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

N.B. When citing this work, cite the original published paper.

(article starts on next page)

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

Cecilia Tullberg[a,*], Gerd Vegarud[b], Ingrid Undeland[a]

[a] *Food and Nutrition Science, Department of Biology and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden*
[b] *Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Science, Ås, Norway*

ABSTRACT

The formation of malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE), 4-hydroxy-2-nonenal (HNE), and 4-oxo-2-nonenal (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-ox® 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-Lista, 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 (body) 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 microalgae *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 microalgae 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

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-unsaturated aldehydes, such as malondialdehyde (MDA), 4-hydroxy-*trans*-2-hexenal (HHE), 4-hydroxy-*trans*-2-nonenal (HNE), and 4-oxy-2-nonenal (ONE). MDA is derived from both n-3 and n-6 PUFAs, 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 derives 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 assume that the stability of different marine oils might differ. Ryckebosch 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 antioxidative 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 (Schnelldorf, Germany). RGL with an activity of 70 U/mg (according to the manufacturer) was purchased from Germe (Marseille, France). Covi-ox® T 70 EU was obtained from BASF (Lyngby, Denmark); containing D-α-tocopherol (92.4 mg/g), D-β-tocopherol (15.2 mg/g), D-δ-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, Darmstadt, 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 Lýsi hf (Reykjavík, Iceland). The algae oil was an unrefined microalgae oil (*Schizochytrium* sp.) called *Lifés DHA S35-CO100* 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 Kemikalia (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-, trace metal ion-, and Hb 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 activity 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 3α-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 GS50 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-carbozyethyl) phosphine (TCEP, 0.312 mM) in 1:10 McIlvaine buffer (0.46 M Na_2HPO_4, 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 Aquasil 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, Ängelholm, Sweden), using microcuvettes and human Hb as external standard. The method is based on oxidation of Hb, derivatization with azide, and transmission detection of azidmetHb. 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, Covi-ox® 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 HGF) 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 $CHCl_3$, then again diluted 1:10 with $CHCl_3$. $CHCl_3$: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
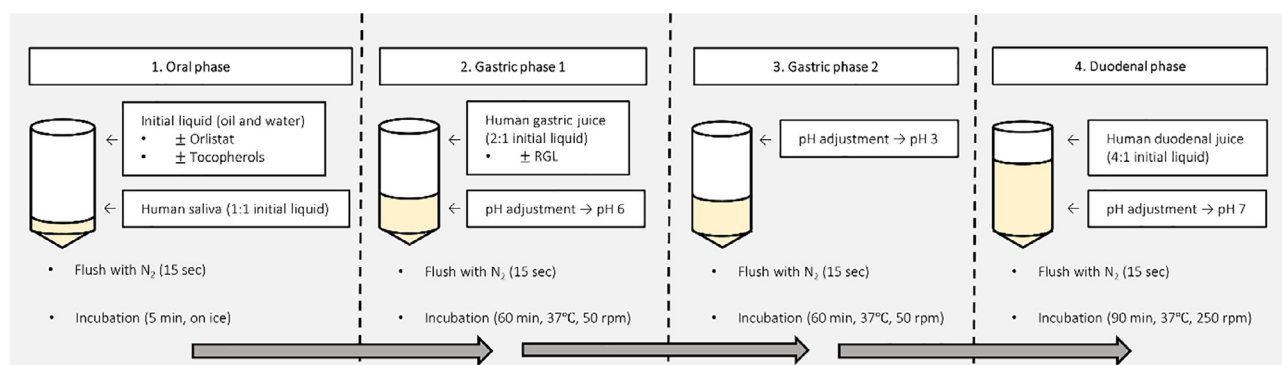
Fig. 1. Flow chart of digestion protocol.

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 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 buffering capacity of lipids during 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), with 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 human digestive fluids were considered more natural than boosting the GI fluids with additional external enzymes. After each addition of digestive fluid, i.e. at the start of the mouth, stomach and intestinal steps, samples were flushed with N_2 gas (15 s) for oxygen reduction. An overview of the changes in pH and dilution during the digestion protocol is presented in Fig. 2. Sampling of digests were done in initial liquid, after addition of saliva, in the gastric phase (t = 0, 60, and 120 min), and in the duodenal phase (t = 0, 45, and 90 min); using
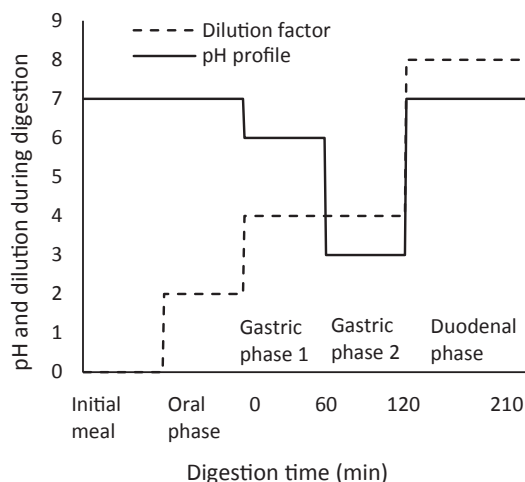
individual test tubes for each collection point, to avoid inflow of external oxygen during sampling. Tubes with digests were flushed with N_2 gas (15 s) and stored in −80 °C immediately after sampling. Digestion was repeated three times for each test condition.

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 $CHCl_3$-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. $CHCl_3$-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) samples, and in ratio 1:2 for low-fat samples (fat content < 2%; samples at end of duodenal digestion). The BHT-enriched $CHCl_3$-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 (N_2 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 NH_2 SPE-colons (500 mg/6 mL; Kinesis, St Neots, UK) and Mega Bond Elut-NH_2 (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

Fig. 2. Changes in pH and initial meal dilution factor over time during static *in vitro* digestion according to the protocol.

Table 1
Fatty acid composition of marine oils, from analysis of total fatty acid methyl esters (FAME), mg FAME/g oil, *n* = 3. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

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[a]	192 ± 0.98	298 ± 5.02	190 ± 1.67	503 ± 0.62
Sum LC n-6 PUFA[a]	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

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

dissolved in $CHCl_3$-MeOH (2:1). Lipid extracts, FFA-extracts and oils were methylated by the in-house methanolic-HCl 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 Supplementary 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 digestion blanks, algae-, anchovy-, and krill oil digests; aldehyde analysis) were replaced with mean values ((max + min)/2) to adjust the residual degrees of freedom (df) to allow for analysis. A significance level of *p* < 0.05 was used. Data presented in the figures is non-transformed.

3. Results

3.1. Lipid oxidation and lipolysis of marine oils of different origin during in vitro digestion

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 (p = 0.03; p = 0.02, respectively) was observed. During duodenal digestion (Fig. 3e), an overall visual trend of increasing formation of HHE was shown. Again, the development of HHE in cod liver oil 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- 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).

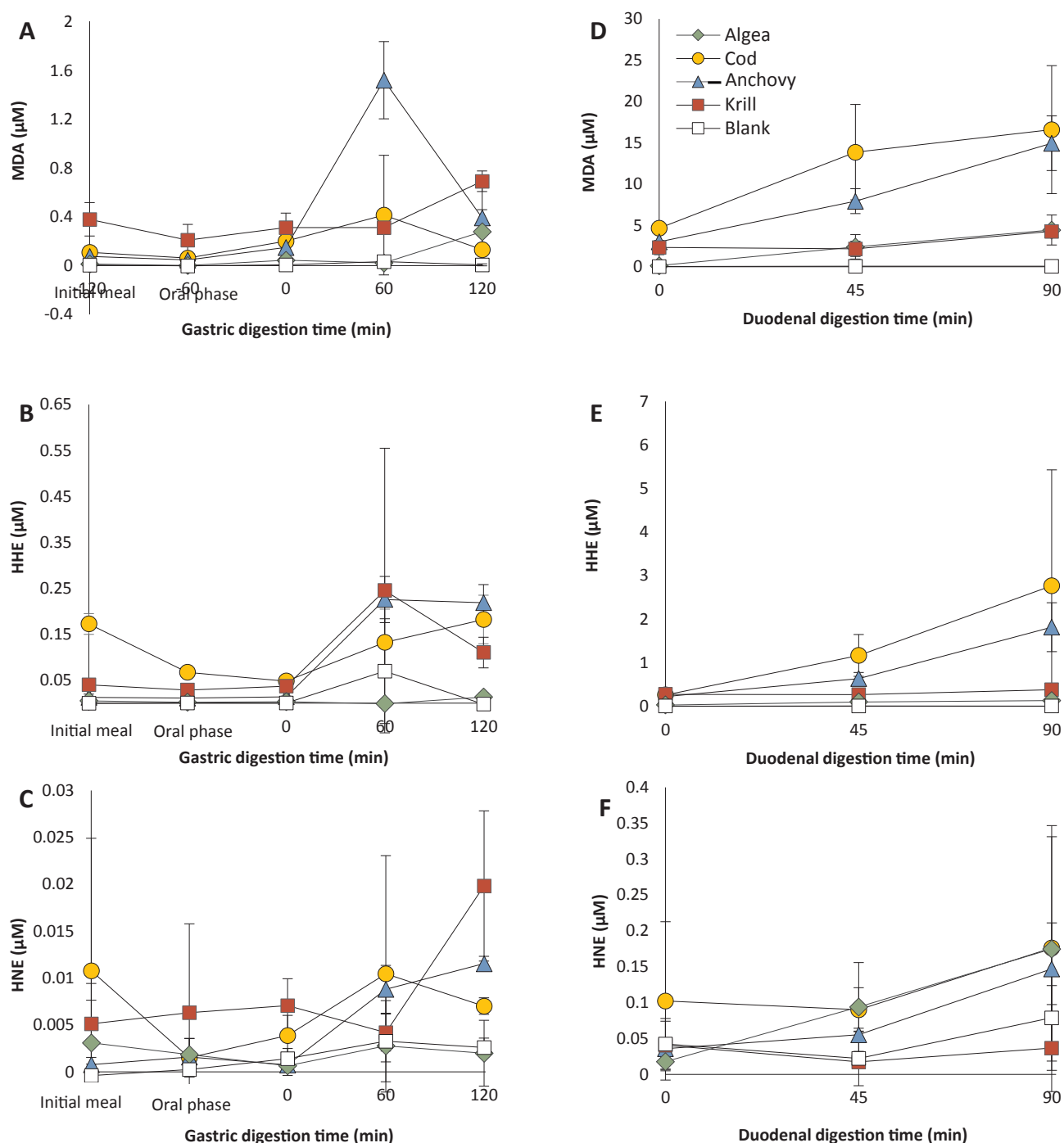For HHE and HNE, no significant differences were seen between

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).

gastric or duodenal digests with pure cod liver oil and addition of or- listat (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, ex- cept 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 ob- served 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
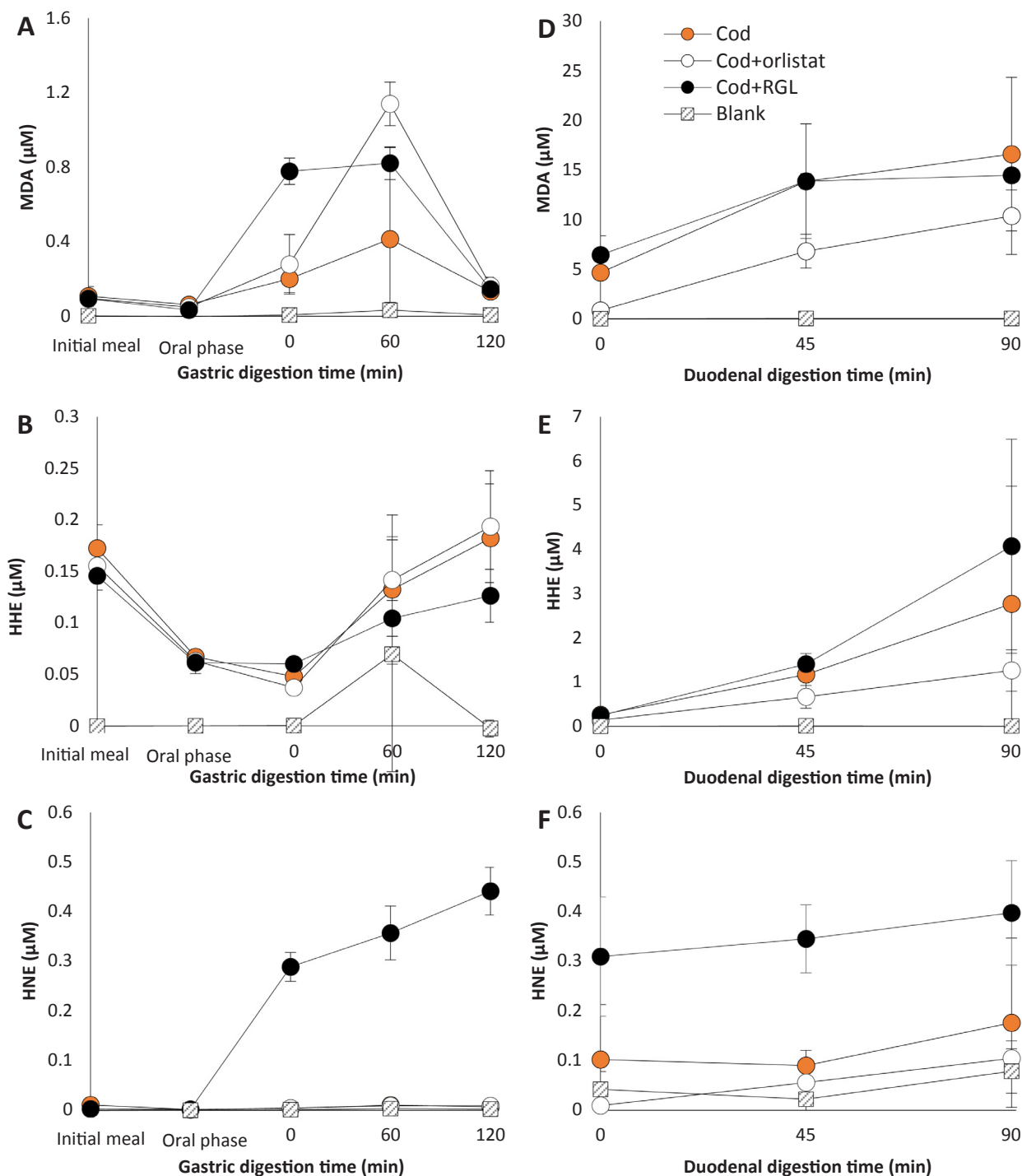
Fig. 4. Effect of addition Orlistat and rabbit gastric lipase (RGL) to cod liver oil on the formation of reactive aldehydes during gastric (0–120 min) and duodenal (120–210 min) static *in vitro* digestion. Malondialdehyde (MDA), 4-hydroxy-*trans*-2-hexenal (HHE), and 4-hydroxy-*trans*-2-nonenal (HNE). 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).

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
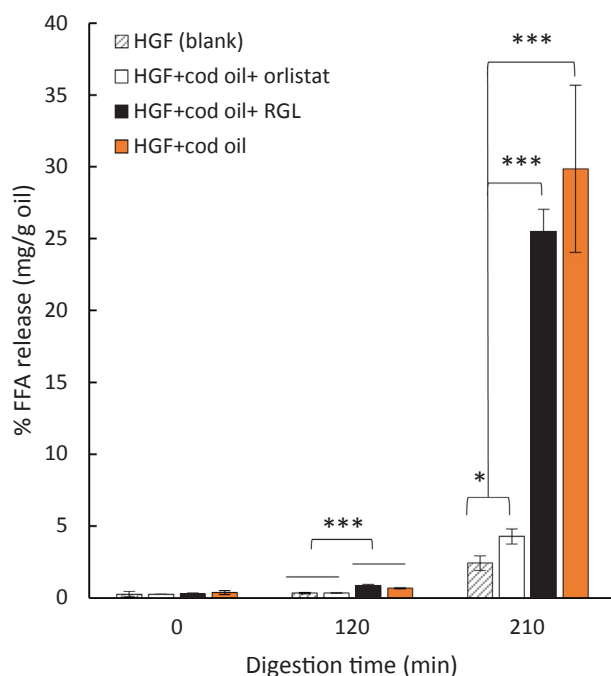
Fig. 5. Release of free fatty acids (FFA) during gastric (120 min) and duodenal (210 min) digestion of cod liver oil, including addition of orlistat and rabbit gastric lipase (RGL). Results are shown as % of FFA per detected total fatty acids (FA) in oil. Error bars are shown as mean ± SD (n > 2) or (max–min)/2 (n = 2). Significant differences are in the figures shown as * = p < 0.05, and *** = p < 0.001.

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

3.3. Effect of addition of α-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 digestion was shown (Fig. 6a). However, during duodenal digestion there was a visual trend that MDA formation was inhibited by both α-tocopherol and Covi-ox® T 70 EU, the latter being most effective (Fig. 6d). At the start of duodenal digestion, t = 0 min, a significant difference was seen between pure cod liver oil digests and those with Covi-ox® T 70 EU (0.00002), and α-tocopherol (p = 0.0005) added. At the end of digestion, a significant difference was also seen between pure cod liver oil and Covi-ox® T 70 EU-fortified cod liver oil (t = 90 min, p = 0.004); the MDA levels detected were 3.5, 10.2, and 16.6 μM in digests with Covi-ox® T 70 EU, α-tocopherol, and without tocopherol, respectively.

For HHE, a reduced development both during gastric and duodenal digestion was observed, with the strongest effect again seen from Covi-ox® T 70 EU. The levels detected at the end of gastric digestion were 0.06, 0.12 and 0.18 μM; and after duodenal digestion 0.30, 0.90 and 2.77 μM in digests with Covi-ox® T 70 EU, α-tocopherol, and no tocopherol, respectively (Fig. 6b and 6e). A significant difference between pure cod liver oil digests and those fortified with Covi-ox® T 70 EU was seen at the end of duodenal digestion (t = 90 min, p = 0.01).

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 digests containing the highest amount of total oil (mg) were those with cod liver oil, but the oxidation profiles for MDA, HHE and HNE for cod liver oil and anchovy oil were still very similar, limiting the likely impact from this factor. Krill oil is rich in astaxanthin and PLs (130 ppm and 40 g/100 g oil, respectively, according to supplier), which both could act protectively against lipid oxidation (Cui & Decker, 2016), although the surface active PLs additionally can act pro-oxidatively (Waraho, McClements, & Decker, 2011) and are sometimes ascribed as more susceptible to oxidative attack than TG (Love & Pearson, 1971). In a previous study, feeding rats with krill oil had an inhibiting effect on oxidative stress and inflammation levels (Grimstad et al., 2012), which indicates that some components in krill oil could have a protective effect against lipid oxidation. We hypothesize the astaxanthin played a significant protective effect under GI-conditions, however, evidence for this should be a subject for future studies. Another possibility which would need to be explored further is that the PLs in the krill oil changed the oxidation route compared to that taking place in oils rich in TG (Thomsen et al., 2013). Alternative oxidation products that can form in PL-rich oils via Schiff base formation are pyrroles (Lu, Nielsen, Baron, & Jacobsen, 2012) and Strecker aldehydes (Lu, Bruheim, Haugsgjerd, & Jacobsen, 2014). In turn, algae oil contains high levels of inherent antioxidants such as polyphenols and tocopherols (Lv et al., 2015), which could explain the low lipid oxidation development seen in the algae oil digests. This was confirmed by the analysis of total inherent tocopherols, which ranked the oils as algae > krill > cod liver > anchovy oil (Supplementary material, Table S4). Worth noting is that anchovy and cod liver oils found in the store commonly contain added antioxidants, e.g. tocopherols, to prevent lipid oxidation from occurring.

The relative amount of PUFA in each meal ranked the four marine oils as algae > krill > anchovy ~ cod, and normalization towards EPA and DHA levels ranked them as algae > anchovy > cod > krill. The individual ranking orders on basis of LC n-3 and n-6 PUFAs did neither match with the susceptibility of the four oils to HHE and HNE development. This indicates that the total FA unsaturation degree has a minor impact on aldehyde formation during GI-conditions. We hypothesize instead that endogenous antioxidants plays an important role for the formation of MDA, HHE and HNE during static *in vitro* digestion of marine oils. Another possibility which would explain that the ranking order of LC n-3 and n-6 PUFA did not match with the HHE and HNE development, could be that other oxidation products than those measured here were formed, especially from krill- and algae oil. The results from this study are in agreement with what was reported by Ryckebosch et al. who found that krill oil and algae oil were less susceptible to lipid oxidation, compared to fish oil, during 8 weeks of storage at 37 °C (Ryckebosch et al., 2013).

At 60 min gastric digestion, the pH was decreased from 6 to 3, which could explain an enhancement in lipid oxidation in the digests with anchovy oil. 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
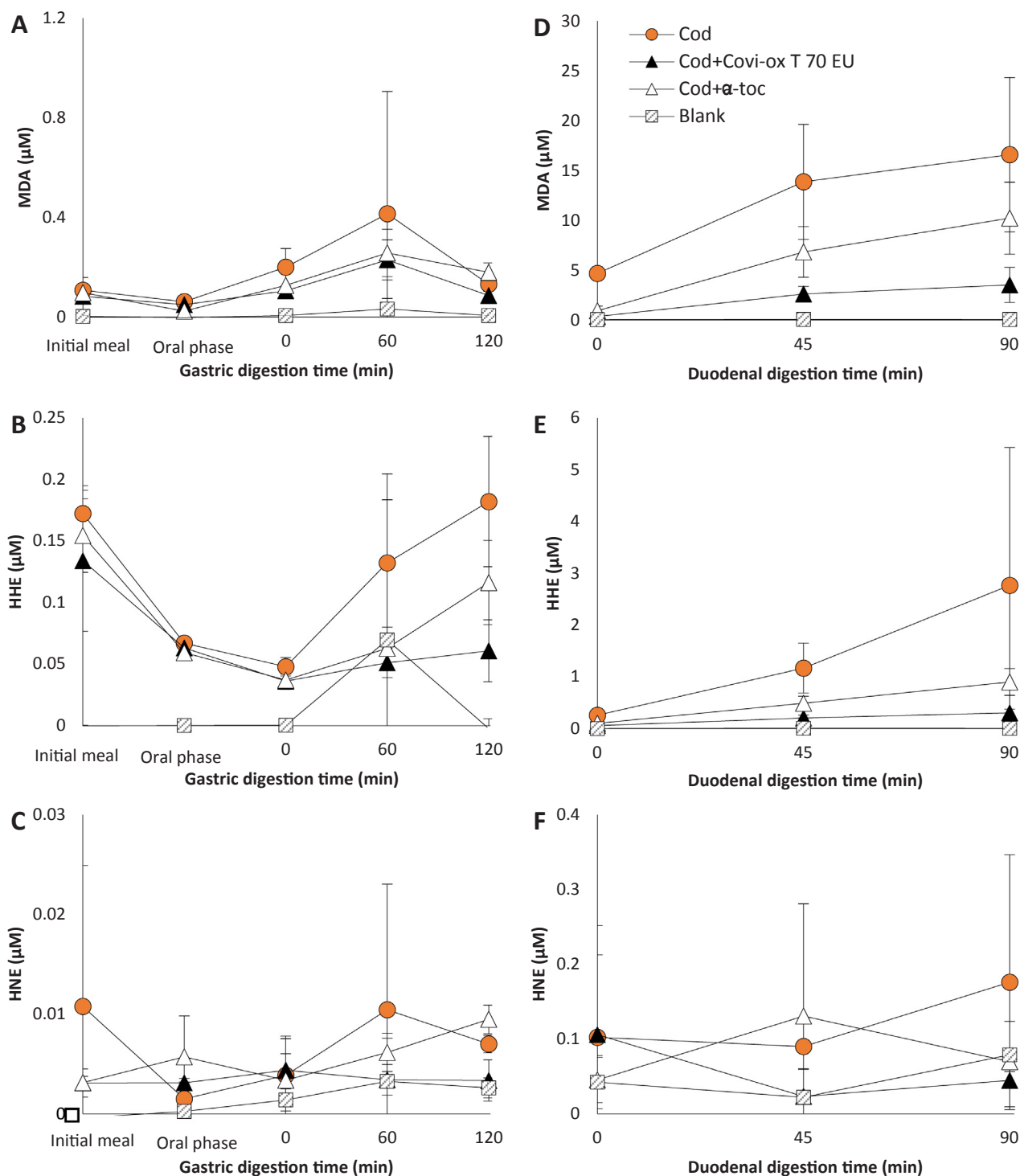
Fig. 6. Effect of addition of Covi-ox® T 70 (tocopherol mix), α-tocopherol (α-toc) to cod liver oil on the formation of reactive aldehydes during gastric (0–120 min) and duodenal (120–210 min) static *in vitro* digestion. Malondialdehyde (MDA), 4-hydroxy-*trans*-2-hexenal (HHE), and 4-hydroxy-*trans*-2-nonenal (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).

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. However, Larsson et al. also observed that the initial oxidative status of cod liver oil mattered for which final *levels* of lipid oxidation products that 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

Nieva-Echevarría, 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 interphase (Waraho 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 lipase inhibitor orlistat would reduce lipid oxidation during digestion. Results showed that orlistat inhibited lipolysis and delayed the onset of duodenal lipid oxidation, which confirms the hypothesis that there is a link between lipolysis and lipid oxidation during GI-conditions. The addition of RGL had no major effect on lipid oxidation, with the exception of HNE formation, which was stimulated. When looking into the specific FFA released with versus without RGL, total n-3 PUFA release was unaffected, while, although non-significant, approximately 2 times more n-6 PUFA was released with RGL. This could explain the difference in formation of HHE and HNE, which originate from n-3 PUFA and n-6 PUFA, respectively. This also further confirms the importance of lipolysis in relation to lipid oxidation, and illustrates the importance of including relevant lipases; we would recommend researchers working with digestion models and lipid containing matrixes to include gastric lipase. To our knowledge, there is not yet any commercial human gastric lipase available on the market, however using gastric lipase of other origin, such as rabbit- or dog gastric lipase, have been suggested as suitable substitutes (Sams et al., 2016).

The release of DHA from the algae oil was significantly higher than from the other marine oil digests at the end of digestion. This can be expected, considering the high levels of DHA found in the algae oil from start. At the end of digestion, algae oil digests also had the highest level of free n-3 PUFA; 2-times higher compared to krill- and anchovy oil digests, and 6.5 times higher than cod liver oil digests. However, as discussed in Section 4.1, the high levels of free n-3 PUFA did not translate into a high development of HHE in the algae oil digests, suggesting that the net effect of endogenous antioxidants was larger than the effect of lipolysis.

When adding tocopherols to the cod liver oil (4.5 mg/g oil) we here saw that lipid oxidation could be delayed. Based on the fact that addition of 1 mg α-tocopherol/g oil to cod liver oil subjected to static *in vitro* digestion previously did not shown any effect on lipid oxidation (Larsson et al., 2012), there seem to be a dose-response effect from tocopherols on GI lipid oxidation. This is further strengthened by the

study of Van Hecke et al. (2016), who showed that, lipid oxidation during digestion of high fat beef was reduced with increasing amounts of α-tocopherol from 0 to 4.5 mg/g (Van Hecke et al., 2016). The addition of a tocopherol mix was expected to work more efficiently compared to addition of pure α-tocopherol, due to synergistic effects. Kenmogne Domguia et al. reported that at low levels (< 2 ppm), δ- and γ-tocopherols were still detectable after static *in vitro* digestion, while α-tocopherol quickly was consumed (Kenmogne-Domguia et al., 2014). Similarly, Ha and Igarashi found that α-tocopherol was more susceptible to oxidation, compared to γ- and δ-tocopherol when added to methyl linoleate (Ha and Igarashi, 1990). The Covi-ox® T 70 EU contains 14% α-, 2% β-, 60% γ-, and 24% δ-tocopherol, and it is well known that the homologues of the tocopherols have different actions, explained by differences in the chemical structure of the homologues. The active groups, the chromanol ring of the tocopherols, have the methyl substitutions placed in different positions in the different homologues (Muggli, 2012). The antioxidative activity of the different tocopherol isomers has previously been found to be ranked as α- > γ- > β- > δ-tocopherol (T = 20–60 °C) in lard (Telegdy and Berndorfer, 1968) while they were ranked as δ- > β- > γ- > α-tocopherol in vegetable oils (rapeseed-, soybean-, corn- and olive oil) (Yoshida, Hirooka, & Kajimoto, 1991), Seppanen, Song, and Saari Csallany (2010) described how both the specific food matrix and the tocopherol concentration highly influences the antioxidative effect, which together with the other findings illustrate how the prevention of lipid oxidation by tocopherols must be carefully optimized for each specific product and condition.

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 lipase 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-ox® T 70 EU, which consist of several tocopherol isomers, was found to be more protective than α-tocopherol.

6. Conflict of interests

References

Adarme-Vega, T. C., Thomas-Hall, S. R., & Schenk, P. M. (2014). Towards sustainable sources for omega-3 fatty acids production. *Current Opinion in Biotechnology, 26*, 14–18. https://doi.org/10.1016/j.copbio.2013.08.003.

Cui, L., & Decker, E. A. (2016). Phospholipids in foods: Prooxidants or antioxidants? *Journal of the Science of Food and Agriculture, 96*(1), 18–31. https://doi.org/10.1002/jsfa.7320.

Del Rio, D., Stewart, A. J., & Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism & Cardiovascular Diseases, 15*(4), 316–328. https://doi.org/10.1016/j.numecd.2005.05.003.

Delgado-Lista, J., Perez-Martinez, P., Lopez-Miranda, J., & Perez-Jimenez, F. (2012). Long chain omega-3 fatty acids and cardiovascular disease: A systematic review. *British*

Journal of Nutrition, 107 Suppl 2(S2), S201–S213. https://doi.org/10.1017/S0007114512001596.

EFSA-Panel on Dietetic Products, Nutrition and Allergies (2012). Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). *EFSA Journal, 10*(7), 2815. https://doi.org/10.2903/j.efsa.2012.2815.

Frankel, E. N., Satué-Gracia, T., Meyer, A. S., & German, J. B. (2002). Oxidative stability of fish and algae oils containing long-chain polyunsaturated fatty acids in bulk and in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 50*(7), 2094–2099. https://doi.org/10.1021/jf0111458.

Gobert, M., Remond, D., Loonis, M., Buffiere, C., Sante-Lhoutellier, V., & Dufour, C. (2014). Fruits, vegetables and their polyphenols protect dietary lipids from oxidation during gastric digestion. *Food & Function, 5*(9), 2166–2174. https://doi.org/10.1039/C4FO00269E.

Grimstad, T., Bjørndal, B., Cacabelos, D., Aasprong, O. G., Janssen, E. A. M., Omdal, R., ... Berge, R. K. (2012). Dietary supplementation of krill oil attenuates inflammation and oxidative stress in experimental ulcerative colitis in rats. *Scandinavian Journal of Gastroenterology, 47*(1), 49–58. https://doi.org/10.3109/00365521.2011.634025.

Ha, K.-H., & Igarashi, O. (1990). The oxidation products from two kinds of tocopherols co-existing in autoxidation system of methyl linoleate. *Journal of Nutritional Science and Vitaminology, 36*(4-SupplementI), 411–421. https://doi.org/10.3177/jnsv.36.4-SupplementI_411.

Hu, M. (2016). Introduction. In M. Hu, & C. Jacobsen (Eds.). *Oxidative stability and shelf life of foods containing oils and fats* (pp. xvii–xxxviii). London, UK: AOCS Press.

Kanner, J., & Lapidot, T. (2001). The stomach as a bioreactor: Dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biology and Medicine, 31*(11), 1388–1395. https://doi.org/10.1016/S0891-5849(01)00718-3.

Kassis, N. M., Gigliotti, J. C., Beamer, S. K., Tou, J. C., & Jaczynski, J. (2012). Characterization of lipids and antioxidant capacity of novel nutraceutical egg products developed with omega-3-rich oils. *Journal of the Science of Food and Agriculture, 92*(1), 66–73. https://doi.org/10.1002/jsfa.4542.

Kenmogne-Domguia, H. B., Moisan, S., Viau, M., Genot, C., & Meynier, A. (2014). The initial characteristics of marine oil emulsions and the composition of the media inflect lipid oxidation during *in vitro* gastrointestinal digestion. *Food Chemistry, 152*, 146–154. https://doi.org/10.1016/j.foodchem.2013.11.096.

Kostewicz, E. S., Abrahamsson, B., Brewster, M., Brouwers, J., Butler, J., Carlert, S., ... Augustijns, P. (2014). *In vitro* models for the prediction of *in vivo* performance of oral dosage forms. *European Journal of Pharmaceutical Sciences, 57*, 342–366. https://doi.org/10.1016/j.ejps.2013.08.024.

Kristinova, V., Storrø, I., & Rustad, T. (2013). Influence of human gastric juice on oxidation of marine lipids – *in vitro* study. *Food Chemistry, 141*(4), 3859–3871. https://doi.org/10.1016/j.foodchem.2013.06.011.

Larsson, K., Almgren, A., & Undeland, I. (2007). Hemoglobin-mediated lipid oxidation and compositional characteristics of washed fish mince model systems made from cod (Gadus morhua), herring (Clupea harengus) and salmon (Salmo salar) muscle. *Journal of Agricultural and Food Chemistry, 55*(22), 9027–9035. https://doi.org/10.1021/jf070522z.

Larsson, K., Cavonius, L., Alminger, M., & Undeland, I. (2012). Oxidation of cod liver oil during gastrointestinal *in vitro* digestion. *Journal of Agriculture and Food Chemistry, 60*(30), 7556–7564. https://doi.org/10.1021/jf301444x.

Larsson, K., Tullberg, C., Alminger, M., Havenaar, R., & Undeland, I. (2016). Malondialdehyde and 4-hydroxy-2-hexenal are formed during dynamic gastrointestinal in vitro digestion of cod liver oils. *Food & Function, 7*(8), 3458–3467. https://doi.org/10.1039/c6fo00635c.

Lee, S. H., & Blair, I. A. (2000). Characterization of 4-oxo-2-nonenal as a novel product of lipid peroxidation. *Chemical Research in Toxicology, 13*(8), 698–702. https://doi.org/10.1021/tx000101a.

Love, J. D., & Pearson, A. M. (1971). Lipid oxidation in meat and meat products—A review. *Journal of the American Oil Chemists' Society, 48*(10), 547–549. https://doi.org/10.1007/bf02544559.

Lu, F. S., Bruheim, I., Haugsgjerd, B. O., & Jacobsen, C. (2014). Effect of temperature towards lipid oxidation and non-enzymatic browning reactions in krill oil upon storage. *Food Chemistry, 157*(Supplement C), 398–407. https://doi.org/10.1016/j.foodchem.2014.02.059.

Lu, F. S. H., Nielsen, N. S., Baron, C. P., & Jacobsen, C. (2012). Oxidative degradation and non-enzymatic browning due to the interaction between oxidised lipids and primary amine groups in different marine PL emulsions. *Food Chemistry, 135*(4), 2887–2896. https://doi.org/10.1016/j.foodchem.2012.07.008.

Lv, J., Yang, X., Ma, H., Hu, X., Wei, Y., Zhou, W., & Li, L. (2015). The oxidative stability of microalgae oil (*Schizochytrium aggregatum*) and its antioxidant activity after simulated gastrointestinal digestion: Relationship with constituents. *European Journal of Lipid Science and Technology, 117*(12), 1928–1939. https://doi.org/10.1002/ejlt.201400588.

Lykkesfeldt, J. (2007). Ascorbate and dehydroascorbic acid as reliable biomarkers of oxidative stress: Analytical reproducibility and long-term stability of plasma samples subjected to acidic deproteinization. *Cancer Epidemiology Biomarkers & Prevention, 16*(11), 2513–2516. https://doi.org/10.1158/1055-9965.epi-07-0639.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., ... Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food – An international consensus. *Food & Function, 5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j.

Miyashita, K., & Takagi, T. (1986). Study on the oxidative rate and prooxidant activity of

free fatty acids. *Journal of the American Oil Chemists' Society, 63*(10), 1380–1384. https://doi.org/10.1007/bf02679607.

Muggli, R. (2012). Applications in food products. In H. Breivik (Ed.). *Long-chain Omega-3 specialty oils* (pp. 165–195). Woodhead Publishing.

N'Goma, J.-C. B., Amara, S., Dridi, K., Jannin, V., & Carrière, F. (2011). Understanding the lipid-digestion processes in the GI tract before designing lipid-based drug-delivery systems. *Therapeutic Delivery, 3*(1), 105–124. https://doi.org/10.4155/tde.11.138.

Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2017a). Effect of liquid smoking on lipid hydrolysis and oxidation reactions during in vitro gastrointestinal digestion of European sea bass. *Food Research International, 97*, 51–61. https://doi.org/10.1016/j.foodres.2017.03.032.

Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2017b). Polyunsaturated lipids and vitamin A oxidation during cod liver oil in *vitro* gastrointestinal digestion. Antioxidant effect of added BHT. *Food Chemistry, 232*, 733–743. https://doi.org/10.1016/j.foodchem.2017.04.057.

Pryor, W. A., & Porter, N. A. (1990). Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids. *Free Radical Biology and Medicine, 8*(6), 541–543. https://doi.org/10.1016/0891-5849(90)90153-A.

Rindgen, D., Nakajima, M., Wehrli, S., Xu, K., & Blair, I. A. (1999). Covalent modifications to 2′-deoxyguanosine by 4-oxo-2-nonenal, a novel product of lipid peroxidation. *Chemical Research in Toxicology, 12*(12), 1195–1204. https://doi.org/10.1021/tx990034o.

Ryckebosch, E., Bruneel, C., Termote-Verhalle, R., Lemahieu, C., Muylaert, K., Van Durme, J., ... Foubert, I. (2013). Stability of omega-3 LC-PUFA-rich microalgal oils compared to commercially available omega-3 LC-PUFA oils. *Journal of Agricultural and Food Chemistry, 61*(42), 10145–10155. https://doi.org/10.1021/jf402296s.

Sams, L., Paume, J., Giallo, J., & Carriere, F. (2016). Relevant pH and lipase for in vitro models of gastric digestion. *Food & Function.* https://doi.org/10.1039/C5FO00930H.

Sayre, L. M., Lin, D., Yuan, Q., Zhu, X., & Tang, X. (2006). Protein adducts generated from products of lipid oxidation: Focus on HNE and ONE. *Drug Metabolism Reviews, 38*(4), 651–675. https://doi.org/10.1080/03602530600959508.

Schaich, K. M. (2013). Challenges in analyzing lipid oxidation: are one product and one sample concentration enough? In A. Logan, U. Nienaber, & X. Pan (Eds.). *Lipid oxidation* (pp. 53–128). AOCS Press.

Schuchardt, J. P., & Hahn, A. (2013). Bioavailability of long-chain omega-3 fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA), 89*(1), 1–8. https://doi.org/10.1016/j.plefa.2013.03.010.

Schuchardt, J. P., Schneider, I., Meyer, H., Neubronner, J., von Schacky, C., & Hahn, A. (2011). Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3 fatty acid formulations – A comparative bioavailability study of fish oil vs. krill oil. *Lipids in Health and Disease, 10*, 145. https://doi.org/10.1186/1476-511X-10-145.

Seppanen, C. M., Song, Q., & Saari Csallany, A. (2010). The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. *Journal of the American Oil Chemists' Society, 87*(5), 469–481. https://doi.org/10.1007/s11746-009-1526-9.

Shewfelt, R. L. (1981). Fish muscle lipolysis – A review. *Journal of Food Biochemistry, 5*(2), 79–100. https://doi.org/10.1111/j.1745-4514.1981.tb00663.x.

Telegdy, & Berndorfer (1968). On the antioxidative mechanism of tocopherols (α-, β-, γ-, δ-) in Lard. *Nahrung, 12*, 407–414.

Thomsen, B. R., Haugsgjerd, B. O., Griinari, M., Lu, H. F. S., Bruheim, I., Vogt, G., ... Jacobsen, C. (2013). Investigation of oxidative degradation and non-enzymatic browning reactions in krill and fish oils. *European Journal of Lipid Science and Technology, 115*(12), 1357–1366. https://doi.org/10.1002/ejlt.201300141.

Tullberg, C., Larsson, K., Carlsson, N.-G., Comi, I., Scheers, N., Vegarud, G., & Undeland, I. (2016). Formation of reactive aldehydes (MDA, HHE, HNE) during the digestion of cod liver oil: Comparison of human and porcine *in vitro* digestion models. *Food & Function, 7*(3), 1401–1412. https://doi.org/10.1039/c5fo01332a.

Undeland, I., Hultin, H. O., & Richards, M. P. (2002). Added triacylglycerols do not hasten hemoglobin-mediated lipid oxidation in washed minced cod muscle. *Journal of Agricultural and Food Chemistry, 50*(23), 6847–6853. https://doi.org/10.1021/jf0201982.

Van Hecke, T., Wouters, A., Rombouts, C., Izzati, T., Berardo, A., Vossen, E., ... De Smet, S. (2016). Reducing compounds equivocally influence oxidation during digestion of a high-fat beef product, which promotes cytotoxicity in colorectal carcinoma cell lines. *Journal of Agricultural and Food Chemistry, 64*(7), 1600–1609. https://doi.org/10.1021/acs.jafc.5b05915.

Van Kuijk, F. J., Holte, L. L., & Dratz, E. A. (1990). 4-Hydroxyhexenal: A lipid peroxidation product derived from oxidized docosahexaenoic acid. *Biochimica et Biophysica Acta, 1043*(1), 116–118. https://doi.org/10.1016/0005-2760(90)90118-H.

Waraho, T., McClements, D. J., & Decker, E. A. (2011). Mechanisms of lipid oxidation in food dispersions. *Trends in Food Science and Technology, 22*(1), 3–13.

Ville, E., Carrière, F., Renou, C., & Laugier, R. (2002). Physiological study of pH stability and sensitivity to pepsin of human gastric lipase. *Digestion, 65*(2), 73–81.

Yao, L., Gerde, J. A., Lee, S.-L., Wang, T., & Harrata, K. A. (2015). Microalgae lipid characterization. *Journal of Agricultural and Food Chemistry, 63*(6), 1773–1787. https://doi.org/10.1021/jf5050603.

Yoshida, H., Hirooka, N., & Kajimoto, G. (1991). Microwave heating effects on relative stabilities of tocopherols in oils. *Journal of Food Science, 56*(4), 1042–1046. https://doi.org/10.1111/j.1365-2621.1991.tb14637.x.