ng/g emulsion] determined by dynamic headspace GC–MS analysis. Emulsion names refer to **Table 1**.

Table 5. Results of the Sensory Evaluation of Fishy and Milky Taste [0–9] of the Emulsions Containing the Different Antioxidant Combinations

	fishy taste[a]			milky taste[b]		
	day 1	day 4	day 7	day 1	day 4	day 7
F + RN(2)	0.4 a	0.4 a	0.9 a	4.0 c	4.5 c	3.5 ab
F + AP	1.5 ab	0.8 a	1.0 a	3.7 c	4.1 c	3.9 ab
F + AP + T	1.7 b		1.0 a	3.6 bc		4.1 b
F + AP + EDTA	1.8 b	1.1 a	1.0 a	3.5 abc	3.8 c	3.7 ab
F + AP + T + EDTA	1.8 b	1.2 a	1.1 a	3.4 abc	3.6 bc	3.8 ab
F + EDTA	3.1 c	2.9 b	3.4 b	2.4 a	2.7 ab	2.8 ab
F	3.0 c	3.1 b	3.3 b	2.6 ab	2.6 a	2.6 ab
F + T + EDTA	2.5 c		3.8 b	3.0 abc		2.7 ab
F + T	3.4 c	3.8 b	3.7 b	2.5 ab	2.4 a	2.3 a

[a] Average standard deviation of 1.1. [b] Average standard deviation of 0.9. Emulsion names refer to **Table 1**.

ments described in the present work [F + RN(1) versus F + RN(2)]. In the first experiment, natural rapeseed oil was added to the fish oil just before emulsification. In the second experiment, we used an oil mixture, where the rapeseed and the fish oil had been deodorized together and thus also stored as a mixture before emulsion preparation. This emulsion was significantly less oxidized and fishy than the emulsion prepared in the first experiment. The results obtained in the second experiment are in accordance with our previous experiments, where we also used a rapeseed and fish oil mixture, which had been deodorized and stored as a mixture (*7*, *8*). In one of these studies, a consumer panel was not able to discriminate the milk containing a mixture of fish oil and rapeseed oil from milk without fish oil (*7*). The fishy taste of the milk containing fish oil and rapeseed oil in that study was similar to the fishy taste observed in the present study. On the basis of the present data, we are, however, not able to assess whether a consumer panel would be able to discriminate milk samples with AP, which had slightly higher fishy taste scores than the milk with fish oil and rapeseed oil, from milk without fish oil.

Overview of Results from Experiment 1 and 2 by PARAFAC Modeling. PARAFAC modeling was employed to provide an overview of the overall results obtained in the two first storage experiments. The model was based on the PV, volatile concentrations, and the sensory residuals, as described previously and illustrated in **Figure 1**. Investigations of core consistency plots and also cross-validation showed that a two-component model was optimal. The scores and loadings of this two-component model are presented in **Figure 4**.

In the loadings plot, the sensory descriptors were separated in a triangular shape. Rancid odor and taste were located to the right near the first component axis. Fishy and metallic odor and taste were located near the second component axis above the odor and taste of milk. PV, 2-pentenal, 2-penten-1-ol, and (*E*,*E*)-2,4-heptadienal were located close to the first component axis in the positive direction, while 1-penten-3-one and 2-hexenal were located above these variables. The data points for storage days moved from left to right and upward with an increasing number of days.

In the scores plot, emulsions were arranged in three different areas, which reflected the location of the sensory descriptors in the loadings plot. Furthest to the right were the emulsion with chromatographed rapeseed oil (RC) and, to the left of this, the emulsion with chromatographed rapeseed oil and fish oil (F + RC). Above the origin along the second component axis were located emulsions from both experiments. The emulsion with fish oil and those with fish oil, tocopherols, and/or EDTA were located together with the emulsion with chromatographed rapeseed oil [F(2), F + T(1), F + T(2), F + EDTA, F + T + EDTA, F + RC + T]. Below these were the emulsions with fish oil and with fish oil and rapeseed oil from the first experiment [F(1), F + RN(1)]. Finally, all of the emulsions containing AP in any mixture and the emulsion with rapeseed and fish oil mixture [F + RN(2)] were clustered in one group in the third quadrant.

This distribution of emulsions first of all showed that emulsions with chromatographed rapeseed oil with and without fish oil separated clearly in the first component direction. In comparison to the loadings plot, this separation was based on their higher PV, volatile concentrations, and their rancid taste and odor, and these parameters increased in concentration or intensity during storage. The second component seemed to separate emulsions with AP and fish and rapeseed oil mixture from the remaining emulsions with fish oil, tocopherols, or EDTA. This separation was mainly based on differences between fishy and metallic odor and taste and milky odor and taste of the emulsions. Additionally, the emulsions with fish

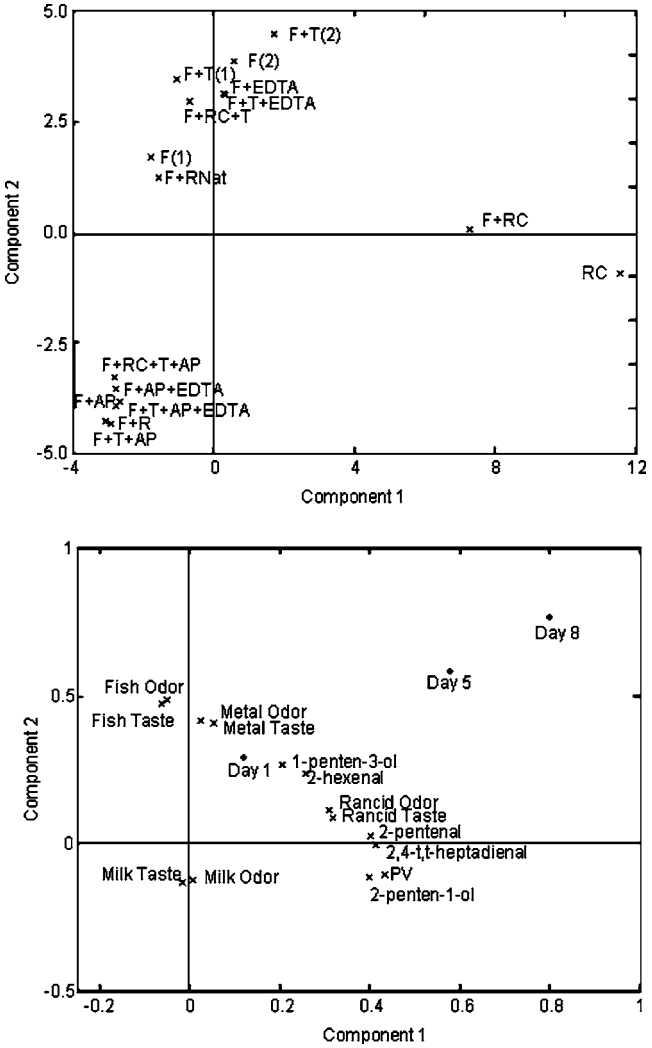

Figure 4. Plot of scores and loadings in the PARAFAC model (component 1 versus component 2). The loadings of the PARAFAC model are illustrated for both the second mode (chemical and sensory data) and the third mode (storage days). Emulsion names refer to **Table 1**.

oil and with fish oil and rapeseed oil from the first experiment were located together, indicating that addition of rapeseed oil (with a PV = 0.3 meq/kg) to fish oil (PV = 0.2 meq/kg) just before emulsification had only a minor influence on oxidation. Both emulsions with fish oil and tocopherols [F + T(1) and F + T(2)] were located above their respective emulsions without tocopherols [F(1) and F(2)], indicating the slight prooxidative effect of tocopherols when added to fish oil alone before emulsification.

Effects of Antioxidants. *Tocopherols.* The hypothesized protective effect of tocopherols added to the stripped rapeseed oil was clearly shown by comparing PV and concentrations of volatiles of the emulsions containing the chromatographed rapeseed oil and fish oil with and without tocopherols (F + RC, F + RC + T, **Table 3**). Despite an increased PV of the highly unstable chromatographed rapeseed oil, the PV or the concentration of volatiles in the resulting emulsion with tocopherol (F + RC + T) was not significantly different from the emulsion containing the natural rapeseed oil throughout the storage period [F + RN(1)].

A prooxidant effect of adding tocopherols directly to fish oil was also indicated in both experiments, as increasing PV, levels of volatiles, and increasing fishy off-flavor were observed.

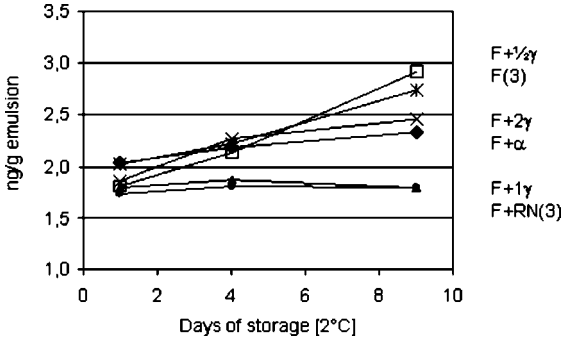

Figure 5. Concentration of (*E*,*E*)-2,4-heptadienal [ng/g emulsion] determined by dynamic headspace GC–MS analysis. Emulsion names refer to **Table 1**.

Several experiments have shown an inversion of the antioxidative effect of mainly α-tocopherol, when used in high concentrations in bulk oils and emulsions (*30*−*32*). In the present experiment, the total concentration of natural and added α-tocopherol in the fish oil was 590 ppm (F + T in both storage experiments). We therefore hypothesize that, together with the γ-tocopherol, the total α-tocopherol concentration in the emulsions might have reverted the antioxidative properties of the α-tocopherol and thus have exerted prooxidative effects on both PV and volatiles during storage. In experiment 3, we investigated the individual effects of the α- and γ-tocopherol isomers in fish-oil-enriched milk. These results indicated that γ-tocopherol added in the concentration determined in the natural rapeseed oil (330 ppm) most efficiently inhibited oxidation of the fish oil in the milk emulsions (**Figure 5**). γ-Tocopherol in half of the concentrations of the natural level (165 ppm) did not inhibit oxidation, while doubling the concentration (660 ppm) inhibited oxidation slightly. The α-tocopherol added in half of the concentrations of that found in the rapeseed oil (110 ppm) also inhibited oxidation slightly. These results could indicate that γ-tocopherol was the most important tocopherol isomer present in the rapeseed oil regarding antioxidative activity.

Ascorbyl Palmitate. All emulsions containing AP, regardless of any other antioxidant addition, were equally stable. The prooxidant effect observed when the tocopherols were added alone was therefore also prevented by the AP. It thus seemed that the presence of AP in the emulsions almost completely blocked oxidation, and apparently, AP did not show any interactive effects with EDTA or tocopherols. In the literature, it has been hypothesized that the prooxidative effect of high concentrations of tocopherols in fish oils is caused by an increased concentration of the tocopheroxyl radicals, which may take part in and enhance the propagation of the lipid autoxidation (*33*, *34*). It has subsequently been proposed that the synergistic effect of AP with tocopherols is based on AP regenerating tocopherols by donating a hydrogen to a tocopheroxyl radical, thus preventing the tocopheroxyl radicals from propagating the autoxidation (*31*). However, it has also been shown that AP acts as an antioxidant in emulsions based on corn oil stripped from tocopherols and thus has an independent antioxidative potential, which possibly results from the radical-scavenging potential of the AP itself (*12*, *16*, *17*). The fish oil used in these experiments contained 330 ppm α-tocopherol, and therefore, the emulsions with added AP only did in fact contain a substantial amount of tocopherol (**Table 1**). Therefore, we could not distinguish whether the effect of AP, including the inhibition of the prooxidant effect of the tocopherols by AP, was based on regeneration of the tocopherols or an independent radical-scavenging effect of the AP.

As previously mentioned, oxidation in o/w emulsions is likely to occur at the interface between the oil and water phase (*4*). Additionally, it has also been proposed that hydroperoxides, especially those from long-chain PUFA, which are slightly more polar than the lipids, also move toward the interface (*35*). The hydrophobic palmitic acid tail of the ascorbic acid makes AP an oil-soluble amphiphilic molecule. In the present emulsions of fish-oil-enriched milk, we suggest that it is likely that AP would orient itself toward the interface of the fat globules. Hence, AP will most likely be located closer to the interfacial region than tocopherols and thus closer to where oxidation occurs, which support the stronger antioxidative effect of AP compared to tocopherols observed in this study.

EDTA. In contrast to tocopherols and AP, EDTA is a water-soluble molecule and the antioxidative mechanism is based on metal chelation of trace metals in the aqueous phase. In milk, the trace metals iron and copper are mainly bound to soluble proteins, caseins, citric acid, and membrane proteins (*36*). In the present storage experiment, no antioxidative effects of EDTA were observed. This indicated either that EDTA was not able to bind the metals sufficiently to prevent them from mediating the degradation of the lipid hydroperoxides or that the trace metals present in the milk were not promoting the oxidation. We have previously observed a protective effect of EDTA in a fish-oil-enriched milk emulsion (*10*). We suggest that the lack of an effect of EDTA in the present study is a result of a high oil quality (i.e., a low PV), which made the oil in the stable emulsions less susceptible to trace-metal-mediated degradation of hydroperoxides.

In the present work, a molar ratio of EDTA/iron of 3.4−4.5:1 was used. Contradicting results on the optimum EDTA/iron ratio have been reported in the literature because some studies suggest that ratios higher than 1:1 are required, whereas other studies have shown that lower ratios such as 1:2 were sufficient (*38*, *39*). Overall, it seems that the optimum ratio between EDTA and iron depends on the composition of the particular emulsion systems. More data are thus required to predict the necessary concentration of EDTA required to retard lipid oxidation in different food systems. However, our results indicated that the lack of effect of EDTA in the present experiment was less likely to be caused by a concentration of EDTA too low to affect oxidation.

Comparison of Tocopherol, AP, and EDTA Effects. Overall very different effects were observed for the different antioxidants employed in these experiments, and most obviously, AP retarded oxidation during storage almost completely. Because AP worked irrespective of addition of extra tocopherols, it seems that the antioxidative mechanism may be both direct scavenging of free radicals and tocopherol regeneration. Metal chelation is less likely. Similarly, tocopherols, which are more efficient radical chain-breaking antioxidants than AP did not show any antioxidant activity in the emulsions based on fish oil alone when both α- and γ-tocopherol were added together. Rather, a slightly prooxidant activity was observed, which could be caused by too high concentrations of the tocopherols (*30*, *32*). However, when added individually, it seemed that the γ-tocopherol isomer was more efficient as an antioxidant in these milk emulsions. The γ-tocopherol isomer seemed to be inhibiting oxidation to a similar extent as AP, although more work would be needed to clarify the antioxidative properties of γ-tocopherol compared to α-tocopherol in emulsions. When these and previous results were taken together, they have shown that (1) it is possible to incorporate 0.5% omega-3 rich fish oil into milk and obtain a satisfactory sensory quality and shelf life and (2) protection of fish-oil-emulsified milk may be accomplished via different antioxidant mechanisms.

ABBREVIATIONS USED

AP, ascorbyl palmitate; EDTA, calcium disodium ethylenediaminetetraacetate; T, tocopherol; RN, rapeseed oil natural; RC, rapeseed oil chromatographed; F, fish oil.

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