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Oxidation of Cod Liver Oil during Gastrointestinal in Vitro Digestion

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ABSTRACT: Oxidation of cod liver oil rich in long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) was investigated during a gastrointestinal (GI) in vitro digestion. The digestion stimulated TBA-reactive substances (TBARS) formation in both the gastric and intestinal steps, whereas levels of lipid hydroperoxides remained nearly constant. The presence of digestive compounds was decisive for the TBARS development because TBARS did not change when the cod liver oil was subjected only to the temperature and pH gradient of the GI model. Preformed oxidation products in the cod liver oil resulted in further elevated TBARS levels during the digestion. Addition of hemoglobin (11.5 μM) to emulsified cod liver oil dramatically increased TBARS and lipid hydroperoxide levels during GI digestion, whereas 1 mg α-tocopherol/g oil did not show any protection against oxidation. Specific concern thus needs to be taken in the design of foods containing LC n-3 PUFA to preserve these lipids and avoid harmful oxidation, both before and after consumption.

KEYWORDS: *n-3 polyunsaturated fatty acids, fish oil, lipid oxidation, gastrointestinal, digestion, TBARS, lipid peroxidation, cod liver oil, hemoglobin*

INTRODUCTION

Fish oils rich in long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) have been associated with reductions in cardiovascular diseases (CVD),[1] inflammation,[2] and cancers.[3] However, evidence has not been conclusive, and, in fact, several meta-analyses[4−6] have shown no or limited effects of fish oil on sudden death, markers for CVD, total mortality, combined cardiovascular events, or cancer. In 2006, Turner et al.[7] were among the first to highlight how these contradictory results could be due to lipid oxidation and discussed how preformed oxidation products in fish oils could nullify the beneficial effects of LC n-3 PUFA, for example, on anti-inflammatory, endothelial, and vascular functions.

Besides lipid free radicals and lipid hydroperoxides, the oxidation products that can form in a polyunsaturated lipid system such as fish oil include certain biologically reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-hexenal (HHE); the latter is a product specific to n-3 PUFA. MDA and HHE are ascribed cytotoxic, mutagenic, and neurotoxic properties and can promote cancer development in the gastrointestinal (GI) tract and liver,[8,9] two regions that usually are exposed to higher levels of oxidation products than plasma and body tissues.[10]

To date, nearly all research on fish lipid oxidation has focused on changes taking place in the food or oil *before* their intake, for example, during processing and storage. Positive and negative health effects of fish oils and their degradation products, respectively, have therefore mainly been discussed in relation to levels found in food products prior to consumption. On the basis of scattered publications in the past 10−15 years, it appears, however, as if lipid oxidation does not stop at the point of ingestion. Halliwell et al.,[10] for example, described the GI tract as pro-oxidative due to the presence of iron and copper ions, heme proteins, preformed oxidized lipids, reactive nitrogen species, hydrogen peroxide, sulfite, and nitrite either entering from ingested food or secreted by digestive organs. In combination with the low pH of the stomach, the action of digestive enzymes, and the disintegration of the food structure, the GI tract appears to be a reactive environment promoting further lipid oxidation during digestion. Gastric oxidation has also been highlighted by Kanner and Lapidot,[11] who described the stomach as a bioreactor. These authors, and later also others,[12−17] have followed lipid peroxidation in turkey meat and linoleic acid emulsions during a simulated gastric digestion step. For example, the influence of certain dietary pro- and antioxidants, such as metmyoglobin,[11,14,15] free iron,[11,15] β-carotene,[11,15,16] α-tocopherol,[16] ascorbic acid,[16] red wine,[11,15] catechin,[11,14,15] and food-derived melanoidins[12,17] has been evaluated. It could be concluded from these studies that lipid oxidation is accelerated during gastric conditions and that co-oxidation of important vitamins occurs.

To the best of our knowledge there is no literature describing the oxidation of fish oil during GI digestion and also none focusing on oxidation in both the gastric and intestinal parts of the digestive tract. Although the pH is raised and the oxygen pressure is further reduced in the intestinal step, the bile-induced increase in lipid droplet surface area and the contact with certain tentatively pro-oxidative bile acids[18] make also this step relevant from an oxidation perspective. In this study, the aims have been to evaluate whether cod liver oil oxidizes during GI in vitro digestion and, if so, whether the oxidation primarily occurs in the gastric step or continues in the intestinal step as well. Furthermore, we have studied how GI oxidation is influenced by the individual digestive compounds, initial oil quality, pre-emulsification, and two dietary oxidation modulators (hemoglobin and α-tocopherol). Lipid oxidation was followed in terms of lipid hydroperoxides and TBARS.

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MATERIALS AND METHODS

Chemicals. Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, bile extract from porcine, and lipase F-AP15 from *Rhizopus oryzae* were all purchased from Sigma-Aldrich (Stockholm, Sweden). All other chemicals used were of analytical grade.

Cod Liver Oil. Refined nonstabilized (without added antioxidants) cod liver oil was supplied by LYSI hf. (Reykjavík, Iceland). Briefly, the oil contained 30% (w/w) PUFA, 52% monounsaturated fatty acids (MUFA), 18% saturated fatty acids (SFA), and 0.04% free fatty acids (FFA) (according to the manufacturer). According to Jonsdottir et al.,[19] who performed detailed fatty acid analyses of this oil, the eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA) contents contributed 8.9, 11.4, and 1.3%, respectively, of total fatty acids. The total LC n-3 PUFA content was 24%. To elucidate the influence from preformed oxidation products on GI oxidation, oxidized cod liver oil was produced by storing the oil in an Erlenmeyer flask for 13 days at room temperature. The oxidized oil hereby obtained an initial TBARS value of 22 μmol/kg oil, whereas the fresh oil had 4 μmol/kg oil. The corresponding amounts of lipid hydroperoxides were 1423 μmol/kg oil (2.8 mequiv/kg oil) and 13529 μmol/kg oil (27 mequiv/kg oil), respectively.

Emulsion Preparation. Oil-in-water (o/w) emulsions were prepared by mixing 4 g of cod liver oil with 16 g of emulsifier solution (17 mM Brij 35, 10 mM phosphate buffer, pH 7.0), giving a final lipid concentration of 20% (w/w). The mixture was homogenized at 24000 rpm for 2 min (Ultra Turrax, model T18 basic, IKA Works, Wilmington, NC, USA) followed by ultrasonication for 5 min at output level 3 and 50% duty cycles (Branson Sonifier 250, Danbury, CT, USA). Emulsions were freshly prepared prior to each in vitro digestion experiment and kept on ice during preparation.

Gastrointestinal in Vitro Digestion. To study the oxidation of cod liver oil during in vitro simulated gastrointestinal digestion, nonemulsified and emulsified oils were subjected to a modified version of the digestion model described by Svelander et al.[20] This is a two-step GI model simulating the stomach and the upper part of the small intestine. One gram of emulsion or 0.2 g of pure oil was mixed with 3 mL of gastric solution (3 g NaCl L^{-1}, 1.06 g KCl L^{-1}, 1.47 g $CaCl_2 \cdot 2H_2O$ L^{-1}, 0.47 g KH_2PO_4 L^{-1}, 0.74 g $MgCl_2 \cdot 6H_2O$ L^{-1}, 2.76 g lipase L^{-1} (40.8 U/mg), and 8.82 g pepsin L^{-1} (1064 U/mg protein)) in a 14 mL screw-capped glass tube. To simulate the initial part of the stomach the samples were adjusted to pH 4.0 with 0.1 M $NaHCO_3$ or 0.1 M HCl. Samples were incubated at 37 °C and agitated on an orbital shaker (400 rpm). After 30 min of incubation, the pH was lowered to 2.0 with 1 M HCl and incubation continued for 30 min. To simulate the small intestinal phase, the pH was raised to 6.9 with 1 M $NaHCO_3$ and 0.6 mL pancreatin/bile extract solution was added (4.0 g pancreatin L^{-1} and 25 g bile extract L^{-1} dissolved in water). Samples were flushed with nitrogen gas for 15 s, vortexed for 15 s, and then flushed again with nitrogen gas for 15 s. Samples were incubated for 2 h in the small intestinal step and thereafter frozen at −80 °C until extraction. For the study with pure bile acids, the bile extract was replaced by a mixture of sodium bile acid salts giving a final concentration in the small intestinal phase of 10 mM bile acids. The mixture was the same as that used by Mel'nikov et al.[21] to simulate human bile based on its six major bile acids: 2.49 mM glycocholate, 2.49 mM glycochenodeoxycholate, 1.66 mM glycodeoxycholate, 1.25 mM taurocholate, 1.25 mM taurochenodeoxycholate, and 0.83 mM taurodeoxycholate. A small modification was made in that 0.03 mM taurolithocholate was also added. This bile acid contributes only to 0.3% of the total bile acids in humans, but was previously identified as the most potent among a series of bile acids tested for their pro-oxidative capacity.[18,22] By using Mel'nikov's mixture with the addition of taurolithocholic acid, 96.3% of the amount of the originally proposed bile acids was covered. To ensure that micelles would form in the intestinal step, emulsified cod liver oil was used in this trial.

Oxygen Concentration Analyses. Oxygen concentration in the headspace of the glass tubes used for in vitro digestion was measured with a PBI Dansensor (CheckMate 11, Ringsted, Denmark). Results are expressed as percent.

Extraction of Lipids and TBARS. Samples taken at the start and after completed gastric and intestinal digestion were subjected to a chloroform/methanol extraction. The entire sample, 4−5 mL, was vortexed with 15 mL of ice-cold chloroform/methanol (1:2, with 0.05% butyl hydroxylated toluene (BHT)) for 60 s. Five milliliters of chloroform (with 0.05% BHT) was added, and samples were vortexed for 15 s. Phase separation was obtained by adding 5 mL of 0.5% NaCl followed by 30 s of vortexing. For start and stomach samples made from nonemulsified oil, the extraction volumes were reduced by 20% due to the lower sample volumes (∼3.2 mL). Finally, samples were centrifuged at 2000*g* for 6 min at 4 °C, and aliquots from both phases were withdrawn and kept at −80 °C until analysis. The chloroform and methanol/water phases were used for the determination of lipid hydroperoxides and TBARS, respectively.

TBARS Measurement. TBARS were determined in the methanol/water phase obtained after the chloroform/methanol extraction as described by Schmedes and Hølmer.[23] 1,1,3,3-Tetraethoxypropane (TEP) was used to prepare a standard curve for quantification. Results were expressed as micromoles of MDA per kilogram of lipid.

Lipid Hydroperoxides Measurement. To determine the amount of lipid hydroperoxides in the chloroform extract obtained after the chloroform/methanol extraction, the ferrithiocyanate method was used as described by Undeland et al.[24] Quantification was done using a standard curve made from cumene hydroperoxide. Results were expressed as micromoles of peroxide per kilogram of lipid.

Free Fatty Acid Measurement. Development of free fatty acids was followed during the static in vitro digestion of nonemulsified oil. The same chloroform/methanol extraction ratio as described above was used but with the modification that start and intestinal samples were acidified with 1 M HCl to pH 1−2 to ensure that the fatty acids partitioned into the chloroform phase. The chloroform phase was directly evaporated under nitrogen for 30 min, and the extracted lipids were redissolved in 1 mL of hexane/acetic acid (99:1). Neutral lipids and free fatty acids were then separated and detected using the HPLC method described by Silversand and Haux.[25]

Transition Metal Analyses. Iron and copper content were determined in digested cod liver oil by ion chromatography following the method of Fredrikson et al.[26] A microwave digestion (Milestone microwave laboratory system EthosPlus, Sorisole, Italy) was done by mixing the digested sample, 0.75 mL of concentrated HNO_3, and 0.15 mL of HCl in a Teflon vial. The sample was digested to a transparent solution by a temperature program reaching 180 °C in 15 min, and this temperature was then kept for 20 min. After cooling, the sample was decanted into a test tube and diluted to a final volume of 10 mL. Ascorbic acid, 0.1 mL (20 mg/mL), was added to 0.9 mL of the digested sample before analysis. The ion chromatography method is based on the formation of mineral complexes by pyridine-2,6-dicarboxylic acid in the mobile phase. The complexes are then postcolumn derivatized with 4-(2-pyridylazo)resorcinol (PAR), resulting in mineral−PAR complexes that are detected by UV−vis absorption at 500 nm.

α-Tocopherol Measurement. The endogenous amount of α-tocopherol in the cod liver oil was determined by high-performance liquid chromatography.[27] Prior to injection, 0.4 g of oil was dissolved in 5 mL of chloroform followed by 50 times dilution in methanol. The α-tocopherol peak was quantified against a DL-α-tocopherol standard (Calbiochem, an affiliate of Merck KGaA, Darmstadt, Germany). Results were expressed as milligrams of α-tocopherol per kilogram of oil of three replicates.

Hemolysate Preparation and Hemoglobin (Hb) Quantification. Blood was obtained from freshly slaughtered farmed Atlantic cod (*Gadus morhua*). As described by Richards and Hultin,[28] a hemolysate was then prepared from the blood, and its Hb concentration was quantified against a bovine Hb standard following conversion to the CO form.

Statistics. Separate digestions were done in triplicates and analyzed in duplicates if not otherwise specified. Statistical analysis was carried out using IBM SPSS statistics 19 (IBM Corp., NY, USA). Differences between groups were determined by ANOVA using Duncan's

multiple-range test as post hoc test with $p = 0.05$ as the significance level.

RESULTS AND DISCUSSION

Effect of the Different Digestive Phases and the Digestive Compounds on the Oxidation of Cod Liver Oil. To study lipid oxidation in cod liver oil during GI digestion, individual digestive compounds as well as the different digestive phases were evaluated in more detail. The TBARS and hydroperoxide results are shown in Figure 1,

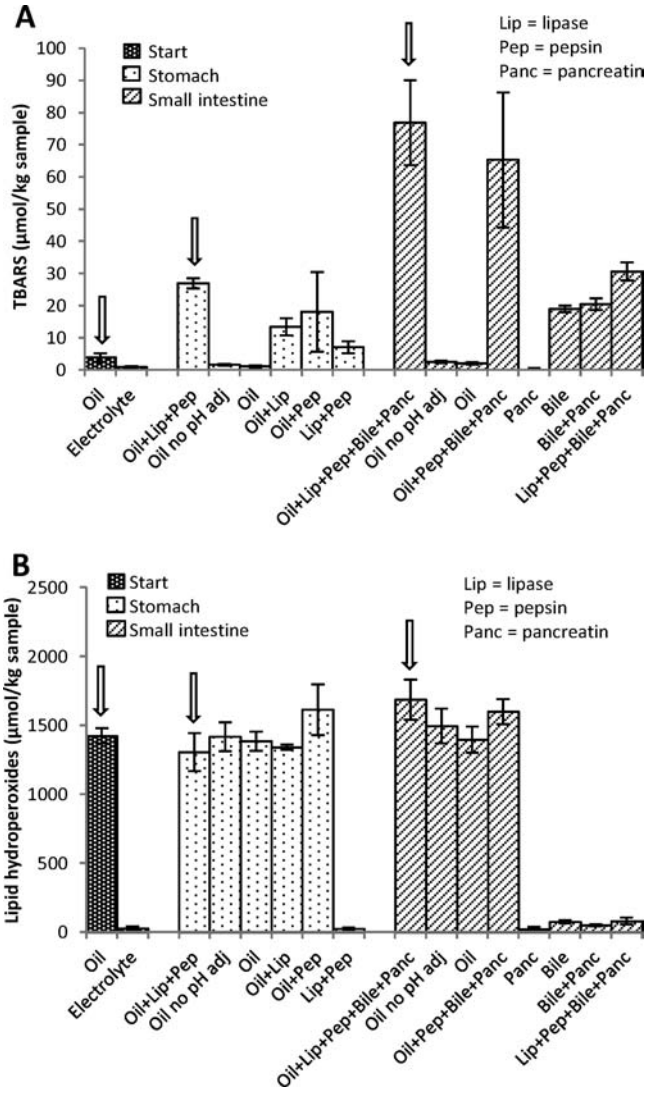

Figure 1. Effect of specific digestive compounds and the combination thereof on (A) TBARS and (B) lipid hydroperoxide formation in cod liver oil during GI digestion. Oil samples subjected to the standard digestion protocol are indicated with an arrow. Contributions of pure digestive compounds without oil to TBARS and lipid hydroperoxides are also shown. Values are the mean ± SD of three to six replicates. Relevant statistical differences ($p < 0.05$) are mentioned in the text.

panels A and B, respectively. Upon digestion of cod liver oil according to the complete digestion protocol (indicated by arrows in Figure 1), TBARS increased gradually in both the gastric and intestinal steps. To exclude that this was an effect solely from storage of unsaturated oil at elevated temperature (37 °C), TBARS were also measured in oils stored for up to 3 h at 37 °C without any addition of digestive compounds or pH adjustments ("Oil no pH adj"). In this case, no significant increase in TBARS was observed. The same was true when this experiment was combined with the pH gradient of the GI model ("Oil"). Thus, the addition of the digestive compounds was clearly responsible for the TBARS increase, which was a basis for looking deeper into the individual contribution from these compounds. In the gastric phase the digestion compounds added were lipase and pepsin, and in the intestinal phase, bile and pancreatin. When following these compounds in the *absence* of oil, it was noted that they gave a certain response in the TBARS test, especially in the intestinal phase ("Lip+Pep +Bile+Panc"). Because of the latter, bile and pancreatin, both alone ("Panc" and "Bile") and together ("Bile+Panc"), were also studied. The results showed that among these two compounds, bile was the main contributor to the TBARS response. The observed impact of the bile extract on TBARS values is further discussed in the text below in relation to the results shown in Figure 2.

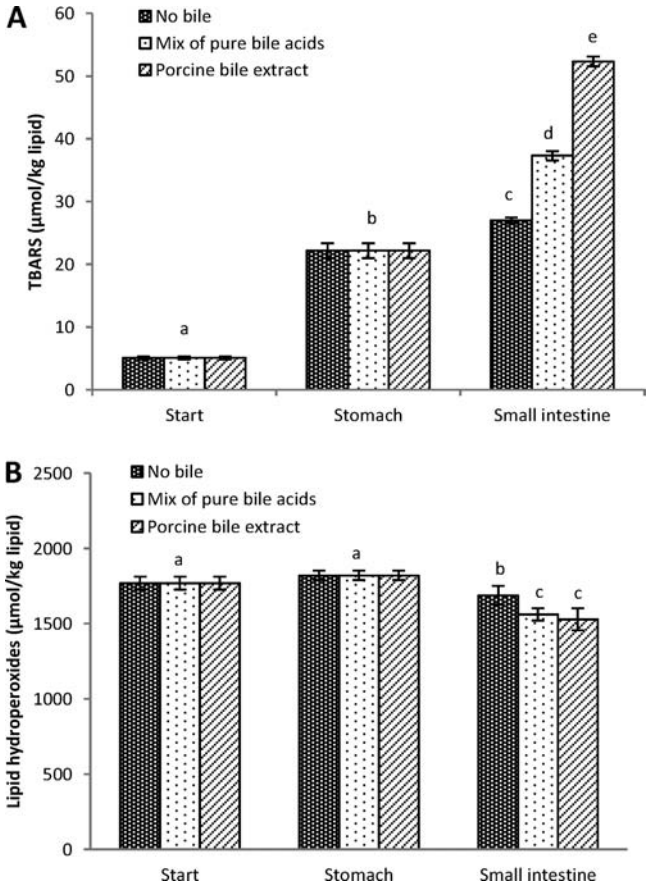

Figure 2. Effect of bile acids on (A) TBARS and (B) lipid hydroperoxide formation in emulsion (20% cod liver oil) during GI digestion. Samples are treated the same up to the intestinal phase. Values are the mean ± SD of four replicates. Bars with different letters indicate statistical differences ($p < 0.05$).

To study whether the development of TBARS in the gastric step was linked to lipase, one sample was "digested" in the absence of lipase (called "Oil+Pep" in the gastric phase and "Oil+Pep+Bile+Panc" in the intestinal phase). The exclusion of lipase caused a very small, nonsignificant TBARS reduction compared to the oil sample digested according to the complete protocol. In accordance with this, the oil sample having only

lipase added ("Oil+Lip") attained higher TBARS values in the gastric step than the oil sample incubated without the addition of any digestion compunds ("Oil").

As shown in Figure 1B, the oil sample subjected to the full GI digestion protocol did not change a lot with respect to the content of hydroperoxides (i.e., primary products of lipid oxidation), even though a small significant increase was seen after the intestinal step. Also with regard to the lipid hydroperoxides, it was verified that the oil without digestion compounds added ("Oil" and "Oil no pH adj") did not change during the 3 h incubation at 37 °C. Furthermore, the full mixture of digestion compounds alone did not contribute to the hydroperoxide data, and when the oil was subjected to the individual digestion compounds, no clear effect on hydroperoxides was seen. The relatively stable hydroperoxide levels, viewed against the differences in TBARS values, of oil samples incubated with different combinations of digestive compounds (Figure 1) indicate that the buildup and decomposition of lipid hydroperoxides may have taken place at nearly the same rates. Lipid hydroperoxide decomposition under stomach conditions has been reported previously.[29] It should be noted, though, that the concentrations of lipid hydroperoxides measured in the oil throughout the digestion were much higher than the concentrations of TBARS reached, indicating that only a small fraction of the hydroperoxides were converted to TBARS. It is, however, also likely that some of the highly reactive compounds responding in the TBARS test are subjected to further reactions and, thus, do not accumulate.

On the basis of the significant contribution from the commercial bile extract to TBARS values in the intestinal step (Figure 1A), a separate set of experiments was performed in which a mixture of pure bile acids was compared with the commercial bile extract (Figure 2). The mixture was designed to mimic human bile, which is known to differ in composition from porcine bile. This aspect was considered to be important because it has previously been shown that individual bile acids can affect lipid oxidation very differently.[18] The results in Figure 2A show that the TBARS values in the intestinal step with the commercial porcine bile extract added were higher than with the pure bile acid mixture. However, it can also be seen that the pure bile acid mixture elevated the TBARS values compared to the sample with no bile acids added. It was confirmed that this elevation was not due to the bile acid mixture per se responding in the TBARS test (data not shown), and it can thus be ascribed to an increase in lipid oxidation. With regard to hydroperoxides (Figure 2B), a small reduction was found in the intestinal step, especially with bile acids or bile extract added. This reduction indicates that breakdown of hydroperoxides into secondary products responding in the TBARS test was stimulated by bile components. Terao et al.[30] showed that a small volume of bile−pancreatic juice from rats containing ∼2 mM bile acids increased TBARS formation during Fe^{2+}-induced oxidation of soybean phosphatidylcholine under simulated intestinal conditions. The same study also showed that TBARS levels increased 2-fold when the effect of ∼4−22 mM sodium deoxycholate was tested and that bile−pancreatic juice could decompose free fatty acid hydroperoxides. It should be noted that in the study by Terao et al.,[30] samples were not subjected to a simulated gastric step prior to addition of bile pancreatic juice or deoxycholate. Using phosphatidylcholine liposomes or an arachidonate solution, it was observed that the hydrophobic bile acid taurodeoxycholic acid (TDC) could enhance the pro-oxidative effect of ferrous iron (100 μM), but TDC was not pro-oxidative in itself.[22] The hydrophilic bile acid tauroursodeoxycholic acid (TUDC), on the other hand, showed antioxidant properties. When tested in a hepatic homogenate and in isolated cell membranes,[18] all bile acids seemed to be pro-oxidative per se, particularly those conjugated with taurine. Only a small enhancing effect on iron-induced oxidation (100 μM $FeCl_3$) was seen by some bile acids. Both studies showed highest pro-oxidative effect with the hydrophobic taurolithocholic acid.[18,22] Suggested explanations for the pro-oxidative activity of bile acids include the interaction between the bile acid and iron enabling the iron ion to promote peroxidation at the lipid membrane or alternatively increasing the permeability of membrane to iron ions.[22] Bile acids might also have an indirect pro-oxidative activity through their ability to increase the lipid surface area and, thus, the exposure to oxidants such as Fe.

That the commercial bile extract gave higher TBARS than the pure bile mixture might be explained by the fact that the former also contained phospholipids and cholesterol. Oxidation of these compounds during production and storage of the commercial bile extract could provide our samples with preformed TBARS. How the other constituents of the commercial bile extract (proteins and bilirubin) could influence oxidation is uncertain. With regard to bilirubin, some authors[31] have ascribed this compound antioxidant properties.

Effect of Different Amounts of Preformed Oxidation Products and Pre-emulsification on GI Oxidation of Cod Liver Oil. The effect of preformed oxidation products and pre-emulsification on GI oxidation of cod liver oil is shown in Figure 3. Whereas the two oxidation levels of the oil were predesigned, the small differences in oxidation levels between the emulsions (5, 8, and 12 μmol TBARS/kg oil) were natural variations induced by different emulsion preparations. The oil with 22 μmol TBARS/kg oil increased more than the fresh oil in the gastric and intestinal steps (Figure 3A). No differences were detected between the emulsions having 8 and 12 μmol TBARS/kg oil, but the one with 5 μmol developed less TBARS. When it comes to peroxide data (Figure 3B), it was only the rancid oil sample that significantly differed from the other ones. However, the starting peroxide value (PV) of this oil was about 10 times higher than the others. The rancid oil obtained lower PV during the digestion, which just like above could indicate an efficient breakdown of peroxides into secondary oxidation products under the gastric conditions. On the basis of the results in Figure 3, no clear effect of pre-emulsification of the cod liver oil could be seen in relation to oxidation under GI conditions.

Possible Influence from Lipolysis on GI Oxidation of Cod Liver Oil. To further explain the increases in TBARS seen in the gastric and intestinal steps during digestion of cod liver oil, the degree of free fatty acid formation during the digestion was recorded. The results in Figure 4 show that 43% of the initial 200 mg of triglycerides was subjected to hydrolysis after a 1 h long gastric phase, and after completion also of the intestinal phase, the corresponding figure was 68%. Thus, in the gastric and intestinal steps, about 35 and 85 mg, respectively, of free fatty acids had been formed. On the basis of these data, it cannot be excluded that the lipolysis process plays a role for the TBARS increases recorded during digestion of cod liver oil. It has previously been described how free fatty acids are more susceptible to oxidation than the intact triglycerides.[32] In addition, free fatty acids can also be pro-oxidative by attracing pro-oxidant metals and co-oxidize triglycerides.[33] The degree of

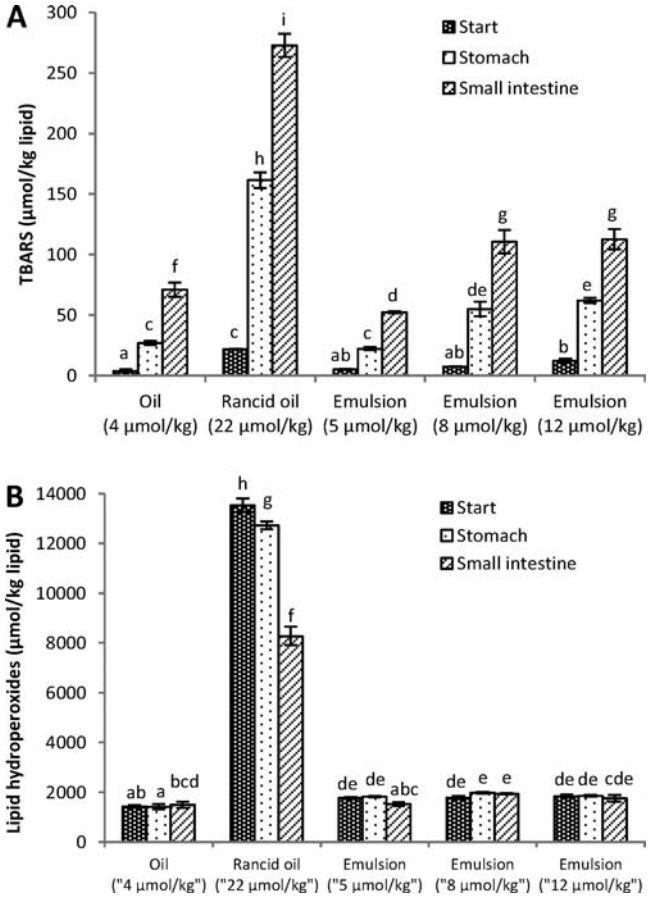

Figure 3. Effect of initial oxidative status and emulsification on (A) TBARS and (B) lipid hydroperoxide formation in cod liver oil and emulsion (20% cod liver oil) during GI digestion. Values are the mean ± SD of triplicates. In panel B, samples are identified according to their initial TBARS value as shown in panel A. Bars with different letters indicate statistical differences ($p < 0.05$).

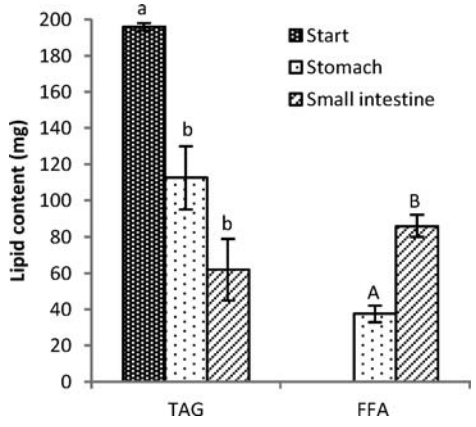

Figure 4. Amount of triacylglycerols (TAG) and free fatty acids (FFA) during GI digestion of cod liver oil. Values are the mean from two digestions, and error bars show (max−min)/2. No free fatty acids were detected in starting samples. Bars with different letters indicate statistical differences ($p < 0.05$).

gastric triglyceride lipolysis is affected, for example, by lipid droplet size, and a wide range of values (5−37%) has been reported.[34] A lipolysis degree of ∼20% was previously determined in humans after 1 h of gastric digestion of a fine emulsion; 73% was deteremined after the addition of pancreatic lipase in the duodenum.[35] An almost complete (98%) absorption of triglycerides in the small intestine of healthy humans has been reported.[34] The results obtained following our in vitro digestion protocol indicate that the degree of lipolysis in the gastric part was probably overestimated and the degree of lypolysis in the intestinal part, underestimated. Plausible reasons for the latter are insufficient incubation time, a lower micelle formation efficiency in the in vitro procedure compared with in vivo conditions, negative feedback inhibition by free fatty acids, or nonoptimal conditions for the pancreatic lipase.

Possible Influence from Trace Elements on GI Oxidation of Cod Liver Oil. Another factor that could play a role for the recorded TBARS formation in the studied GI model is the combination of low pH and the presence of trace elements. Low pH increases the solubility of trace elements, which in turn could facilitate hydroxyl radical ($^{\bullet}OH$) formation via Fenton chemistry as well as alkoxy radical ($LO^{\bullet}$) and aldehyde formation through homolytic or heterolytic lipid hydroperoxide breakdown.[36] According to the above, the literature also indicates a pro-oxidative synergy between iron and bile acids. On this basis, the presence of trace elements was measured in oil samples both prior to digestion and after the gastric and intestinal steps. The results shown in Figure 5

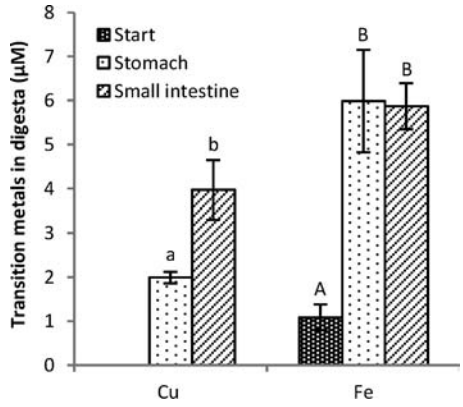

Figure 5. Concentrations of copper (Cu) and iron (Fe) in the digesta during GI digestion of cod liver oil. Values are the mean ± SD of triplicates. No copper was detected in starting samples. Bars with different letters indicate statistical differences ($p < 0.05$).

illustrate that there was a certain contribution of iron and copper from the digestive compounds, which indeed could induce the reactions described above. No copper could be detected at the start, but during digestion there was a gradual buildup by the addition of gastric and intestinal digestive compounds. Cu was present at 2 and 4 μM after the gastric and intestinal phases, respectively. Slightly higher levels were obtained for iron, and a certain amount could be found in the oil itself. In contrast to copper, the major contribution of iron originated from gastric digestive compounds (∼6 μM), and no further increase was seen in the intestinal digesta. To put the iron and copper data in the intestinal step into some perspective, it can be mentioned that they were ∼10−30 and 100%, respectively, of what can be found in herring muscle.[27] Very low concentrations of Fe^{2+} (2 μM) have been shown to catalyze peroxidation in cod roe phospholipid liposomes.[37]

Effect of Hb and α-Tocopherol on GI Oxidation of Emulsified Cod Liver Oil. To study whether the observed GI oxidation of cod liver oil emulsion was affected by well-known

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fish-derived pro- and antioxidants, Hb and α-tocopherol were selected. Hb can mediate oxidation of fish lipids by several different mechanisms and is the dominating heme-protein in both light and dark fish muscle.[28] In herring fillets, which have a relatively large proportion of dark muscle, a concentration of 11.5 μM Hb has previously been determined (unpublished data). α-Tocopherol, on the other hand, is an antioxidant naturally present in cell membranes, where it plays a role in the defense against oxidative stress. To inhibit lipid oxidation in fish oils from liver and muscle during storage, α-tocopherol or other tocopherol derivatives are frequently used. A concentration of 1 mg α-tocopherol/g oil (i.e., ∼2 μM) is commonly used in cod liver oil products aimed for human consumption.

The effect of 1 mg α-tocopherol/g oil and 11.5 μM Hb on the formation of TBARS and hydroperoxides during GI digestion of a cod liver oil emulsion is presented in Figure 6. Addition of Hb resulted in a dramatic and immediate increase in TBARS as seen from the high start values of Hb-containing samples compared to the emulsion without Hb (Figure 6A). During the digestion, TBARS was further increased and especially the gastric step was pro-oxidative. It was found that considerable amounts of TBARS also formed when the digestive compounds were excluded from the Hb-enriched oil, and in this case, it did not make a difference whether the pH-adjustment cycle was applied or not. Possible interference of Hb with the TBARS analysis was dismissed because no substantial contribution to TBARS values was seen in an Hb control without cod liver oil emulsion (data not shown).

As presented in Figure 6B the amount of lipid hydroperoxides increased largely in the Hb-fortified emulsion during the gastric step, but then decreased again after the intestinal step. The same pattern, but less pronounced, was observed for the pH-adjusted Hb sample without digestive compounds. When both digestive compounds and the pH-adjustment cycle were excluded, a gradual buildup in both steps occurred. In all of the Hb-containing samples, a substantial elevation in both lipid hydroperoxides and TBARS was observed already at the first sampling point. At pH 6, which was the starting pH of the cod liver oil emulsion, it is known from earlier studies of fish Hb, that the catalytic effect of the Hb is largely accelerated.[38] Different suggested explanations for this behavior exist. Autoxidation of Hb is accelerated at reduced pH and leads to the accumulation of the met-form ($HbFe^{3+}$),[39] which more efficiently breaks down hydroperoxides than the reduced Hb forms. Moreover, free iron and heme can be released from the metHb molecule and further stimulate lipid hydroperoxide breakdown.[40] At acidic conditions and in the presence of hydroperoxides, metHb can also be activated into a very reactive perferrylHb radical.[41] The very low pH in the gastric step (pH 2) seemed not to further activate Hb as a catalyst because application of the pH gradient to the Hb-containing emulsions deficient in digestive compounds did not further increase oxidation. Some of the digestive compounds as such thus appeared to be responsible for the additional oxidation seen in the Hb-containing sample subjected to the full digestion protocol (Figure 6).

In addition, it has been shown that mildly pepsin proteolyzed metMb had a strongly enhanced pro-oxidative effect in methyl linoleate emulsion compared to nonproteolyzed metMb and hematin.[42] It was suggested that proteolyzed Mb fragments were more nonpolar and thus favor the contact between heme iron and peroxides at the lipid/water interface enabling peroxide cleavage. Furthermore, mild Mb proteolysis seemed

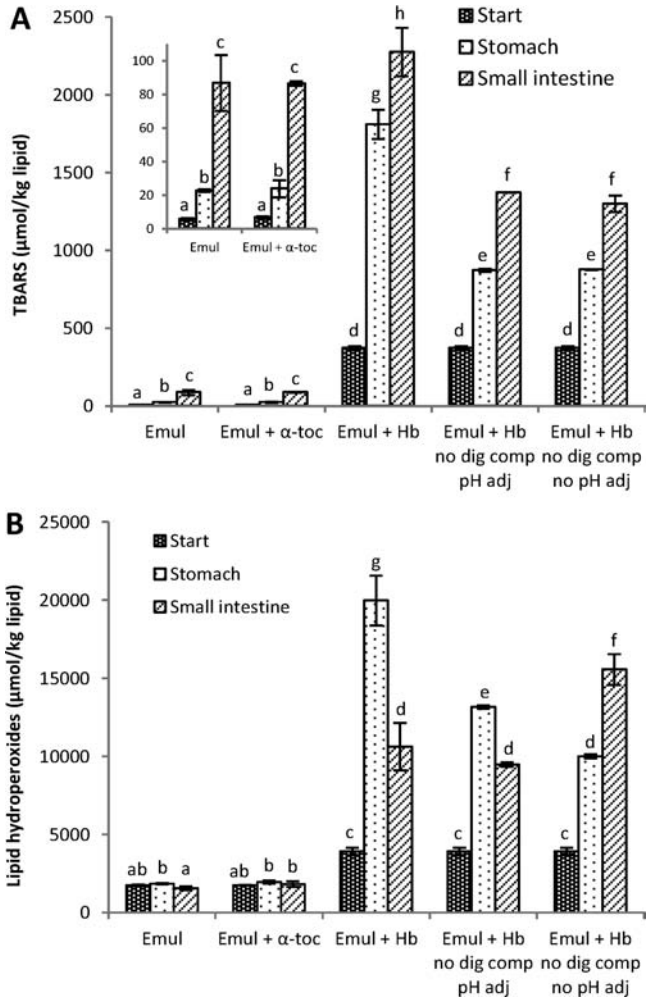

Figure 6. Effect of α-tocopherol (1 mg/g lipid) or hemoglobin (Hb, 11.5 μM on emulsion basis) on formation of (A) TBARS and (B) lipid hydroperoxides in emulsion (20% cod liver oil) during GI oxidation. A magnification of emulsion ± α-tocopherol has been inserted in panel A to show the relatively low TBARS values obtained from these samples compared to samples with Hb. Emul, emulsion; α-toc, α-tocopherol; dig comp, digestive compounds; pH adj, pH adjustments. Values are the mean ± SD of four replicates except for Hb-containing samples, for which values are the mean from duplicates and error bars show (max−min)/2. To stabilize variances, all values were logarithmized before statistical analysis. Bars with different letters indicate statistical differences ($p < 0.05$).

to exert its catalytic activity by a relative stabilization of ferric iron and a Fe^{2+}/Fe^{3+} cycling mechanism.

Apart from an increased rate of lipid hydroperoxide decomposition in the presence of Hb, a possible limitation of accessible oxygen necessary for the very fast propagation of lipid peroxidation could also explain the observed reduction in hydroperoxides in two of the Hb-enriched samples after the intestinal step (Figure 6B). In the used model, the amount of oxygen in the headspace of the intestinal samples was lowered to 0.72 ± 0.68% (∼5.3 Torr; $n = 4$), which is in the same range as what was determined in the mid small intestine of rats (11 Torr).[43]

Figure 6A shows that a concentration of 1 mg α-tocopherol/g oil was not sufficient to inhibit TBARS formation in cod liver oil emulsion subjected to the in vitro GI digestion. In fact, almost no difference in oxidation could be seen between

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emulsions with or without α-tocopherol addition, in terms of neither TBARS nor lipid peroxide formation. The unfortified cod liver oil emulsion naturally contained 0.167 ± 0.005 mg α-tocopherol/g oil. This suggests that rather other types of antioxidants are needed to prevent lipid oxidation of cod liver oil emulsion during digestion. In gastric models, efficient oxidation inhibition was provided, for example, by red wine polyphenols,[11,15] catechin,[11,14,15] and food-derived melanoidins.[12,17]

Potential Consequences of Lipid Oxidation in the GI Tract. Because secondary oxidation products from marine lipids give rise to an unpleasant taste and odor and because some of these oxidation products have very low odor thresholds, it can be claimed that oxidized lipids would not be ingested by humans. However, primary oxidation products are tasteless and odorless, and processing techniques such as encapsulation may mask negative sensory attributes originating from rancid lipids. Today, no governmental regulation of maximum level of lipid oxidation products in food exists. A recent study of marine n-3 supplements on the Norwegian market concluded that about half of the n-3 supplements were above the maximum limit for peroxide value as recommended by The European Pharmacopoeia and as much as 92% exceeded the GOED guidelines.[44] Some oils had even higher peroxide values than the oxidized cod liver oil used in this paper.

The effects of oxidized lipids on health have been discussed extensively because these products are thought to be involved in many widespread diseases such as atherosclerosis, cancers, diabetes mellitus, and Parkinson's disease.[29] Systemic effects as well as local effects can be involved. Nevertheless, controlled studies of oxidized marine oils in humans have been rare. One recent randomized controlled trial on healthy humans showed no effect of a low-quality cod liver oil on markers of lipid peroxidation, oxidative stress, or inflammation in blood and urine compared to a high-quality oil.[45] However, the study period was relatively short (7 weeks), and possible local oxidative damage in the GI tract was not elucidated. Potential biological effects of oxidized marine oil enriched with n-3 PUFA have also been investigated in different cell-based models.[46] With oils containing a high oxidation level they found increased expression of genes involved in inflammation and oxidative stress as well as elevated lipid peroxidation in cell membranes. Moreover, they suggested that the possible inhibitory effect on cancer cells by nonoxidized oil can be lost upon oxidation of the oil.

Negative health effects from individual oxidation products have been more extensively studied than effects from complex oxidized oil mixtures. Particularly MDA has been in focus, which is an important toxic product formed from n-3 fatty acid peroxidation, which also contributes to the TBARS value. It can be partly absorbed by the body and may be involved in the development of, for example, atherogenicity and stiffening of cardiovascular tissue through its ability to react with primary amines and proteins such as collagen.[8,29,47]

According to the above, not only can absorbed lipid oxidation products provide potential harmful effects but also local damage can be of significance, for example, in intestinal cancer development. To combat the high exposure of reactive compounds found in the GI tract, a defense system of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase exists in the mucosal cells,[48] whereof some are even located extracellulary.[49] Upon largely increased levels of oxidative stress, this defense may be insufficient. Furthermore, other dietary compounds such as antioxidants can also be affected by reactive lipid oxidation products in the GI tract, and co-oxidation of, for example, β-carotene, ascorbic acid, and tocopherols may impair the amount of bioaccessible vitamins.[16] More detailed studies on potential local health risks of oxidized lipids in the GI tract are necessary.

The indication of GI oxidation in this study, together with the potential harmful effects of lipid oxidation products, stresses the importance of using well-defined oils when the effects of marine oil on biological markers are evaluated. Such methodological issues have also been highlighted by Dyall,[50] who stated that special precautions need to be taken when studies are performed with n-3 fatty acids to draw correct conclusions. Our results indicated that also fresh marine oils, with and without a commercial level of α-tocopherol, can give rise to toxic oxidation products through reactions taking place during the GI passage. We also showed that relatively small differences in initial oil quality may be enlarged during digestion. Therefore, we hypothesize that unspecified oil quality as well as uncontrolled continued oxidation during GI digestion may partly explain some of the inconsistencies in the results from randomized controlled trials of fish oil and its effects on sudden death, CVD, and cancer.[4−6] Our results also highlight the importance of carefully designing oxidative protection of products containing LC n-3 PUFA that persists along the complete chain from raw material processing to its passage through the GI tract to maintain the positive properties associated with n-3 PUFA on health. It must be stressed, though, that because our data are based on a static GI in vitro digestion model lacking the full complexity of a human body, confirmatory studies are needed in more advanced models, preferably in humans.

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Notes

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