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Oxidation of marine oils during in vitro gastrointestinal digestion with human digestive fluids – Role of oil origin, added tocopherols and lipolytic activity

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Abstract

The formation of malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE), 4-hydroxy-2-nonanal (HNE), and 4-oxo-2-nonanal (ONE) in cod liver-, anchovy-, krill-, and algae oil during in vitro digestion with human gastrointestinal fluids was investigated. Adding rabbit gastric lipase, lipase inhibitor (orlistat) and tocopherols to cod liver oil, lipolysis and oxidation was also studied. Among the marine oils, the highest aldehyde levels (18 µM MDA, 3 µM HHE and 0.2 µM HNE) were detected after digestion of cod liver oil, while the lowest levels were detected in krill and algae oils. Addition of rabbit gastric lipase significantly increased the release of HNE during the digestion. Orlistat significantly reduced lipolysis and MDA formation. Formation of MDA and HHE was delayed by tocopherols, the tocopherol mix Covi-α® T 70 EU being more effective than pure α-tocopherol.

1. Introduction

There is today an increasing interest in marine omega-3, or long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA), e.g. due to the association between intake of marine oil and beneficial effects on cardiovascular diseases (Delgado-List, Perez-Martinez, Lopez-Miranda, & Perez-Jimenez, 2012). However, concerns have been raised regarding the instability of marine oils due to the high susceptibility to lipid oxidation during storage, and the indications that marine oils could oxidize also during digestion (Hu, 2016). Recent studies have shown that lipid oxidation can take place under physiological conditions relevant for in vitro gastrointestinal (GI) digestion of marine oils (Kristinova, Storrø, & Rustad, 2013; Larsson, Cavonius, Alminger, & Undeland, 2012; Larsson, Tullberg, Alminger, Havenaar, & Undeland, 2016; Tullberg et al., 2016).

Marine oils generally contain a high level of LC n-3 PUFA, and the marine LC n-3 PUFA supplement market is rapidly growing, as is the fortification of other foods with marine LC n-3 PUFA. By far the most common marine oils used today are whole fish (bod) oil (often from anchovy) and cod liver oil. The possibility to use alternative marine oils, such as krill- and microalgae oils (in this article referred to as algae oil), has incremented the hope of finding more sustainable substitutes to the traditional fish oils (Adarme-Vega, Thomas-Hall, & Schenk, 2014). The content of LC n-3 PUFA is generally fairly similar between krill-, fish and cod liver oil, but a majority of the LC n-3 PUFAs in krill oil are bound to phospholipids (PLs), while LC n-3 PUFAs mainly are located in the sn-2 position of triglycerides (TG) in cod liver oil and fish oil (Schuchardt & Hahn, 2013). Also, 22 and 21% of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been found to be present in free form in krill oil (Schuchardt et al., 2011). Oil from the microalga Schizochytrium sp. contains a high proportion of DHA compared to other marine oils, while the situation is reversed for EPA (Kassis, Gigliotti, Beamer, Tou, & Jaczynski, 2012). A very low level of the FA in microalga oil from Schizochytrium sp. were found in other forms than TG; 0.07 w/w% as glycolipids and 0.8 w/w% as PLs (Yao, Gerde, Lee, Wang, & Harrata, 2015). The recommended daily intake of

Abbreviations: BHT, Butylated hydroxytoluene; BSA, Bovine Serum Albumin; DHA, Docosahexaenoic acid; DNP, 2,4-dinitrophenolhydrazine; EDTA, Ethylenediaminetetraacetic acid; EPA, Eicosapentaenoic acid; FAME, Fatty acid methyl esters; FA, Fatty acids; FFA, Free fatty acids; GC–MS, Gas Chromatography–Mass Spectrometry; GI, Gastrointestinal; Hb, Haemoglobin; HDF, Human duodenal fluid; HGF, Human gastric fluid; HHE, 4-hydroxy-2-nonenal; HNE, 4-hydroxy-2-nonanal; HPLC, High pressure liquid chromatography; LC/APCI-MS, Liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry; LC n-3 PUFA, Long-chain n-3 polyunsaturated fatty acids; MDA, Malondialdehyde; ONE, 4-oxo-trans-2-nonenal; PV, Peroxide value; RGL, Rabbit Gastric Lipase; SPE, Solid phase extraction; TEP, 1,1,3,3-tetraethoxypropane

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DHA and EPA is 250 mg (Efsa Panel on Dietetic Products and Allergies, 2012), hence the total intake of marine oils to reach this daily level might vary depending on their inherent EPA and DHA composition. Another feature that distinguish marine oils from each other is the naturally occurring antioxidants, krill oil is high e.g. in astaxanthin (Kassis et al., 2012), while algae oils generally are rich in phenolic compounds and β-carotene (Lv et al., 2015). Fish oils are normally enriched with tocopherols, as a way to prevent lipid oxidation.

Marine oils are susceptible to lipid oxidation, a highly complex chain reaction, generating a wide variety of products, and therefore being difficult to monitor. The complexity has led to an increasing demand for quantitative analysis methods, targeting specific lipid oxidation products (Schaich, 2013). Lipid oxidation e.g. leads to the generation of reactive α,β-unaturated aldehydes, such as malondialdehyde (MDA), 4-hydroxy-trans-2-hexenal (HHE), 4-hydroxy-trans-2-nonenal (HNE), and 4-oxo-2-nonenal (ONE). MDA is derived from both n-3 and n-6 PUFA's, and is according to the review by Del Rio, Stewart, and Pellegrini (2005) found e.g. in cell studies to have carcinogenic and genotoxic properties. MDA is furthermore commonly used as a biomarker for lipid oxidation (Del Rio et al., 2005). Other cytotoxic and genotoxic aldehydes of interest when studying lipid oxidation in marine oils are HHE and HNE; HHE is derived from lipid oxidation of n-3 PUFA (Van Kuijk, Holte, & Dratz, 1990), while HNE is connected to n-6 PUFA (Pryor & Porter, 1990). Another lipid oxidation product, ONE, is closely related to HNE, and has been shown to be highly genotoxic and regiospecific when interacting with DNA. ONE has an even higher reactivity compared to HNE (Sayre, Lin, Yuan, Zhu, & Tang, 2006). The exact formation route of ONE is not yet fully understood, but Lee et al. have shown that ONE can be derived from 4-hydroperoxy-2-nonenal (Lee & Blair, 2000), and ONE has also been found to be the major lipid oxidation product from linoleic acid hydroperoxide (Rindgen, Nakajima, Wehrli, Xu, & Blair, 1999). The formation of MDA, HHE and HNE has previously been reported during GI digestion of cod liver oil, both in a static and in a dynamic in vitro digestion model, where it was shown that the formation of aldehydes increased with digestion time (Larsson et al., 2016; Tullberg et al., 2016). ONE has to our knowledge not previously been studied during digestion, however, this would be of interest due to its high reactivity and its close relation to HNE.

Due to their slightly different compositions, one could argue that the stability of different marine oils might differ. Ryckeboesch et al. (2013) showed that oil from krill and microalgae were less prone to oxidation compared to e.g. fish oil during storage. Additionally, Frankel et al. reported significant differences in storage stability when assessing fish and algae oil as crude oils versus as emulsions, and the oils were found to be more prone to oxidation as emulsions (Frankel, Satué-Gracia, Meyer, & German, 2002). No study to date has compared the stability of different marine oils during digestion; a process which leads to natural emulsification in the duodenum, as well as subjection to elevated temperature, peristalsis and lipolysis for several hours. Our hypothesis was that the two oils naturally enriched in endogenous antioxidants (krill and algae oil) would be more stable also during conditions in the GI tract.

A positive relation between degree of lipolysis and oxidation was observed by Larsson et al. during in vitro digestion of cod liver oil (Larsson et al., 2012). To investigate this further, a possible approach would be to add a lipase inhibitor to reduce lipolytic activity, or to add extra lipases. One of the lipases that recently has been reported to be important for human lipolysis is gastric lipase, contributing to 10–25% of the total lipolysis (N’Goma, Amara, Dridi, Jannin, & Carrière, 2011). This enzyme has earlier not been included when designing in vitro model digestions (Kostewicz et al., 2014). Whether additional lipase in the gastric phase has any impact on lipid oxidation during GI digestion of oils has not been reported before. This would, however, be expected due to the known susceptibility of free fatty acids (FFA) to oxidation (Miyashita and Takagi, 1986).

The increasing evidence that lipid oxidation takes place in the GI tract arises the question of how to prevent this reaction. Larsson et al. reported that no effect was seen on lipid oxidation, when adding α-tocopherol at 1 mg/mL to a cod liver oil prior to static in vitro digestion (Larsson et al., 2012). In another study, a slight decrease in aldehydes was detected in the gastric phase when ethylenediaminetetraacetic acid (EDTA; 0.13 mM), was added to emulsified cod liver oil (Larsson et al., 2016). The addition of different antioxidant containing beverages, e.g. red wine and berry juice to herring oil was found to reduce lipid oxidation during gastric in vitro digestion (Kristinova et al., 2013). Similar results were seen in vivo in minipigs when including fruits and vegetables to oxidized vegetable oil in the diet (Gobert et al., 2014). When adding the synthetic antioxidant 2,6-di-tert-butyl-hydroxytoluene (BHT) to cod liver oil (800 ppm), lipid oxidation was shown to be almost completely inhibited during in vitro digestion. A slight anti-oxidative effect was also seen at lower BHT levels (20 ppm) (Nieva-Echevarría, Goicoechea, & Guillén, 2017b). Results are, however, lacking where commercially relevant levels of different types of tocopherols are evaluated as antioxidants under GI-conditions. Tocopherols are declared as food grade and are commonly used by the marine oil industry.

In this study, four marine oils were studied for the formation of specific lipid oxidation-derived aldehydes during GI digestion, using human digestive fluids. The oils used were unrefined krill- and algae oil, and refined cod liver oil and anchovy oil; the two latter thus being stripped from heavy metals and other contaminants. Lipolysis and lipid oxidation during in vitro gastric and duodenal digestion were investigated. The effect of adding rabbit gastric lipase (RGL), orlistat and tocopherol mix Covi-ox® T 70 EU, in addition to pure α-tocopherol to cod liver oil were also studied. The following target aldehydes were chosen as markers for lipid oxidation; MDA, HHE, HNE, and ONE.

2. Materials and methods

2.1. Chemicals and enzymes

Proteins and enzymes used as standards in the enzyme activity assays (porcine pepsin P6887, trypsin from porcine pancreas T0303, human haemoglobin (Hb) H7379, ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, ascorbic acid, bovine serum albumin (BSA), 1,1,3,3-tetraethoxypropane (TEP), orlistat and 2,4-dinitrophenylhydrazine (DNPH) were all purchased from Sigma-Aldrich (St. Louis, USA). Fatty acid (FA) pattern and peroxide value (PV) analyses (porcine pepsin P6887, trypsin from porcine pancreas T0303, human haemoglobin (Hb) H7379, ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, ascorbic acid, bovine serum albumin (BSA), 1,1,3,3-tetraethoxypropane (TEP), orlistat and 2,4-dinitrophenylhydrazine (DNPH) were all purchased from Sigma-Aldrich (St. Louis, USA). RGL with an activity of 70 U/mg (according to the manufacturer) was produced from Germe (Marseille, France). Covi-ox® T 70 EU was obtained from BASF (Lyon, France); containing D-α-tocopherol (92.4 mg/g), D-β-tocopherol (15.2 mg/g), D-Σ-tocopherol (146 mg/g) and D-γ-tocopherol (468 mg/g), according to the supplier. HHE, HNE and ONE standards were purchased from Cayman Chemicals (Ann Arbor, USA). A Milli-Q plus system (Merck Millipore, Dürrnatt, Germany) was used to purify the water used to a resistivity of 18.2 MΩ-cm. All other chemicals used were of analytical grade.

2.2. Marine oils

Oils of different marine origins were used in the digestions. Refined whole anchovy oil (peruvian anchoveta, Engraulis ringens) and cod liver oil (Gadus morhua), without added antioxidants, were supplied by Lysi hf (Reykjavik, Iceland). The algae oil was an unrefined microalgae oil (Schizochytrium sp.) called Lifes DHA S35-C0100 and supplied from DSM (Basel, Switzerland). The unrefined krill oil Superba™ Krill Oil (Aker Biomarine Antarctic AS, Oslo, Norway) was produced from Antarctic krill (Euphausia superba) and provided by Sanpharm AB (Gothenburg, Sweden). Fatty acid (FA) pattern and peroxide value (PV) of the oils were analysed as described in 2.5.1 and 2.8, respectively.
2.3. Human fluids

Saliva was collected from 6 healthy volunteers at Chalmers University of Technology, Sweden. The saliva was collected in the morning prior to breakfast, using sterile straw pipettes for saliva provided by Kemikaliala (Skurup, Sweden). Spontaneous drooling was stimulated by showing pictures of fish dishes to volunteers during collection. Human gastric fluid (HGF) and human duodenal fluid (HDF) were aspirated from 6 volunteers, according to Tullberg et al. (2016). The aspirations were done at Lovisenberg Diakonale Hospital (Oslo, Norway, November 2015), and the study was conducted with approval from the Norwegian Regional Ethics Committee (project no 2012/2230, Biobank no 2012/2210). The Declaration of Helsinki was followed, and all participants in the study signed up as volunteers with informed consent. The pH and colour of the individual aspirates were assayed, to avoid the inclusion of samples were reflux of duodenal fluid had occurred. Gastric fluid aspirates were excluded if the colour was yellow and if pH > 4. Individual aspirates were pooled to eliminate individual effects as much as possible, and samples were stored at −80 °C. Enzyme activities of the pooled aspirates were assayed as described in Section 2.4. The pH of HGF was set to pH 6 at collection time to preserve the human gastric lipase from being degraded by the pepsin (Ville, Carrière, Renou, & Laugier, 2002).

2.4. Characterization of human digestive fluids

Gastric lipase, trypsin and pancreatic lipase activities were assayed in triplicates immediately after aspiration, while other parameters, bile-salt-, calcium ion-, ascorbic acid-, and tocopherol concentrations, were analysed in pooled samples after freezing at −80 °C and thawing. Salivary α-amylase-, pepsin-, trypsin-, and pancreatic lipase activity were all assayed in the pooled human enzyme fluids in accordance with the standardized digestion protocol by Minekus et al. (2014), and the result from the enzymatic activity measurements can be found in the Supplementary material (Table S1). A comprehensive overview of the composition of the human GI fluids can be found in the Supplementary material (Table S2).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.07.049.

2.4.1. Gastric lipase activity

Gastric lipase activity was assayed in HGF using a titrimetric protocol as described by Ville et al. (2002). The assay was performed at pH 6, using tributyrin as substrate. Activity of gastric lipase in HGF was calculated in U/mL.

2.4.2. Bile-salt concentrations

A bile acid kit (DiaSys Diagnostic Systems, Holzheim, Germany) was used to spectrophotometrically determine the bile-salt concentration in the HDF at 540 nm, according to the manufacturer’s instructions. The analysis is based on the combined formation of NADH and the dye formazan, developed due to the action of the enzyme 3a-hydroxysteroid dehydrogenase on bile acids. Taurocholic acid was used as a standard in this assay, and the result are expressed as mmol bile salt/L.

2.4.3. Calcium ion concentration

Calcium ion concentrations in the human digestive fluids were measured using a Dionex high pressure liquid chromatography (HPLC) BioLC system (Thermo Scientific, Sunnyvale, USA) with a CG14 guard column, combined with an IonPac CS14 analytical column (4 × 250 mm). The BioLC was combined with a CD20 conductivity detector, a G50 gradient pump, and a Triathlon autosampler (SparkHolland, Emmen, the Netherlands). Detection was done according to Tullberg et al. (2016).

2.4.4. Ascorbic acid concentration

Ascorbic acid was determined by the method described by Lykkesfeldt (2007), with minor in-house modifications. Briefly, HGF and HDF were centrifuged, diluted 1:1 with meta-phosphoric acid (10% w/v) and disodium EDTA (2 mM), vortexed and stored at −80 °C before analysis. At the time point for analysis, the samples were diluted 1:1 with Tris (2-carboxyethyl) phosphine (TCEP, 0.312 mM) in 1:10 McIlvaine buffer (0.46 M Na₂HPO₄, 0.27 M citric acid, pH 4.5) and 9:10 phosphate buffer (50 mM, disodium EDTA 20% (w/v), pH 2.8). Detection of ascorbic acid was conducted using a Jasco Corporation HPLC system (Tokyo, Japan), including an autosampler (AS-2057) and a plus pump (AS-2080), and the system was coupled with Aqualas C18 analytical column (4.6 × 150 mm, Thermo Scientific, Sunnyvale, USA). Following HPLC separation, electrochemical detection was done with a Decade II system (Antec Leyden, Zoeterwoude, the Netherlands). Data collection was carried out with the software Jasco Chrom Pass (Jasco Corporation, Tokyo, Japan).

2.4.5. Trace metal ions

Analysis of trace metal ions (Cu, Ni, Zn, Co, Mn, and Fe) in saliva, HGF, HDF and orlistat were done as described by Tullberg et al. (2016). Acidic microwave digestion of samples was followed by separation and detection using ion chromatography coupled with UV–vis, quantified with external standards.

2.4.6. Haemoglobin (Hb)

Hb was analysed in the human fluids using a HemoCue® Plasma/Low Hb System (HemoCue AB, Angelholm, Sweden), using microcuvettes and human Hb as external standard. The method is based on oxidation of Hb, derivatization with azide, and transmission detection of azidemetHb. The results are expressed as g Hb/L.

2.5. Levels, treatments and characteristics of oils to be digested

The aim was to normalize the amount of intake oil based on the analyzed total EPA and DHA content of the oils, in order to simulate a daily EPA and DHA intake of 250 mg oil by a human. The amounts of cod liver, anchovy, krill and algae oils digested in the down-scaled digestion model according to this normalization was 32, 21, 29 and 21 mg, respectively. The intake liquid (“meal”) included the oil and water at approximately a 1:10 ratio, to simulate taking an oil capsule together with a mouthful of water. To determine the background level of lipid oxidation products and FFA, digestions with water alone, i.e., without any oil, were also included in the experimental setup and referred to as blanks. When assessing the addition of tocopherols, Covix® T 70 EU (4.5 mg/g oil), and α-tocopherol (4.5 mg/g oil), these were added to cod liver oil just prior to digestion. The lipase inhibitor orlistat (1.75 mg/mL oil) was added to cod liver oil in the same way. RGL (0.68 mg/mL HGF, 48 U RGL/mL HDF) was added to the HGF just prior to the gastric phase of the digestion model.

2.5.1. Initial peroxide value (PV) of the marine oils

The initial peroxide value (PV) of the marine oils before digestion was determined in accordance with Undeland, Hultin, and Richards (2002). In short, 150 mg oil was diluted in 5 mL CHCl₃, then again diluted 1:10 with CHCl₃. CHCl₃:MeOH (1:1) was then added to the samples (1:1.5), followed by addition of an ammonium thiocyanate solution (1:100) and an Fe(II)chloride solution (1:100), before incubation (24 °C, 20 min). PV was then spectrophotometrically determined at 500 nm, quantified with a standard curve from cumene hydroperoxide (80%), and reported as mmol/kg oil.

2.5.2. Initial tocopherols in the marine oils

Naturally occurring tocopherols in the marine oils were analysed according to Larsson, Almgren, and Undeland (2007). Tocopherols were extracted in MeOH, vortexed, sonicated and centrifuged, and then
analysed on a HPLC system (Jasco, Easton, USA) with a Luna (3 mm i.d × 150 mm, 3 μm) C18 column (Phenomenex, Macclesfield, UK). Detection was done spectrofluorometrically at 295 nm (excitation) and 330 nm (emission) by a Shimadzu RF-551 detector (Kyoto, Japan). Quantification was done towards external standards (α, γ-, δ- and α-tocopherols) were reported as mg tocopherol/kg oil.

2.6. **In vitro static digestion**

In this study, a static 3-step in vitro digestion model with human GI fluids was used (see Fig. 1). The model was modified according to the protocol by Minekus et al. (2014), with a special focus on lipid digestion and lipases in the human digestive fluids. This was done by including an additional pH-step during gastric digestion, to better simulate the pH and dilution factor changes over time during static digestion in the human body (Sams, Paume, Giallo, & Carriere, 2016). All the naturally occurring enzymes and respective activities (U/mL) of pepsin, lipases, proteases, amylase were kept and recorded as recommended in the protocol by Minekus et al. (2014), except for the exception of external RGL, which was added in addition to human gastric juice when studying lipolysis of cod liver oil. This was decided since the native enzyme activities in the digestive fluids were considered more natural than boosting the GI fluids with additional external enzymes. Studies of additional digestive fluid, i.e. at the start of the mouth, stomach and intestinal steps, samples were flushed with N₂ gas (15 s) for oxygen reduction. An overview of the changes in pH and dilution during the digestion protocol presented in Fig. 2. Sampling of digests were done in initial sample, in the gastric phase (t = 0, 60, and 120 min), and in the duodenal phase (t = 0, 45, and 90 min); using individual test tubes for each collection point, to avoid inflow of external oxygen during sampling. Tubes with digests were flushed with N₂ gas (15 s) and stored in −80 °C immediately after sampling. Digestion was repeated three times for each test condition.

2.7. **Analysis of aldehydes; MDA, HHE, HNE and ONE**

MDA, HHE, HNE and ONE were determined by liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry (LC/APCI-MS) in the digests, in the undigested crude oil, and in the digestion blanks. This was done according to Tullberg et al. (2016). Briefly, the digests were mixed with butylated hydroxytoluene (BHT), EDTA and HCl, to prevent lipid oxidation during analysis and to precipitate proteins. Aldehydes were derivatized by DNPH, extracted with dichloromethane, and dissolved in MeOH before separation and detection by LC/APCI-MS. Quantification of aldehydes was made against standard curves of MDA, HHE, HNE, and ONE treated in the same way as the samples. Data acquisition for MDA, HHE, HNE and ONE were done in selected ion monitoring (SIM) modes, collecting ions at 234.0, 293.1, 335.1, and 333.0 m/z, and at fragmentor setting 50, 150, 170, and 170, respectively. Measurements were done in single replicates.

2.8. **Lipid extraction, separation of free fatty acids (FFA) and analysis of fatty acid (FA) pattern**

2.8.1. **Lipid extraction and separation of free fatty acids (FFA)**

Lipids were extracted from intake liquid (“meal”) and digested samples (mouth, gastric (120 min) and duodenal (90 min) phase), with CHCl₃-MeOH, as described by Tullberg et al. (2016). Throughout the whole procedure, samples were covered in aluminium foil and kept on ice to prevent oxidation. The FA heptadecanoic acid (C17:0), was added from start to all samples as an internal standard. CHCl₃-MeOH, containing 0.05% (w/v) BHT, in ratio 2:1 was used for high-fat samples (fat content ≥ 6%; initial digestion samples), in ratio 1:1 for medium-fat content samples (2–6%; samples at end of gastric digestion), and in ratio 1:2 for low-fat samples (fat content < 2%; samples at end of duodenal digestion). The BHT-enriched CHCl₃-MeOH was added to the samples in a ratio of 10:1, and after vortexing (10 s), NaCl (0.5%) was added (1:2.75). The tubes were then again vortexed (10 s) and centrifuged (3000 × g, 6 min). The chloroform phase was collected and evaporated to dryness (N₂ gas, 40 °C). In order to analyse the FA pattern of the FFA in intake liquid and digests, lipid classes (TG, PL and FFA) of the extracted lipids were pre-separated on solid phase extraction (SPE) columns. Telos NH₂ SPE-colons (500 mg/6 mL; Kinesis, St Neots, UK) and Mega Bond Elut-NH₂ (500 mg/6 mL; Agilent Technologies, Santa Clara, USA) were used.

2.8.2. **Analysis of total fatty acids (FA) and free fatty acids (FFA)**

Total FA was analysed in crude oils, intake liquid and digested oils using the obtained lipid or FFA extracts from 2.8.1, or the crude oils...
dissolved in CHCl₃-MeOH (2:1). Lipid extracts, FFA-extracts and oils were methylated by the in-house methodocholic-CHCI₃ transesterification as described by Tullberg et al. (2016), and using 3 mL petroleum ether to extract the total FA. The organic phase was analysed using GC–MS with the external FA standard mix GLC 463 (Nu-Chek prep, Inc., Elysian, USA). FA methyl esters were then detected as FAME (mg FAME/g oil). The overall FA composition of the four oils can be found in Table 1, and the more comprehensive results from the FA composition analysis can be found in the Supplemental material (Table S3).

2.9. Calculations and statistics

Results are shown as mean values, and error bars represents standard deviation in cases where n > 2, and (max — min)/2 where n = 2. Statistical differences between treatments over the whole digestion period were determined by mixed model with repeated measurements after logarithmic transformation of aldehyde data. Time was chosen as the within-subject factors, MDA, HHE and HNE were chosen as dependent factors, respectively, and condition*time was chosen as the interaction term in the mixed model. Statistical differences between groups in each digestion time point was determined by general linear model univariate analysis, using Tukey’s HSD as post-hoc test (IBM SPSS Statistics 19, IBM Corp., New York, USA), again after logarithmic transformation of data. The general linear model was performed with the effect of gastric lipase and orlistat in one model, and effect of tocopherols in another model. Missing replicates (in time t = 0, n = 2, for the e

| FAME                  | Cod liver oil | Anchovy oil | Krill oil | Algae oil |
|-----------------------|---------------|-------------|-----------|-----------|
| Sum SFA               | 166 ± 1.37    | 247 ± 3.66  | 192 ± 3.31| 222 ± 6.77|
| Sum MUFA              | 327 ± 4.46    | 137 ± 2.47  | 78 ± 3.2  | 6.6 ± 0.41|
| Sum PUFA              | 242 ± 1.94    | 377 ± 6.78  | 256 ± 4.31| 619 ± 20.4|
| Sum LC n-3 PUFA*     | 192 ± 0.98    | 298 ± 5.02  | 190 ± 1.67| 503 ± 0.62|
| Sum LC n-6 PUFA*     | 11.2 ± 0.44   | 13.5 ± 0.41 | 1.4 ± 0.37 | 109 ± 3.58|
| C20:5 n3 (EPA)       | 76.9 ± 0.1    | 150 ± 2.3   | 120 ± 1.19| 7.7 ± 0.27|
| C22:6 n3 (DHA)       | 96.2 ± 0.5    | 124 ± 2.25  | 60.6 ± 0.26| 484 ± 15.59|

* Chain length > C18, > 1 double bond starting in n-3 or n-6 position.

The results from the analysis of MDA, HHE and HNE are shown in Fig. 3. Data on ONE did not differ from the digestion blank, hence these results are not included in the figure. Significant differences in MDA, HHE and HNE levels between the digested marine oils and digestion blanks were observed based on the mixed model with repeated measurements on logarithmic data including the whole digestion time, the only exception being algae oil digests compared to digestion blank for HNE. Also the algae oil digests differed significantly over time compared to digests with the other marine oils (p < 0.05), both for MDA and HHE. The MDA levels detected were quite stable throughout the gastric digestion (Fig. 3a). The exception was the anchovy oil digests, where increased levels of MDA were seen after 60 min digestion time, being significantly different from the digestion blank (p = 0.015) and algae oil (p = 0.03). However, this levelled off at the end of the gastric digestion (t = 120 min). An overall increase in MDA development was seen in all oil digests during the course of duodenal digestion, with the highest levels detected in the cod liver oil digests (t = 90 min; 16.6 µM) (Fig. 3d). Overall, more pronounced differences between the oils were seen in the duodenal phase, with the anchovy- and cod liver oil digests being subjected to significantly higher MDA development than the algae oil and krill oil digests; the latter having equal MDA levels at the end of digestion.

During gastric digestion (Fig. 3b), the levels of HHE formed were quite low, with cod liver oil and anchovy oil digests having the highest levels at the end of digestion. At the end of the gastric digestion, a significant difference between on the one hand algae- and cod liver oil digests, and on the other hand algae- and anchovy oil digests showed similar pattern, with the highest mean HHE level found in the cod liver oil digests (2.8 µM; t = 90 min). Likewise, the algae oil and the krill oil digests both had significantly lower HHE levels compared to the digests with anchovy oil and cod liver oil (p < 0.05 at t = 45 and 90 min).

No significant differences were detected when comparing the different HNE levels in the marine oil digests (Fig. 3c and 3f). During duodenal digestion there was a general increase in HNE formation in digests over time; with the exception of krill oil. Algae-, cod liver- and anchovy oil digests all contained similar levels of HNE at the end of the duodenal digestion, t = 90 min, with the highest levels detected in the cod liver oil digests (0.18 µM).

Analysis on HPLC showed that the naturally occurring tocopherols in the marine oils differed, with the highest total levels detected in algae- and krill oil, while only α-tocopherols could be detected in the cod liver oil and anchovy oil (Supplementary material, Table S4).

Among the four marine oils, it was seen that krill oil gave rise to the highest total FFA release (51%) at the end of digestion (t = 210 min), although not significantly different from the FFA release from the other oils. The highest levels of free EPA was found in the krill and the anchovy oil at the end of digestion. Further, at the end of digestion, 14 times more free DHA was observed in algae oil digests compared to cod liver oil digests, and 5 times more in algae oil compared to krill and anchovy oil digests (data not shown).

3.2. Effect of addition of a lipase inhibitor (orlistat) and rabbit gastric lipase (RGL) on lipolysis and gastrointestinal (GI) oxidation of cod liver oil

The results from the analysis of MDA, HHE and HNE after addition of orlistat and RGL to cod liver oil are shown in Fig. 4. Data on ONE did not differ from the digestion blank, hence these results are not included in the figure. Significant differences in MDA, HHE and HNE levels between the cod liver oil digests and digestion blank were observed based on the mixed model with repeated measurements on logarithmic data including the whole digestion time, the only exception being that no significant difference was seen between orlistat addition and digestion blank for HNE levels. Also the cod liver oil digests with RGL differed significantly over time compared to cod liver oil digests without RGL (p < 0.05) for HNE.

When studying the addition of orlistat to cod liver oil during gastric digestion, we observed a peak in MDA formation at t = 60 min; reaching 0.16 µM which was higher, although not significantly, compared to the MDA level found in digests with pure cod liver oil at the same time point. The MDA levels in the gastric digests with orlistat however decreased again during duodenal digestion (p = 0.001, t = 0) and continued to stay low compared to pure cod liver oil throughout the duodenal digestion (Fig. 4a and d). When analyzing trace metal ions in the orlistat powder, no contamination was detected (data not shown).

For HHE and HNE, no significant differences were seen between
gastric or duodenal digests with pure cod liver oil and addition of orlistat (Fig. 4b-c, 4e-f). However some visual trends can be noticed during duodenal digestion (Fig. 4e-f) such as a delay in the onset of HHE and HNE formation in cod liver oil digests when adding orlistat.

Addition of RGL to HGF gave a non-significant increase in MDA formation in cod liver oil digests, compared to non-fortified HGF, at the beginning of the gastric digestion (t = 0, Fig. 4a). During duodenal digestion, we observed no difference in MDA formation with or without addition of RGL to the cod liver oil digests (Fig. 4d). Also for HHE there were no apparent differences with or without the addition of RGL, except for the slightly higher HHE levels detected at the end of digestion with RGL (4.1 µM versus 2.8 µM, Fig. 4b and 4e). The development of HNE in cod liver oil digests was, however, significantly higher when boosting HGF with addition of RGL during gastric digestion, compared to non-fortified HGF (p = 0.0004, 0.009, 0.000001; gastric t = 0, 60, 120 min; Fig. 4c). Addition of RGL also gave a non-significant increase in HNE formation during duodenal digestion, compared to non-fortified HGF, Fig. 4f.

When evaluating the FFA release as % of total FA, Fig. 5, we observed that the addition of orlistat significantly reduced lipolysis of cod liver oil both in the gastric and duodenal step (p = 0.0003, t = 120 min; p = 0.000004, t = 210 min). RGL had no significant effect.

Fig. 3. Reactive aldehydes detected in the digests during gastric (0–120 min) and duodenal (120–210 min) digestion of cod liver oil, anchovy oil, krill oil and algae oil. Malondialdehyde (MDA), 4-hydroxy-trans-2-hexenal (HHE), and 4-hydroxy-trans-2-nonenal (HNE). Gastric step is shown in panels (A) MDA, (B) HHE, and (C) HNE, and duodenal step shown in panels (D) MDA, (E) HHE, and (F) HNE. Error bars are shown as mean ± SD (n > 2) or (max − min)/2 (n = 2).
on gastric or duodenal lipolysis (Fig. 5), however the mean % FFA release based on total FA was approximately 25% higher, although difference was still non-significant, in presence of RGL at the end of the gastric digestion. Data from the analysis of specific PUFA release can be found in the Supplementary material (Fig. S1). From this it can be seen that similar levels of total n-3 PUFA were released during gastric digestion with and without addition of RGL. However, out of the total n-3 PUFA, although non-significant, about 10% more EPA, and 20% more DHA were released from cod liver oil digested with the non-fortified HGF, compared to after RGL addition, at the end of gastric digestion (t = 120 min). Additionally, on average 2.2-times more total n-6 PUFA was released from cod liver oil with RGL at t = 120 min, compared to without RGL (non-significant difference). Addition of orlistat to cod liver oil led to 20 times less n-3 PUFA, released at the end of digestion, compared to pure cod liver oil (p < 0.001). More specifically, 18.7-times less EPA and 13.5-times less DHA were released from cod liver oil

Fig. 4. Effect of addition Orlistat and rabbit gastric lipase (RGL) to cod liver oil on the formation of reactive aldehydes during gastric (0–120 min) and duodenal (120–210 min) static in vitro digestion. Malondialdehyde (MDA), 4-hydroxy-trans-2-hexenal (HHE), and 4-hydroxy-trans-2-nonenal (HNE). Gastric step is shown in panels (A) MDA, (B) HHE, and (C) HNE, and duodenal step shown in panels (D) MDA, (E) HHE, and (F) HNE. Error bars are shown as mean ± SD (n > 2) or (max − min)/2 (n = 2).
at the end of duodenal digestion ($t = 90$ min, $p = 0.01$). Pure cod liver oil digests and those forti-

HNE was developed after Covi-ox® digestion was observed, with the strongest e-

was seen between pure cod liver oil digests and those forti-

The initial levels of total EPA + DHA differed in the marine oils, with anchovy oil and algae oil (mainly DHA) containing the highest con-

During duodenal digestion, the aldehyde formation increased, which is

During duodenal digestion, a signi-

significant differences are in the figures shown as * = $p < 0.05$, and ** = $p < 0.001$.

with orlistat at $t = 210$ min, compared to without orlistat.

3.3. Effect of addition of $\alpha$-tocopherol and Covi-ox® T 70 EU on gastrointestinal (GI) oxidation and lipolysis of oils

When looking at MDA development in cod liver oil digests with and without tocopherols added, no significant difference during gastric diges-

A possible explanation for the decrease in pH could decrease the protective ef-

The initial levels of total EPA + DHA differed in the marine oils, with anchovy oil and algae oil (mainly DHA) containing the highest con-

For HHE, a reduced development both during gastric and duodenal diges-

For HHE, a reduced development both during gastric and duodenal diges-

very low levels of HNE were detected in the gastric phase with tocopherol addition (Fig. 6c). During duodenal digestion, no significant differences were seen between the treatments, however slightly less HNE was developed after Covi-ox® T 70 EU addition to the cod liver oil.

4. Discussion

4.1. Why do marine oils of different origin oxidize differently during in vitro digestion?

The findings in this study supports the hypothesis that crude marine oils (refined cod liver- and anchovy oil, vs. unrefined krill and algae oil) have different susceptibility towards lipid oxidation during digestion. The initial levels of total EPA + DHA differed in the marine oils, with anchovy oil and algae oil (mainly DHA) containing the highest concentrations, followed by krill oil and then cod liver oil. Hence, the di-

As described e.g. by Kanner and Lapidot (2001), a low pH could decrease the protective effect of antioxidants, and hence promote lipid oxidation. A possible explanation for the decrease in MDA levels detected in the anchovy oil digests after 120 min gastric digestion is that MDA reacted with e.g. proteins present in the HGF. During duodenal digestion, the aldehyde formation increased, which is in line with previous studies on lipid oxidation during digestion of

Fig. 5. Release of free fatty acids (FFA) during gastric (120 min) and duodenal (210 min) digestion of cod liver oil, including addition of orlistat and rabbit gastric lipase (RGL). Results are shown as % of FFA per detected total fatty acids (FA) in oil. Error bars are shown as mean ± SD (n > 2) or (max – min)/2 (n = 2). Significant differences are in the figures shown as * = $p < 0.05$, and ** = $p < 0.001$.
marine oils (Kenmogne-Domguia, Moisan, Viau, Genot, & Meynier, 2014; Larsson et al., 2012; Larsson, Tullberg, Alminger, Havenaar, & Undeland, 2016; Tullberg et al., 2016). An explanation to this could be changes in the matrix composition with the presence of bile salts and pancreatic enzymes, which in turn e.g. could influence the emulsification of the oil. The presence of already pre-formed lipid oxidation products from the gastric phase or the increased stirring rate in the duodenal digestion phase could also play important roles. Since it is still unknown what contributes the most to the increasing lipid oxidation during duodenal digestion compared to the gastric digestion, we believe that it is important to include both a gastric and a duodenal digestion step, when studying lipid oxidation during in vitro digestion.

Several of our previous studies (Larsson et al., 2012, 2016) observed like this study that oxidation of marine oils increased during in vitro digestion. The presence of already pre-formed lipid oxidation products from the gastric phase or the increased stirring rate in the duodenal digestion phase could also play important roles. Since it is still unknown what contributes the most to the increasing lipid oxidation during duodenal digestion compared to the gastric digestion, we believe that it is important to include both a gastric and a duodenal digestion step, when studying lipid oxidation during in vitro digestion.

**Fig. 6.** Effect of addition of Covi-ox® T 70 (tocopherol mix), α-tocopherol (α-toc) to cod liver oil on the formation of reactive aldehydes during gastric (0–120 min) and duodenal (120–210 min) static in vitro digestion. Malondialdehyde (MDA), 4-hydroxy-trans-2-hexenal (HHE), and 4-hydroxy-trans-2-nonenal (HNE). Gastric step is shown in panels (A) MDA, (B) HHE, and (C) HNE, and duodenal step shown in panels (D) MDA, (E) HHE, and (F) HNE. Error bars are shown as mean ± SD (n > 2) or (max – min)/2 (n = 2).
digestion. However, Larsson et al. also observed that the initial oxidative status of cod liver oil mattered for which final levels of lipid oxidation products developed during in vitro digestion (Larsson et al., 2012, 2016). PV data from the present oils indeed showed that the cod liver oil was the most oxidized oil prior to digestion, and the algae oil the least oxidized oil, while the oxidation status for anchovy- and krill oil were in between. Being substrates for secondary oxidation product formation, initial lipid hydroperoxide levels thus could be an additional factor explaining the results of this study. On the other hand, krill oil had the highest initial levels of MDA prior to digestion, and these high initial MDA levels obviously did not boost a fast progression of MDA during digestion, which again could be explained e.g. by alternative routes of oxidation in the krill oil.

4.2. Role of lipolytic activity and added tocopherols on lipid oxidation development during digestion

Nieves-Echavarria, Goicoechea, and Guillén (2017a) investigated the effect of liquid smoking on lipid oxidation and lipolysis of fish muscle during GI in vitro digestion, and saw that lipolysis was not affected, while smoke could prevent lipid oxidation. To our knowledge, no one has however previously investigated the direct effect of lipolysis during digestion on lipid oxidation. The release of FFA from triglycerides is known to increase lipid oxidation during storage and processing (Miyashita and Takagi, 1986). FFA have been shown to induce lipid oxidation by attracting transition metal ions which could act as pro-oxidants at the oil/water interface (Warahoh et al., 2011), and by catalyzing the generation of hydroxyl radicals from hydroperoxides (Miyashita and Takagi, 1986). Furthermore, studies done on fish muscle have suggested that hydrolysis of PLs have a protective effect on lipid oxidation, while hydrolysis of TGs would induce lipid oxidation (Shewfelt, 1981). Based on this we hypothesized that addition of the oxidation, while hydrolysis of TGs would induce lipid oxidation have suggested that hydrolysis of PLs have a protective e
catalyzing the generation of hydroxyl radicals from hydroperoxides

Results showed that orlistat inhibited lipolysis and delayed the onset of lipid oxidation during digestion of the cod liver oil. The results showed that krill- and algae oils remained less oxidized compared to anchovy- and cod liver oils, and that reduced level of lipolysis reduced development of lipid oxidation during the digestion. It should be emphasized that this was measured in krill- and algae oils with their natural antioxidants preserved, since these oils were unrefined; the refined anchovy- and cod liver oil found in the store would have e.g. external tocopherols added to them. The addition of an external lipase, RGL, did not significantly increase lipolysis, but it increased the levels of HNE detected during overall GI digestion. The lipid inhibitor orlistat showed an inhibitory effect on lipid oxidation during duodenal digestion. Finally, lipid oxidation during static in vitro GI digestion could be reduced by the addition of tocopherols at levels relevant for industrial use. Covi-α® T 70 EU, which consists of several tocopherol isomers, was found to be more protective than α-tocopherol.

5. Conclusion

In this study, the origin of marine oils played an important role in the formation of lipid oxidation-derived aldehydes during static in vitro GI digestion. The results showed that krill- and algae oils remained less oxidized compared to anchovy- and cod liver oils, and that reduced level of lipolysis reduced development of lipid oxidation during the digestion. It should be emphasized that this was measured in krill- and algae oils with their natural antioxidants preserved, since these oils were unrefined; the refined anchovy- and cod liver oil found in the store would have e.g. external tocopherols added to them. The addition of an external lipase, RGL, did not significantly increase lipolysis, but it increased the levels of HNE detected during overall GI digestion. The lipid inhibitor orlistat showed an inhibitory effect on lipid oxidation during duodenal digestion. Finally, lipid oxidation during static in vitro GI digestion could be reduced by the addition of tocopherols at levels relevant for industrial use. Covi-α® T 70 EU, which consists of several tocopherol isomers, was found to be more protective than α-tocopherol.

6. Conflict of interests

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