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Role of lipolysis and tocopherols in the formation of primary and secondary oxidation products during simulated gastrointestinal digestion of n-3 PUFA-rich oils

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ABSTRACT

Oxidation of n-3 PUFA-rich oils in the gastrointestinal (GI) tract leads to the formation of potentially harmful compounds such as 4-hydroxy-2-hexenal (HHE), 4-hydroxy-2-nonenal (HNE), and malondialdehyde (MDA). This study investigated the interplay between fatty acids release, tocopherol concentration, and formation of hydroperoxides, HHE, HNE, and MDA during simulated digestion of *Schizochytrium* sp. oils, in comparison with cod liver and linseed oils. Free DHA showed a stronger correlation with tocopherol consumption than esterified DHA. Hydroperoxide formation showed non-linear relationship with tocopherol amounts and free PUFAs, though also bound PUFAs correlated to hydroperoxide formation. Key fatty acids explaining increases in oxidation markers other than DHA, such as 20:3n-6, were identified. While HHE formation correlated with oil unsaturation and esterified DHA, MDA had linear relationship with hydroperoxide formation. These findings enhance the understanding of lipid oxidation mechanisms during digestion, providing valuable insights for improving the stability of n-3-rich oils in food applications.

1. Introduction

Omega-3 long-chain polyunsaturated fatty acids (n-3 PUFAs), especially eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids are essential for human health, contributing to critical functions such as brain development, cardiovascular health, and regulation of inflammation. For example, increased dietary intake of EPA and DHA has been linked to improved cognitive function and a lower risk of heart disease (Saini & Keum, 2018). Marine foods are the main traditional source of n-3 PUFAs but these resources are facing significant challenges, such as overfishing (FAO, 2022), price fluctuations (Oliver et al., 2020), and increase in ocean water temperature (Colombo et al., 2020). Modifications of the aquatic food supply system would noticeably increase yearly n-3 PUFAs stock but still at inadequate levels to address an increasing world population (Shepon et al., 2022).

Among the primary marine producers of DHA- and EPA-rich oils, the heterotroph *Schizochytrium* is considered among the best alternatives to fisheries. Besides minimization of potential environmental contaminants, *Schizochytrium* is made attractive by lower energy requirements compared to photosynthetic microorganisms and its high DHA contents (up to 50%) (Demets & Foubert, 2021). Moreover, *Schizochytrium* oil is produced in triacylglycerol (TAG) form, allowing the application of traditional refining practices (Demets & Foubert, 2021). *Schizochytrium* is the dominant microbial DHA source in the EU, as safety and regulatory acceptance of *Schizochytrium* sp. oils are supported by EFSA opinions (Turck et al., 2021).

Oils with high PUFA content are prone to oxidation due to their high degree of unsaturation. In presence of pro-oxidants, unsaturated lipids generate radicals able to react with oxygen to form hydroperoxides (primary oxidation products) and another lipid radical, able in turn to propagate the reaction. Hydroperoxides decompose through radical scission which, besides propagating the reaction, generates multiple

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secondary volatile and non-volatile oxidation products such as aldehydes, ketones, epoxides, oxo-fatty acids. Among the aldehydes, malondialdehyde (MDA) is formed through the β-scission of cyclical hydroperoxides formed in PUFAs, irrespective of their class. On the other hand, 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE) originate from n-3 and n-6, respectively, with multiple formation pathways possible (Frankel, 2005). Due to their high reactivity, these aldehydes have been considered agents in chronic inflammation, neurodegenerative diseases, atherosclerosis, and cytotoxicity (Esterbauer et al., 1991; Tullberg et al., 2016).

The exposure of n-3-rich oils to prooxidants continues from production and storage to consumption. The gastrointestinal tract is a recognized prooxidative environment due e.g., to the oxygen incorporated during mastication, low pH of the stomach, and the presence of transition metal ions. A few studies (Beltrame et al., 2023; Larsson et al., 2012; Tullberg et al., 2019) have revealed that lipolysis also plays a major role for the development of lipid oxidation during the digestion of marine oils. Lipid hydrolysis begins in the stomach with the action of gastric lipase, specific to *sn*-3 position, leading to the formation of diacylglycerols (DAGs) and free fatty acids (FFAs). While the lipolysis extent in the stomach is low, compared to the small intestine, it is considered essential for initiating intestinal lipolysis, as DAGs and proteins begin lipid emulsification with the aid of stomach peristalsis. Main enzymatic breakdown of TAG occurs in the small intestine with pancreatic lipase, which hydrolyzes *sn*-1,3 positions, aided by digestion products acting as emulsifiers. Pancreatic lipase adsorbs to the lipid–water interface and hydrolyzes TAGs into FFAs and 2-monoacylglycerols (2-MAGs). Bile salts incorporate digestion products in mixed micelles, promoting lipolysis and aiding absorption. Released fatty acids are generally considered more labile to oxidation, compared to acylglycerols, but the role of specific liberated fatty acids in lipid oxidation during digestion is unclear. Studies from our team noticed increases in lipid oxidation in n-3 PUFA-rich oil after incrementing lipolysis by supplementing additional lipase (Tullberg et al., 2019) and increases in hydroperoxide addition to 20:5n-3 in position *sn*-2 when position *sn*-1,3 of structured lipids was hydrolyzed during digestion (Beltrame et al., 2023).

Linear models explaining oil storage stability with their fatty acid composition have been published (Kerrihard et al., 2015). Although differences in composition and positional distribution of fatty acids in oils can influence lipolysis, which in turn can influence the formation of oxidation products, such models are currently unavailable for simulated digestion conditions.

Tocopherols are antioxidants commonly found in and added to oils, including *Schizochytrium*. In an oil-in-water emulsion, e.g. the typical physical form of oils in the gastrointestinal tract, tocopherols are typically located at the interface, which is a crucial location for lipid oxidation (Losada-Barreiro et al., 2014). The formation of micelles during the intestinal phase and release of FFA make the behavior of tocopherols worth attention, as different environments and non-covalent interactions can alter their antioxidant activity (Hennebelle et al., 2024). Moreover, differences in the tocopherol content of oils can influence the oxidation process.

We hypothesize that fatty acid composition of n-3 PUFA-rich oils determines the FFA release during digestion, which in turn influences lipid oxidation. Therefore, the present study for the first time details the interplay between fatty acid composition, lipolysis, and tocopherol concentration in the formation of primary and secondary oxidation products during simulated digestion of n-3 PUFA-rich oils with a particular focus on *Schizochytrium sp* oils.

2. Materials and methods

2.1. Chemicals and enzymes

All solvents, salts, and chemicals were supplied by SigmaAldrich (Saint Louis, MO, USA) unless specified otherwise. Concentrated hydrochloric acid was purchased from Avantor (Radnor, PA, USA). Tris (hydroxymethyl)aminomethane (Tris) was purchased from ThermoFisher (Waltham, MA, USA).

Amylase, porcine pepsin, porcine pancreatin, and bile salts were purchased from SigmAldrich. Rabbit gastric lipase (RGE15-1G) was obtained from Lipolytech (Marseille, France). Proteins and enzymes used as standards in the enzyme activity essays were all purchased from SigmaAldrich.

Standards of α-, β-, γ-, and δ-tocopherol were purchased from SigmaAldrich. Standards of HHE and HNE were purchased from Cayman Chemicals (Ann Arbor, MI, USA), while 1,1,3,3-tetraethoxypropane (TEP) was purchased from SigmaAldrich.

2.2. Oils

Oils of different origins were used in the digestion experiments. Fish oil from cod liver (FO), restructured cod liver oil (RO) and linseed (LO) oils were obtained from commercial sources. Four different *Schizochytrium sp* oils were obtained from commercial sources and were labeled S1–4 in increasing order of measured total PUFAs (Section 2.8 and Table 1). RO was solely employed to validate the PLS model of oil digestion (Section 2.9). Oils were stored at −80 °C prior to experiments as bulk or hexane solutions. All experiments were performed within one year.

2.3. Measurement of enzymatic activity

Lipase activity of porcine pancreatin and rabbit gastric extract (RGE) was measured according to Grundy (Grundy et al., 2021) using a 907 Titrando (Metrohm, Herisau, Switzerland) in pH-stat titration mode controlled with *Tiamo* software. Amylase activity and protease activity of porcine pepsin and RGE were measured according to the Supplementary Information of Brodkorp (Brodkorb et al., 2019).

2.4. Static in vitro digestion

2.4.1. Effect of emulsification on FFA release

The effect of emulsification and oil concentration on lipolysis was tested with LO by comparing released FFA after digestion of bulk and emulsified oil at different chyme concentrations. Sodium caseinate was selected as lipid-free emulsifier. One day before the experiments, sodium caseinate 3% solution was prepared with deionized water at 100 °C. After cooling, the solution was added with sodium azide 0.05% and let to stir overnight. Prior to simulated digestion, LO was emulsified with sodium caseinate solution (20% LO, 53% caseinate solution, 27% deionized water) using an UltraTurrax homogenizer (IKA, Staufen, Germany) at speed 6/6 for 2 min twice while keeping the mixture on ice.

2.4.2. Simulated digestion

The composition of simulated digestion fluids followed the INFOGEST 2.0 protocol (Brodkorb et al., 2019). The required amounts of HCl (1 M) and NaOH (1 M) to bring simulated oral bolus, gastric bolus, and intestinal chyme to pH 7, 3, and 7, respectively, were tested with blank digestions. Experiments were carried in dim light, while digestion themselves were carried in an orbital shaker placed inside a Ehret BK 426 oven (Freiburg, Germany). Aliquotes of oil solutions in hexane were dried with N_2 flow at 37 °C, while LO emulsions were added right after preparation. For simulated oral phase, oils or emulsions were added with simulated saliva fluid pretempered to 37 °C to reach a total bolus volume of 1 mL. After addition of $CaCl_2$ to achieve a total bolus concentration of 1.5 mM, mastication was simulated by hitting the mixture with a glass rod 32 times, followed by incubation for 2 min at 37 °C. Then, the oral bolus was added 1:1 *v*/v with preheated simulated gastric fluid. After addition of $CaCl_2$ to a total gastric bolus concentration of 0.15 mM and pH adjustment, the mixture was incubated at 37 °C for 2 h.

Table 1
Fatty acid composition and initial peroxide value (PV_i) of the oils utilized in this study. Oils are reported by increasing order in relative PUFA content.

Fatty acid	FO	S1	S2	S3	S4	RO	LO
w/w%							
12:0	n.d.	0.9	n.d.	tr.	tr.	tr.	n.d.
14:0	4.09	9.93	8.49	0.33	0.39	0.23	tr.
15:0	0.34	0.7	0.57	0.06	0.11	tr.	n.d.
16:0	9.93	19.76	21.39	9.83	18.27	2.55	3.93
16:1(n-7)	9.4	5.58	4.56	0.14	0.09	0.75	n.d.
18:0	2.09	0.63	0.74	1.44	1.56	4.1	3.3
18:1(n-9)	14.99	5.91	0.29	27.01	14.98	8.38	13.32
18:1(n-7)	4.78	0.27	5.25	0.38	0.3	2.78	n.d.
18:2(n-6)	2.22	tr.	0.13	2.07	1.82	0.9	13.49
18:3(n-6)	0.23	tr.	0.13	0.08	0.11	0.16	n.d.
18:3(n-3)	0.64	tr.	n.d.	0.2	0.28	0.69	65.36
18:4(n-3)*	2.91	0.23	0.3	0.24	0.22	1.97	n.d.
20:0	n.d.	n.d.	tr.	0.12	0.21	0.32	tr.
20:1(n-9)	14.52	n.d.	n.d.	0.09	tr.	1.62	0.17
20:2(n-6)	0.44	n.d.	n.d.	n.d.	n.d.	0.26	n.d.
20:3(n-6)	0.1	tr.	n.d.	0.07	0.13	0.28	n.d.
20:4(n-6)	0.37	0.22	0.3	0.09	0.16	1.69	tr.
20:4(n-3)*	0.76	0.47	0.49	0.46	0.61	1.42	n.d.
20:5(n-3)	9.86	1.49	1.89	0.36	0.61	35.99	n.d.
22:0	tr.	n.d.	tr.	0.33	0.32	0.15	0.11
22:1(n-9)	7.05	n.d.	n.d.	tr.	tr.	0.26	n.d.
22:5(n-6)	n.d.	7.73	8.61	9.14	11.01	1.06	n.d.
22:5(n-3)/24:0	1.23	0.21	0.43	0.24	0.34	4.18	n.d.
22:6(n-3)	13.29	45.72	46.17	47.25	48.31	27.73	n.d.
24:1(n-9)	0.39	n.d.	0.11	n.d.	n.d.	0.51	n.d.
ΣSAFA	16.49	31.92	31.34	12.18	20.96	9.2	7.48
ΣMUFA	51.13	11.77	10.2	27.62	15.43	14.31	13.62
ΣPUFA	32.38	56.32	58.46	60.2	63.61	76.49	78.9
n-3/n-6	8.3	5.91	5.37	4.26	3.81	16.56	4.83
ΣC12–16	23.76	36.87	35.01	10.46	18.96	3.73	4.03
ΣC18	27.86	7.34	6.84	31.42	19.27	18.98	95.47
ΣC20	26.05	2.28	2.76	1.19	1.82	41.58	0.37
ΣC22	21.67	53.66	55.28	57.06	60.08	33.38	0.11
C_{avg}	18.8	19.24	19.4	20.11	20.07	19.79	17.93
ECN	16.7	15.86	15.93	16.41	16.32	16.08	15.56
$C{=}C_{avg}$	2.11	3.38	4.12	3.68	3.74	4.13	2.37
PV_i (meq/kg oil)	8.43 ± 0.65	9.82 ± 0.19	4.63 ± 0.21	0.46 ± 0.07	0.10 ± 0.03	5.96 ± 1.00	1.62 ± 0.23

* identified with literature.

The amount of porcine pepsin required to reach 2000 U/mL was corrected with the protease activity of RGE. At the end of incubation, preheated simulated intestinal fluid was added 1:1 v/v. After addition of $CaCl_2$ to a total chyme concentration of 0.6 mM and pH adjustment, the simulated chyme was incubated for 2 h at 37 °C. Pancreatin amount in simulated intestinal fluid corresponded to a lipase activity of 2000 U/mL in the final chyme.

2.5. Analysis of aldehydes (HHE, HNE, and MDA)

The amounts of HHE, HNE, and MDA in digesta were determined in undigested oils and at the end of simulated digestion but prior to lipid extraction. This was done by employing the liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry (LC/APCI-MS) protocol published by (Tullberg et al., 2016). Briefly, right at the end of intestinal phase incubation, tubes were placed on ice, vortexed for 5 s, and 250 uL of digesta were taken. Aliquotes were stored at −80 °C until analysis. For the analysis, digesta aliquotes were mixed with BHT, EDTA, and HCl, to prevent lipid oxidation and to precipitate proteins. Aldehydes were derivatized by 2,4-dinitrophenylhydrazine (DNPH), extracted with dichloromethane, and dissolved in MeOH before separation and detection by LC/APCI-MS. The same procedure was followed for undigested oils, which were mixed with simulated saliva fluid in replication of the conditions at the beginning of the mouth phase. Calibration curves were built with standards of HHE, HNE, and MDA, the latter obtained by hydrolysis of TEP in 1% sulfuric acid. Qualifier ions for MDA, HHE, and HNE were *m/z* 234.0, *m/z* 293.1, and *m/z* 335.1, while quantifier ions were *m/z* 159, *m/z* 46, and *m/z* 46, respectively. Measurements were made in single replicates.

2.6. Lipid extraction from digesta

Lipids were extracted by adding hexane:isopropanol 2:1 *v*/v immediately after sampling for aldehyde analysis. The solvent contained BHT (0.05%) to prevent further oxidation. Throughout the procedure, samples were kept on ice. The solvent was added to the digestates in ratio 1:1 v/v and tubes were vortexed for 10 s and centrifuged at 1610 ×*g* for 3 min. The extraction was performed twice. Upper phases were combined and evaporated to dryness with N_2 at 37 °C. Samples were recovered with 3 mL of chloroform:methanol 1:1 *v*/v and stored at −80 °C. Oil yield was measured gravimetrically and corrected with the results of fatty acid analysis (Section 2.8).

2.7. Separation of free fatty acids (FFA)

Separation of FFA from the neutral lipid fraction (NL) of lipid digesta was performed with Mega Bond Elut-NH2 SPE columns (Agilent Technologies, SantaClara, CA, USA). After column conditioning with hexane, aliquotes of digesta were eluted with chloroform:isopropanol 2:1 v/v to collect the NL fraction and 2% AcOH in Et_2O to collect the FFA fraction. After drying with N_2 flow at 40 °C, the fractions were recovered with chloroform:methanol 2:1 and stored at −80 °C until analysis.

2.8. Fatty acid composition

Aliquots of lipid digestates, undigested oils, and FFA fractions were

dried, added with 2 mL of methanolic hydrochloric acid mixture (prepared by adding acetyl chloride to methanol in 1:10 v/v ratio), and incubated overnight at 50 °C. Analysis of resulting fatty acid methyl esters (FAME) was carried out with a Shimadzu GC-2030 equipped with autoinjector and FID. Heptadecanoic acid (17:0) was used as internal standard. The mixtures FAME 37 from Supelco Inc. (Bellafonte, PA, USA) and GLC-490 from Nu-Chek Prep (Elysian, MN, USA) were used as external standards. Analytical details have been reported elsewhere (Damerau et al., 2020).

2.9. Peroxide value (PV)

The peroxide value (PV) of undigested oils and lipid digesta was determined adapting the protocol of Undeland (Undeland et al., 2002). Oil drops were dissolved in $CHCl_3$:MeOH 2:1 BHT 0.05%. Aliquots of oil solutions and lipid digesta solutions were brought to 2 mL with $CHCl_3$. Samples and blanks ($CHCl_3$) were added with 1.33 mL of ice-cold $CHCl_3$:MeOH 1:1, followed by addition of 33.4 μL of ammonium thiocyanate solution 0.3 g/mL and 33.4 μL of Fe(*II*)chloride solution 2 mg/mL (prepared fresh by reacting $BaCl_2 \bullet 2\ H_2O$ with $FeSO_4 \bullet 7\ H_2O$), and then incubated for 20 min at room temperature. The UV absorption at 500 nm was measured to quantify the PV. Cumene hydroperoxide (20 mM solution in $CHCl_3$:MeOH (2:1)) was used to build the calibration curve. The PV was reported as meq/kg oil or meq/kg PUFA.

2.10. Tocopherol content

The concentrations of tocopherols in the different samples and digestates were analyzed with NP-HPLC-FLD, using a Shimadzu Nexera XR LC-30 system equipped with RF-20 A fluorescence detector (292–325 nm excitation-emission wavelengths) and a Phenomenex OOG-4162-EO Luna 3 μm silica column (Aitta et al., 2023). A mixture of heptane and 1,4-dioxane (98:2) was used as mobile phase in isocratic mode. Column was kept at 30 °C, while the autosampler was kept at 4 °C. Tocopherol standard curves were prepared using α-, β-, γ-, and δ-tocopherol stock solutions.

Digestates were filtrated with 0.45 μm PTFE filters, dried with N_2 at room temperature in dim light and recovered with heptane. Undigested oils were diluted in heptane.

2.11. Statistical analysis

The statistical analysis was performed with RStudio (RStudio, 2020). Shapiro-Wilk and Levene tests (*dlookr* and *car* packages) were used to assess normality of the data and homogeneity of data variance. The analysis of variance was performed with the functions *aov* and TukeyHSD (*stats* package) for ANOVA test or *oneway* for Games-Howell posthoc test (*userfriendlyscience* package). Spearman ρ correlations were computed with *cor* function (*stats* package). In alternative, distance correlations were computed with *correlation* function (*correlation* package) with Holm *p*-value adjustment and with or without Windsor normalization of data (*datawizard* package). The confidence level of 95% ($p < 0.05$) was used for statistical significance.

Multivariate partial least squares (PLS) models of oil digestion in relation to oil composition and increase in oxidation markers (PV, HHE, HNE, and MDA) in relation to oil and lipid digesta composition were computed with *opls* function of *ropls* package (Thévenot et al., 2015) after logarithm transformation and Pareto-scaling of data.

Linear and non-linear fitting of data was performed with OriginPro 2022b (OriginLab Corp., Northampton, MA, USA) utilizing 1000 iterations for the latter.

3. Results and discussion

3.1. Hydrolysis of bulk and emulsified LO

The first step of the present work compared the hydrolysis of LO in bulk or emulsified form at different oil concentrations in chyme during simulated digestion. The obtained degrees of lipolysis, expressed as FFA % (*w*/w of total oil), are reported in **Supplementary Fig. 1**. Emulsification with sodium caseinate had a positive effect on lipolysis only at the lowest oil concentration. Therefore, all further digestion experiments were conducted with bulk oil. **Supplementary Fig. 1** compares the FFA release at different oil concentration in chyme with the reports of Sabet (Sabet et al., 2022) and Okuro (Okuro et al., 2023). Noticeably, despite the differences in oil (rapeseed) and measurement method for FFA (pH-stat), the degree of lipolysis was in general agreement. Moreover, the lipolysis degree of bulk oil was in agreement with the lipolysis degree of rapeseed oil measured previously in our lab (Beltrame, Linderborg, & Damerau, 2025). As indicated by the trend in **Supplementary Fig. 1**, the degree of hydrolysis had a power law ($y = a \cdot x^b$) relationship (Adj. R^2 0.854) with the oil concentration in the chyme. A power relationship between FFA% and corn oil concentration was also visible (Adj. R^2 0.919) in the simulated digestion experiments reported by Tan (Tan et al., 2020).

3.2. Effect of fatty acid composition on oil hydrolysis

The effect of fatty acid composition on the hydrolysis degree of different omega-3-rich oils was investigated following in vitro digestions. The degree of lipolysis after simulated digestion of bulk oils, expressed as FFA% (*w*/w), is reported in Fig. 1a. At the present experimental conditions, LO had highest digestibility (65.4 ± 3.6%), while S2 and RO the lowest (27.4 ± 0.5% and 25.0 ± 4.0%, respectively). The lipolysis of the other oils oscillated around 37% (Fig. 1a) which was equivalent to 58% of lipolysis taking place in LO.

After the observation of the power law relationship between FFA release and the amount of oil subjected to digestion reported in **Supplementary Fig. 1**, impact of oil concentration was further investigated at different oil/pancreatine/chyme (mg/mg/mL) ratios with LO, assuming these three variables influenced the final hydrolysis. The results, reported in **Supplementary Fig. 2**, corresponded again to a power law relationship (Adj. R^2 0.959). The coefficient obtained by rearranging the trend equation (in bold in **Supplementary Fig. 2**) was then computed from the experimental results of all the digested oils. The relationship of this coefficient with fatty acid composition and PV of the oil prior to digestion was then investigated with multivariate PLS. The results are reported in Fig. 1b-c. The comparison between FFA% from PLS model coefficients and experimental FFA% (Fig. 1b) shows only slight deviation (identity slope 0.96), indicating good quality of the model. Fig. 1c shows the PLS coefficient for all the variables used for model computation. Oil lipolysis was positively affected by 16:0, 18:2n-6, and 18:3n-3 among single fatty acids and by the total saturated (SAFA), total 12 to 16-carbon FAs, and total 18-carbon FAs. According to our results, 12:0 and 16:0 had positive influence on lipolysis, while 14:0 had no influence and 15:0 and 16:1n-7 had negative influences. The initial peroxide value (PV_i) had a very small positive coefficient and Variable Importance in Projection (VIP) < 1 (0.47). Likewise, a previous report found no effect of initial oxidation status on digestibility of different commercial soybean oils (Martin-Rubio et al., 2019). Neither oxidation of linseed oil at 70 °C for 36 h nor of sunflower oil at 70 °C for 96 h had significant effects on lipolysis degree (Nieva-Echevarría, Goicoechea, & Guillén, 2017; Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2017). Lipolysis was negatively affected mainly by total 22-carbon FAs and 22:6n-3 (VIP > 1), followed closely in relevance by total n-3 (VIP 0.81) and total PUFA (VIP 0.79) (Fig. 1c). An increased n-3/n-6 ratio had negative impact on lipolysis, as previously observed (Beltrame, Valta, et al., 2025) but had VIP < 1. Average carbon chain

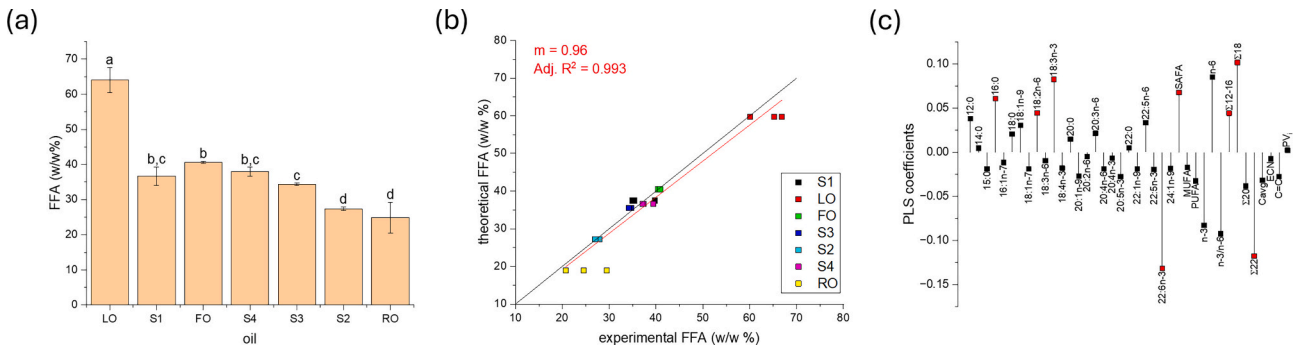

Fig. 1. (a) FFA in lipid extracts of simulated digesta expressed as relative percentage of total lipid extract (b) FFA according to PLS model in comparison with experimental results. Black and red lines respectively represent identity and linear fitting of data (c) Variable coefficients in the PLS model. Red marks represent most influential variables to the model (VIP > 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

length (C_{avg}) and average number of double bonds ($C{=}C_{avg}$) had negative impact on lipolysis but VIP < 1. The equivalent carbon number of oils (ECN) has very small negative coefficient and VIP < 1 (Fig. 1c).

It is generally believed that the rate of TAG hydrolysis decreases with increasing length of the carbon chain (Acevedo-Fani et al., 2021) but only a few studies highlighted the influence of the fatty acid composition. For example, Ozturk noticed higher release of FFA from 8:0–10:0 mixture TAG compared to corn oil (55% 18:2n-6). However, in their study fish oil (unreported composition) had similar digestibility compared to corn oil, despite the likely higher amount of 20- and 22-carbon FAs (Ozturk et al., 2015). Verkempinck and colleagues (Verkempinck et al., 2018) submitted to simulated digestion different emulsions obtained with three olive, soybean, and linseed oils and the same emulsifier. According to the reported fatty acid composition, only 16:0 and total SAFA had a positive effect on lipolysis, while 18:2n-6 had no visible influence and total 18-carbon FAs and 18:3n-6 had a negative effect. Similarly to our results, the report of Verkempinck shows no significant effect of 18:1n-9 on lipolysis (Verkempinck et al., 2018). However, the oils utilized in this work lacked in 18:1n-9, as our focus was on n-3 PUFAs. Present results confirm the belief that TAG unsaturation has no significant impact on lipolysis (Acevedo-Fani et al., 2021) as the influence of the oil average number of C=C ($C{=}C_{avg}$) in the PLS model was modest. LO and FO had similar $C{=}C_{avg}$ (2.37 and 2.11, Table 1) but significantly different lipolysis. We could conclude that singular fatty acids rather than ECN, C_{avg}, or $C{=}C_{avg}$ drove lipolysis. Zheng and coworkers noticed that higher 20:5n-3/22:6n-3 ratios favored lipolysis of fish oil emulsions, although complete fatty acid composition was absent from their report (Zheng et al., 2024). Higher hydrolysis of TAGs containing 20:5n-3 rather than 22:6n-3 was noticed previously in our lab (Beltrame et al., 2023) but the present PLS model assigned no significant effect to the presence of 20:5n-3. One factor scarcely taken into account in these studies is the location of these fatty acids in the TAG, as positions *sn*-1 and *sn*-3 are those targeted by pancreatic lipase (Rogalska et al., 1990). Our previous investigation showed a negative non-linear relationship between lipolysis and 22:6n-3 in position *sn*-1,3. Moreover, an apparent linear relationship (Adj. R^2 0.978) between lipolysis and relative MUFA content in position *sn*-1,3, while there was no clear trend with SAFA (Beltrame, Valta, et al., 2025).

3.3. Tocopherol decrease after digestion

The tocopherol composition of undigested oils and lipid digesta is reported in Fig. 2a. The most relevant tocopherol in the oils used in the present study was γ-tocopherol, except for S3, which contained mainly α-tocopherol. Highest total tocopherol levels were found in the S-oils, with S2 standing out, having a total content of about 6000 ng/mg oil. As shown in Fig. 2a, simulated digestion affected tocopherol concentrations but at different extents in different oils. Total tocopherol decrease was statistically significant ($p < 0.05$) for S2, S1, and S3, while for LO, FO, and S4 it lacked significance. Decreases in γ-tocopherol were statistically significant ($p < 0.05$) for S2 and S1, while for α-tocopherol were significant for S3 and S2, respectively. Differently from our results, Nieva-

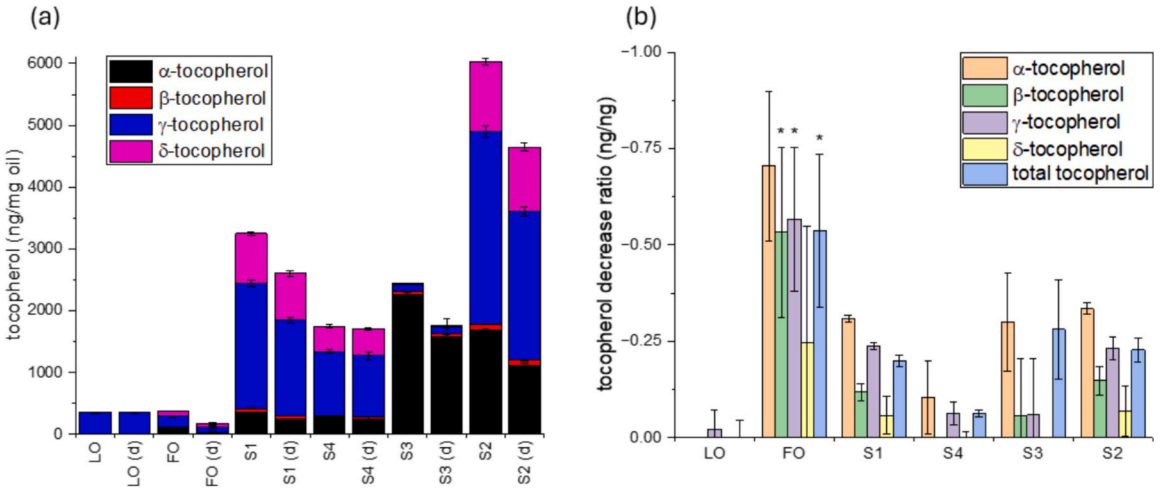

Fig. 2. (a) tocopherol composition and concentration in undigested oils and in lipid extracts from simulated digesta, marked with "(d)" (b) Ratio between tocopherol decrease and its respective concentration prior to digestion. Asterisk marks significant difference ($p < 0.05$) in tocopherol decrease between oils.

Echevarria and coworkers have reported a substantial depletion of γ-tocopherol after simulated digestion (from 0.32 mmol/mol TAG to below detection limit) of linseed oil. Moreover, such depletion was also observed after the addition of α-tocopherol to the oil, which was as well largely consumed (Nieva-Echevarría et al., 2019). High consumption levels on γ-tocopherol were also observed by Martin-Rubio (Martin-Rubio et al., 2019).

The consumption rates of tocopherol expressed as ng/ng are reported in Fig. 2b, which shows a different behavior of FO compared to the other oils. In particular, the consumption rates of α-, β-, γ-, and total tocopherol in FO were significantly higher ($p < 0.05$) from all the others, while δ-tocopherol showed no significant differences. High consumption rates of tocopherol in fish oil were previously also observed (Kenmogne-Domguia et al., 2014). In addition, two oils, S1 and S2, showed more clearly differences between the consumption of different tocopherol isomers during digestion, i.e. α-tocopherol was consumed faster, followed by γ-, and then by β- and δ-tocopherol. This was in agreement with previous observations from storage trials of fish (Kulås & Ackman, 2001) and soybean (Player et al., 2006) oils. Noticeably, in their fish oil-enriched food model during a storage trial at room temperature, Horn et al. have reported a protective effect of added γ-tocopherol on the α-tocopherol present (Horn et al., 2009).

Spearman correlation was computed to investigate the relationship between tocopherol consumption and composition of NL and FFA fractions of digesta. The results are reported in Fig. 3. The 22:6n-3 and 20:5n-3 present in FFA fraction had a stronger correlation with tocopherol consumption compared to NL fraction, indicating an higher rate of oxidation of the cleaved fatty acids compared to the glycerol-bound ones. Both n-3 PUFAs had stronger correlations with γ- and δ-tocopherol consumption, compared to consumption of α- and β-tocopherol. Compared to glyceryl structures, FFAs are the most susceptible to oxidation in bulk oils (Lyberg et al., 2005). Therefore, PUFAs in FFA form were expected to have a stronger correlation with tocopherol degradation. When reaching the emulsion interface, FFAs are exposed to oxidative damage, and tocopherols, located at the interface, exert antioxidant activity preferentially with FFA. However, this observation was obvious only for the aforementioned 22:6n-3 and 20:5n-3. On the other hand, the presence of MAGs and DAGs could explain the correlations between n-3 PUFAs in the NL fraction and tocopherol consumption. DAGs and MAGs are more susceptible to oxidation than TAGs, due to lower steric hindrance. Moreover, their higher polarity leads to their orientation towards emulsion interface (Goñi & Alonso, 1999). Lipid structures differing in class and fatty acid composition interact differently with tocopherols (Ahonen et al., 2022; Cao et al., 2015; Yanishlieva et al., 2002). At the same time, it is possible that tocopherol behavior in NL and FFA fraction changed according to its concentration, which was noticed after simulated digestion of oxidized rapeseed oil (Tarvainen et al., 2012). Changes in tocopherol consumption rates at different initial concentrations (linear increases for α- and γ- and asymptotic increase for δ-tocopherol) were noticed also during storage of fish oil at 30 °C (Kulås & Ackman, 2001).

The relationship between tocopherol consumption and starting oil PV (PV_i) lacked discernible trends (**Supplementary Fig. 3**) but showed negative Spearman correlations of $\rho = -0.69$, $\rho = -0.74$, and $\rho = -0.66$ for β-, γ-, and δ-tocopherol, respectively. This observation can be explained by higher oxidation initiation and therefore higher scavenging activity. A similar finding was observed after simulated digestion of soybean oils with different initial hydroperoxide content (Martin-Rubio et al., 2019).

3.4. Increase in PV after digestion

The formation of hydroperoxides is the first step of the lipid oxidation cascade and therefore it is of high importance. The effect of simulated digestion on PV of lipid digesta is reported in Fig. 4a, where PVs were normalized to the PUFA concentration of the oil. Fig. 4a includes data from part of this team, which to our knowledge were the first ones to investigate the oxidative fate of cod liver oil during simulated digestion, although using a protocol diverging from the present study, for example due to the use of fungal lipase (Larsson et al., 2012). Larsson et al. already noticed that lipid oxidation of cod liver oil proceeds at a faster rate when undigested lipids are more oxidized. The same was noticed by Nieva-Echevarria and coworkers after digestion of sunflower oils (Nieva-Echevarría, Goicoechea, Manzanos, et al., 2017). However,

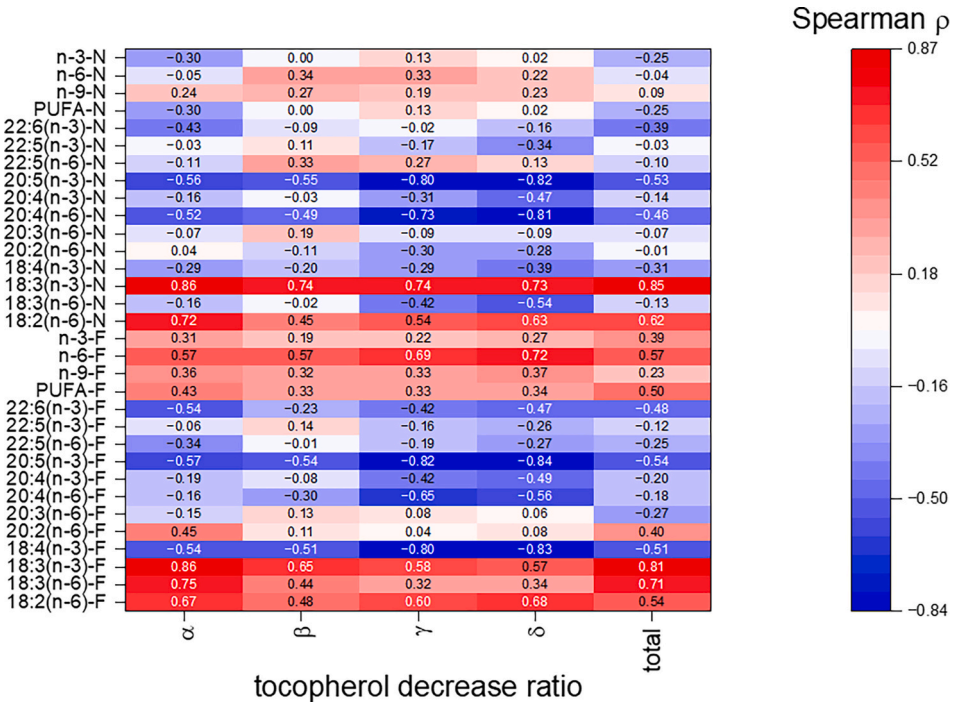

Fig. 3. Spearman correlation between PUFAs in FFA and NL fractions of digesta and decrease in α-, β-, γ-, δ-, and total tocopherol. Fraction acronyms are shortened to F and N respectively for clarity of the figure.

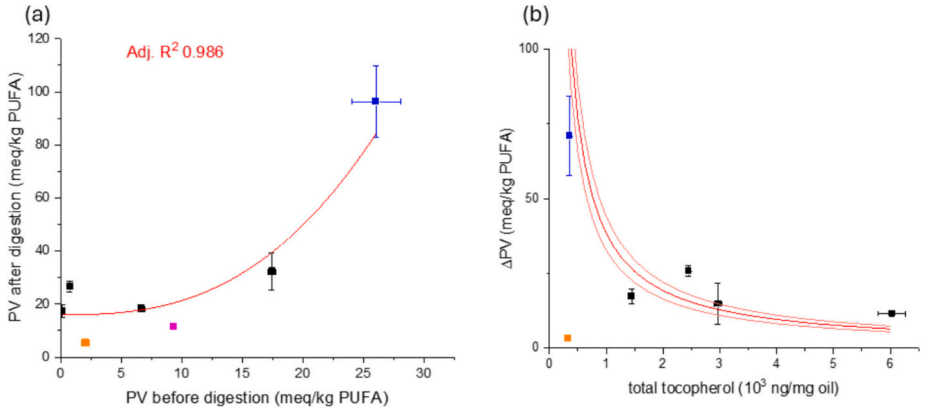

Fig. 4. (a) non-linear relationship between PV measured in undigested oils and digesta extracts and reported as meq/kg PUFA (b) Non-linear relationship between PV increase after digestion and total tocopherol concentration in oil prior to digestion according to the PUFA content of FFA. Rationale of the trend is reported in Supplementary Fig. 4. LO (orange mark) was excluded from fitting. Magenta mark in Fig. 4a reports Larsson et al. (2012) and was excluded from non-linear fitting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

an outline of the trend was missing from the literature. The trend here reported remarkably followed a non-linear power function (Adj. R^2 0.986). The following discussion focuses on explaining the difference between final and initial PV (ΔPV). The ΔPV observed with FO and LO were statistically significant from the rest of the oils ($p < 0.05$), while among the *Schizochytrium sp* oils, only S2 and S3 differed significantly from the others ($p < 0.05$). Fitting a non-linear trend in the data led to an acceptable result (Adj. R^2 0.703, not shown) but as discussed in Section 3.3, both FFA and tocopherols have a role in lipid oxidation. The relationship between ΔPV and the ratio between the total released PUFA (FPUFA) and total tocopherol was linear (Adj. R^2 0.902, **Supplementary Fig. 4**). On this basis, a trend in hydroperoxide formation was computed considering both initial total tocopherol concentration and FPUFA. The trend is reported in Fig. 4b. The results showed rapid accumulation of hydroperoxides at low concentrations of tocopherol in the oils and slow accumulation at high concentrations, instead of absence of accumulation. At higher concentrations, tocopherols could have exerted pro-oxidant effect (Frankel, 2005) or could have partitioned in the emulsion bulk, where their activity is minimal (Losada-Barreiro et al., 2014).

Our results confirmed previous information on the inability of tocopherols to fully prevent lipid oxidation during simulated digestion. For example, the addition of α-tocopherol in sunflower oil attenuated hydroperoxide formation after digestion only at highest dosage (2%), from a 5-fold to a 3-fold increase in sunflower oil (Nieva-Echevarría et al., 2019). Moreover, in the present work, a certain degree of linearity was observed in the relationship between ΔPV and tocopherol consumption (**Supplementary Fig. 5a**), in addition to $\rho = -0.68$ (Fig. 6a). Since, in the absence of tocopherols, hydroperoxides undergo degradation during simulated digestion (Beltrame et al., 2023), we interpreted these findings as stabilization of formed hydroperoxides by tocopherols through radical quenching and inability to prevent the formation of new ones.

In the present work, we aimed to understand the influence of the fatty acid composition on the oxidative fate of oils during simulated digestion. Spearman correlation between ΔPV and fatty acid composition of undigested oil and lipid digesta are reported in **Fig. 6a** and **b**. Correlations were also calculated excluding FO from the dataset to check the skewedness of the results caused by the high ΔPV observed in FO. For the same reason, distance correlation was computed to consider non-linear associations among variables (**Supplementary Fig. 7**). While Spearman results in Fig. 6a were influenced by FO composition, Fig. 6b showed much less skewed correlations between fatty acid composition of lipid digesta and ΔPV. Released 22:5n-3, 20:3n-6, 18:1n-7, 18:1-n9, total MUFA, and 20:4n-3 had a confirmed positive correlation with ΔPV (Fig. 6b). Released 22:6n-3 had a confirmed positive correlation, although it remained marginal ($\rho = 0.2$). Distance correlation deviated from these findings only in assigning positive correlation (0.49) to released PUFAs (**Supplementary Fig. 7**). The positive correlation of released MUFAs with increase in hydroperoxides, despite their lower proneness to oxidation, can be interpreted as deprotective effect of lipolysis on PUFAs still esterified to glycerol. In fact, a positive correlation between PUFAs in the NL fraction and ΔPV was observed. Expectedly, the main correlations shifted from C20 to C22 PUFAs after exclusion of FO, but correlations with glycerol-bound 18:4n-3, 20:3n-6, and 20:4n-3 were still observed (Fig. 6b). Although more conservatively, distance correlation confirmed the findings (**Supplementary Fig. 7**).

The relationship between accumulation of hydroperoxides and fatty acid composition of the lipid digesta was further evaluated by computing a PLS model, whose quality and results are reported in Table 2. Variance in both free and glycerol-bound FA explained the variance in ΔPV. Among the main loadings having positive correlation with hydroperoxide formation, only free 20:2n-6 and bound 22:6n-3 had VIP > 1. Nevertheless, the model ascribes relevance to free 18:1n-9 and 22:5n-3, which are among the species mentioned above. The high relevance of MUFAs can only partly be explained by FO, as it was preserved after sample removal. At the same time, the comparison of Spearman correlation and VIP of the PLS model (Table 2) shows that the

Table 2
Multivariate PLS model of increase in oxidation markers in relation to composition of FFA and NL fractions.

	ΔPV	ΔHHE[a]	ΔHNE[a]	ΔMDA
Main positive loadings on p1 in decreasing order				
	20:2(n-6)-F*	22:6(n-3)-N†	20:2(n-6)-F*	20:2(n-6)-F*
	22:6(n-3)-N*	22:6(n-3)-F	22:5(n-6)-N	22:6(n-3)-N*
	22:5(n-6)-N†	22:5(n-3)-F*	20:4(n-6)-N*	22:6(n-3)-F*,†
	18:1(n-9)-N†			22:5(n-6)-N
	22:6(n-3)-F			24:1(n-9)-F
	22:5(n-3)-F†			20:1(n-9)-N
	24:1(n-9)-F			22:5(n-3)-F*,†
	18:1(n-9)-F			20:4(n-6)-N†
Fatty acid variance – Oxidation marker variance for p1				
	49% - 78%	92% - 30%	61% - 23%	38% - 67%
Model quality				
R^2X	0.863	0.970	0.757	0.633
R^2Y	0.809	0.505	0.458	0.880
Q^2Y	0.746	0.306	0.113	0.733
RMSEE	3.87E-06	0.004	0.001	1.46

Fraction acronyms are shortened to F and N respectively for clarity of the table; the model excludes FO from the dataset; [a]computed with only n-3 and n-6 fatty acids, respectively; *VIP > 1, †ρ > 0.5 (excluding FO from dataset).

observed correlations between 22:5(n-6)-N and ΔPV ($\rho > 0.5$) may partly reflect shared variance driven by changes in 20:2(n-6)-F. We ascribed such correlation to DAGs and MAGs migrating towards the surface of the lipid drop or micelle due to increased polarity after MUFA release (Goñi & Alonso, 1999), and consequent enhanced exposure to oxidation.

Kenmogne-Domguia ascribed the oxidative fate of rapeseed oil (Kenmogne-Domguia et al., 2012) and fish oil (Kenmogne-Domguia et al., 2014) during simulated digestion to the chemical environment, i.e. to the acidic pH of the gastric phase. Contribution of gastric pH to accumulation of hydroperoxides was confirmed by previous results from our group, which showed that the gastric phase had higher incidence on the tocopherol consumption during simulated digestion compared to the intestinal phase (Beltrame, Linderborg, et al., 2025), although other authors reported no effect of pH on tocopherol stability in emulsion (Kim et al., 2018). Low pH can act as pro-oxidant by increasing the solubility of trace metals, which in turn generate radicals through the Fenton reaction (Larsson et al., 2012). Noticeably, lipolysis extent is low during gastric phase (Beltrame, Linderborg, et al., 2025; Okuro et al., 2023). However, our results corroborate previous findings (Beltrame et al., 2023; Tullberg et al., 2019) showing that lipolysis correlated with the extent of lipid oxidation. Arguably, the noticeable increase in O_2 uptake and tocopherol consumption during intestinal phase observed by Kenmogne-Domguia can be explained by higher fish oil lipolysis during the intestinal phase (Kenmogne-Domguia et al., 2014).

3.5. Formation of aldehydes during digestion

The concentrations of aldehydes in simulated fluid at the beginning and at the end of the digestion process are reported in Fig. 5. Aldehydes were quantified without technical replication. Therefore, the observed HHE, HNE, and MDA trends should be interpreted cautiously. While HNE formation during simulated digestion was negligible and lacked statistical significance (Fig. 5b), HHE had a different behavior. Increases in HHE concentration were observed with *Schizochytrium* oils except S4, but while LO had no significant change in concentration, a decrease in concentration was observed with FO (Fig. 5a). On the other hand, MDA significantly increased after digestion of all the oils. For marine oils, the final concentrations of MDA were higher than those obtained by Tullberg (Tullberg et al., 2019) but comparable to those of Kenmogne-Domguia (Kenmogne-Domguia et al., 2014). At the end of the digestion, LO had the lowest amounts of MDA, while the differences in MDA concentration among the other digesta had small significance (S2 and S4 differing from FO). Differences between start and endpoint can be explained by the dilution during the digestion and formation-degradation cycle (visible for example in (Tullberg et al., 2019)) due to high reactivity towards nucleophilic groups. While the earlier could apply solely to HHE in FO, the latter was investigated by correlating the aldehyde increases to composition of undigested oil and lipid digesta. The correlation with tocopherol consumption was negative except for ΔHHE. **Supplementary Fig. 5** shows linear trends among aldehydes and tocopherol consumption and that the observed $\rho \sim 0$ with ΔHHE (Fig. 6) was due to the FO sample (**Supplementary Fig. 5b**). Tullberg et al. found that adding α-tocopherol or a tocopherol mix to cod liver oil at 4.5 mg/g oil reduced the amounts of MDA in digesta by 38% and 79%. Noticeably, such decreases were observed mainly during intestinal phase, when most lipolysis occurs (Tullberg et al., 2019). Incidentally, the presence of lipid oxidation at so high tocopherol dosages during simulated digestion provides further support to the trend computed in the present work and shown in Fig. 4b.

As expected, ΔHHE correlated with oil 22:6n-3 ($\rho = 0.51$), total C22 ($\rho = 0.51$), C_{avg} ($\rho = 0.64$), and $C{=}C_{avg}$ ($\rho = 0.58$), indicating that higher amounts of DHA in the oil correlate to higher increases in HHE after digestion. While the correlation was absent in the FFA fraction, correlation of $C{=}C_{avg}$ and ΔHHE was observed in the NL fraction ($\rho = 0.61$, **Supplementary Fig. 6b**) hinting at a correlation between HHE formation and oxidation of acylglycerols. Such correlation was also visible in Fig. 6b ($\rho = 0.70$ with n-3-N and PUFA-N) and **Supplementary Fig. 7**. This could be explained by steric constraint imposed by esterification, favoring the Hock cleavage required for HHE formation (Frankel, 2005). On the other hand, the lack of linearity in the relationship between C=C and ΔHHE (**Supplementary Fig. 6b**) could be explained by the increased bending in lipid structures with increasing DHA content, which disfavored intermolecular radical transfers (Schaich et al., 2013). In the current experimental setup, HNE formation was negligible and therefore we failed to observe the correlation between HNE and n-3/n-6 noticed by Van Hecke (Van Hecke et al., 2019), probably due to the higher stability of the observed n-6 FA compared to n-3 FA (Schaich et al., 2013) and the lower amounts of n-6 FA (Table 1) compared to Van Hecke work. On the other hand, the correlation between ΔMDA and ΔPV was high ($\rho = 0.77$) and showed good degree of linearity (Adj. R^2 0.798, **Supplementary Fig. 6d**) and a slope (0.31 after unit normalization) close to previous results (R^2 0.878, slope 0.47) on simulated gastric digestion of red meat combined with different oils (Tirosh et al., 2015). Due to this high proportionality, we observed correlations between ΔMDA and fatty acid composition similar to those discussed for ΔPV (Section 3.4). Differently from ΔPV, correlations with C20 PUFA such as 20:5n-3 and 20:3n-6 remained high in both N and F fractions after removal of FO (Fig. 6b). Also, while ΔPV marginally correlated with 22:6n-3 (but was assigned relevance by PLS model, Section 3.4), ΔMDA correlated with free 22:6n-3 (Fig. 6b). Such fatty acid increased in importance in the PLS model, when compared to ΔPV (Table 2). Therefore, while bound 22:6n-3 was relevant for the formation of both hydroperoxides and MDA, free 22:6n-3 had slightly higher relevance for the formation of MDA. The observation can be explained by the protic environment required by the first scission of cyclic internal hydroperoxides for the generation of MDA at lower temperatures (Schaich et al., 2013). The comparison of Spearman correlation values and PLS loadings showed again that 20:2(n-6)-F could have driven the correlation between 20:4(n-6)-N and ΔMDA (Table 2). Therefore, as discussed in

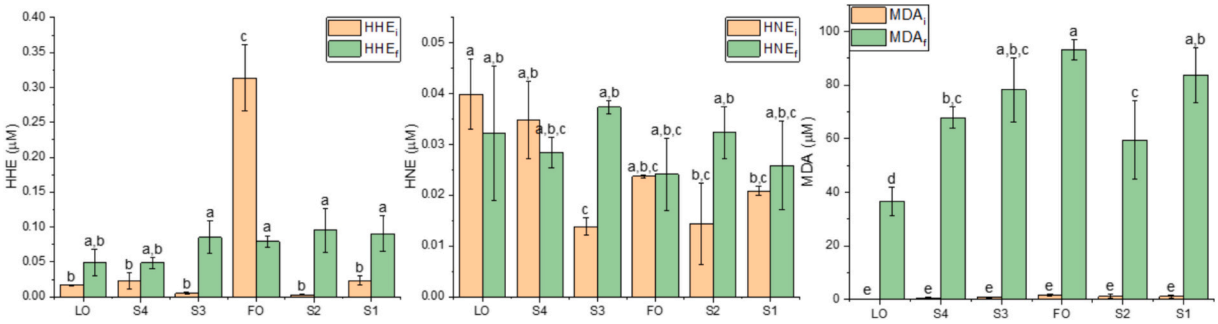

Fig. 5. Concentrations of 4-hydroxy-2-hexenal (HHE), 4-hydroxy-2-nonenal (HNE), and malondialdehyde (MDA) in simulated saliva prior to simulated digestion and in simulated chyme prior to lipid extraction. Different letters mark significant difference ($p < 0.05$).

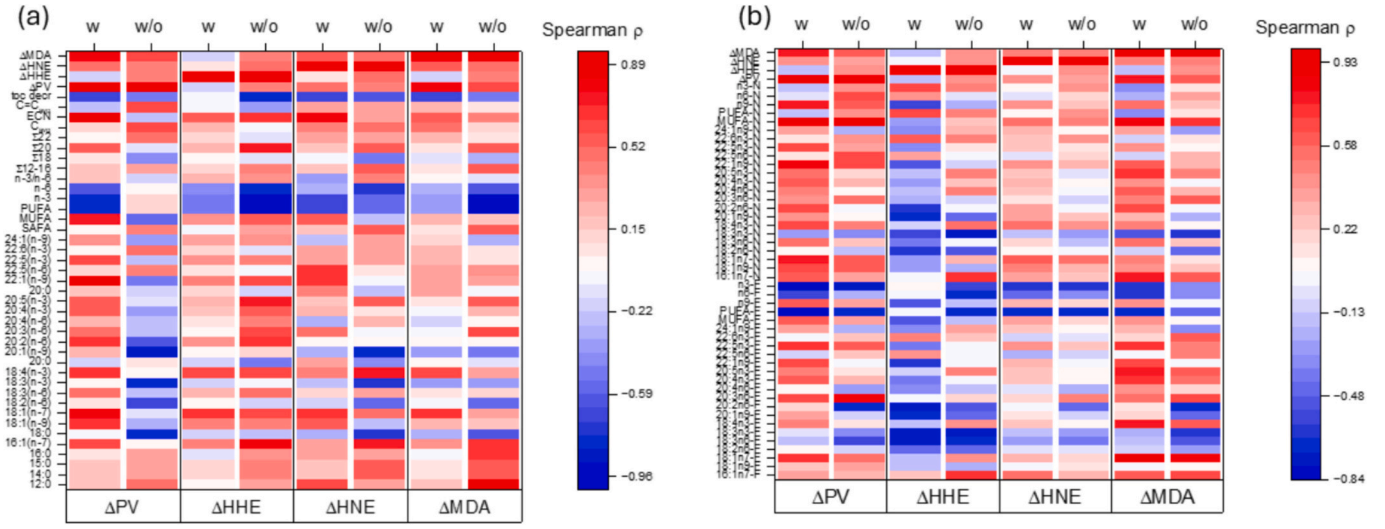

Fig. 6. (a) Spearman correlation between increase in oxidation markers after digestion and fatty acid composition of the undigested oil (b) Spearman correlation between increase in oxidation markers after digestion and PUFA composition of FFA and NL fractions of lipid extracts from digesta. Fraction acronyms are shortened to F and N respectively for clarity of the figure. Results including (w) or excluding (w/o) of FO digesta from the dataset are shown.

Section 3.4, fatty acids that are more easily cleaved drive the oxidation of glycerol-bound fatty acids.

3.6. Overall view on n-3 PUFA-rich oil oxidation during gastrointestinal lipolysis

Free fatty acids are known promoters of oxidation at high temperatures or during processing and storage of lipids, mainly due to their metal-chelating properties (Frankel, 2005). Tullberg et al., focusing on gastrointestinal conditions, reported that inhibition or enhancement of marine oil lipolysis in turn decreased or enhanced oxidation. When lipolysis was enhanced with addition of a different lipase, increase in oxidation was unequal among oxidation markers (Tullberg et al., 2019), likely because different fatty acids were released. In the study of Van Hecke, different muscle foods of different fatty acid compositions were subjected to simulated digestion, but no attention was paid on how lipolysis influenced oxidation markers (Van Hecke et al., 2019). Also, the application of the standardized simulated digestion protocol INFOGEST 2.0 is more recent than these studies.

Our observations integrated previous findings by detailing the effects of fatty acid composition on lipolysis and oxidation markers. Lipolysis is determined by the fatty acid composition of the oil. In this work, focusing on marine n-3 PUFA-rich oils, lipolysis was mainly driven by the relative amounts of 12–16-carbon SAFA and 18-carbon FA. While making acylglycerols more susceptible to oxidation, released fatty acids are exposed to the oxidative environment and to tocopherols located at the interface. Different tocopherols have different radical scavenging effects, according to their type (α-tocopherol consumed first, δ-tocopherol almost undegraded), the initial oxidation status of the oil, and the nature of the fatty acids. In this work, 22:6n-3 and 20:5n-3 had the highest correlation with tocopherol degradation. Here we report for the first time the non-linear relationship, governed by the amount of released PUFAs, between development of hydroperoxides and initial tocopherol content of the oil submitted to simulated digestion. Moreover, our results showed that the more tocopherol is consumed, the more hydroperoxides and aldehydes are formed during simulated digestion. Different fatty acids, whether in glycerol or free form, have different susceptibility to oxidation. Our results highlighted the correlation between formation of hydroperoxides and released 22:6n-3, 22:5n-3, 20:3n-6, 20:4n-3 and glycerol-bound 18:4n-3, 20:3n-6, and 20:4n-3, while either free or bound 20:5n-3 and 20:3n-6 and free 22:6n-3 correlated with the formation of MDA. The correlation of aldehyde formation (HHE and MDA) with fatty acids in acylglycerols (such as 22:6n-3, 22:5n-3, and 20:4n-6) further confirms that DAGs and MAGs are susceptible to oxidative damage and highlights that while 12–16-carbon SAFA and 18-carbon FA are less susceptible per se to oxidation, they aid oxidation on one hand by generating DAGs and on the other by facilitating further lipolysis.

4. Conclusions

The present work examined the relationship between tocopherol concentration and lipolysis of n-3 PUFA-rich oils during simulated digestion and the development of primary and secondary oxidation products. The presented data fitted different models which explained the oxidative behavior. Tocopherol consumption during digestion correlated with released 22:6n-3 and 20:5n-3, in line with the critical antioxidant role at the emulsion interface, where digestion products are located. We observed strong proportionality between increase in PV after digestion and tocopherol consumption, while released PUFAs explained the non-linear relationship between increase in PV and initial tocopherol concentration. Some free PUFAs (22:5n-3, 20:3n-6, and 20:4n-3) had a higher correlation with PV increase than others. On the other hand, the positive correlation with highly cleavable FAs such as MUFAs was interpreted as a deprotective effect of lipolysis on PUFAs still esterified to glycerol, in line with previous observations, and a driver of more unsaturated FAs towards the interface and therefore higher exposure to oxidation. The high proportionality between increase in PV and increase in MDA, which confirmed previous observations, was reflected in the similarity in the fatty acids driving their formation. However, a more relevant role of C20 PUFAs was observed. Moreover, released 22:6n-3 had a more pronounced correlation with the formation of MDA than hydroperoxides. Conversely, the formation of HHE showed a stronger correlation with esterified PUFAs, which could be tentatively ascribed to a favorable conformation. Overall, our results have deepened our understanding of lipid oxidation during simulated digestion of n-3 PUFA-rich oils. The presented work paves the way for further studies on lipid oxidation at the micellar phase of digestion and. Results can be applied in the design of n-3 PUFAs supplements based on structured TAGs.

CRediT authorship contribution statement

Gabriele Beltrame: Writing – original draft, Visualization,

Investigation, Funding acquisition, Formal analysis, Conceptualization. **Annelie Damerau:** Writing – review & editing, Investigation. **Karin Larsson:** Writing – review & editing, Methodology, Conceptualization. **Ingrid Undeland:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Kaisa M. Linderborg:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.149454.

Data availability

Data will be made available on request.

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