### Title

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# Quantitation of Oxylipins in Fish and Algae Oil Supplements Using Optimized Hydrolysis Procedures and Ultra-High Performance Liquid Chromatography Coupled to Tandem Mass-Spectrometry

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**ABSTRACT:** Fish and algae oil supplements are enriched with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are precursors to oxidized fatty acids, known as oxylipins. Here, we optimized a base hydrolysis method for measuring oxylipins in oil with ultrahigh-performance liquid chromatography coupled to tandem mass-spectrometry (UPLC-MS/MS) and quantified them in fish and algae oil supplements. Hydrolysis of 2 μL of oil with sodium carbonate resulted in greater oxylipin concentrations and minimal matrix effects, compared to higher oil volumes (10, 20, and 30 μL). Oxylipin yield was higher when oil was hydrolyzed in methanol containing 0.1% acetic acid and 0.1% butylated hydroxytoluene, compared to no methanol, and using sodium hydroxide versus sodium carbonate. Oxylipins extracted from 2 μL of oil using sodium hydroxide in solvent showed that EPA-derived oxylipins were most abundant in fish oil (84−87%), whereas DHA-oxylipins were abundant in algae oil (83%). This study shows that fish and algae oils are direct sources of EPA- and DHA-derived oxylipins.

**KEYWORDS:** *Fish oil, algae oil, oxylipins, EPA, DHA, hydrolysis*



## INTRODUCTION

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) recommend a daily intake of 250 mg of the long-chain omega-3 polyunsaturated fatty acids (n-3 PUFAs), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), to reduce the risk of chronic disease.[1−3] This amount can be achieved in many ways, including the consumption of 2 servings of low-fat fish per day (e.g., cod) or one serving of oily fish (such as salmon) per week.[4] Another way to meet these recommendations is through dietary supplements. In 2007, fish oil/omega-3/DHA supplements were consumed by approximately 37% of American adults.[5] The oil in these supplements is obtained from fish or DHA-synthesizing algae.

The high PUFA content of fish/algae supplements makes them susceptible to auto-oxidation during extraction and storage.[6−8] The oxidation of PUFAs can also occur enzymatically via lipoxygenase (LOX),[9,10] cyclooxygenase (COX),[11] or cytochrome P450 (CYP450)[12] prior to processing (i.e., in the fish or algae itself). PUFA oxidation is typically assessed with qualitative methods such as the peroxide, anisidine, and TOTOX values, which measure primary, secondary, and total oxidation products, respectively.[13] A limitation of qualitative assays is that they do not provide information on the oxidized molecular species present in the sample. This is important to know because PUFAs such as EPA and DHA in fish and algae can oxidize via enzymatic or non-enzymatic pathways[9,11,12] to generate bioactive metabolites, known as oxylipins.[14−16] However, there is limited information on whether fish or algae oil supplements could serve as sources of oxylipins.

A few studies measured PUFA-derived oxylipins in salmon and rainbow trout using ultrahigh-performance liquid chromatography coupled with tandem mass-spectrometry (UPLC-MS/MS). Raatz et al.[17] reported the presence of hydroxy metabolites of EPA and DHA (i.e., resolvins) and prostanoid products of arachidonic acid (AA) in farm-raised salmon. The authors showed that concentrations of these oxylipins decreased after baking. Flaskerud et al.[18] also showed the presence of several oxylipins in rainbow trout (*Oncorhynchus mykiss*), which decreased in concentration after pan-frying. It is not known whether oxylipins end up in fish oil supplements after extraction, which involves heat.[19]

Macro- and microalgae are also sources of oxylipins.[20,21] While several studies have reported the presence of oxylipins in a variety of algae species,[22,23] the oxylipin profile of DHA-synthesizing algae such as *Crypthecodinium cohnii* or *Schizochytrium* has not been investigated.[24]

Previous studies measuring total oxylipins (i.e., the sum of bound and unbound) in food matrices, including oil, have done so by UPLC-MS/MS following base hydrolysis to release bound (i.e., esterified) oxylipins.[25,26] In oil, however, analyte recoveries were low, likely due to incomplete hydrolysis of bound oxylipins.[25] In other matrices such as plasma, lowering the sample volume or increasing the base concentration was reported to increase the concentration of oxylipins including epoxy and hydroxy fatty acid metabolites due to improved

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hydrolysis efficiency.[27] This suggests that similar to plasma, modifying the sample volume (or mass) and base amount may improve oxylipin hydrolysis of oils.

Thus, in the present study, we first tested several modifications of the hydrolysis method on cod liver oil samples, and then used the optimal procedure to quantify oxylipins in commercially available fish and high-DHA algae oil supplements. Oxylipins that were measured in this study included PUFA-derived metabolites of omega-6 linoleic acid (LA, 18:2n-6), dihomo-gamma-linolenic acid (DGLA, 20:3n-6) and AA (20:4n-6), and omega-3 alpha-linolenic acid (ALA, 18:3n-3), EPA and DHA. Because many of these PUFAs are present in fish and algae, we hypothesized that corresponding oxylipin derivatives would be found in the oil extracts.

## MATERIALS AND METHODS

**Materials.** Methanol, chloroform, ethyl acetate, hexane, acetonitrile, and toluene were obtained from Fisher Scientific (Hampton, NH, USA). Methanol and acetonitrile were LC/MS grade, while the other solvents were Optima grade. Acetic acid, butylated hydroxytoluene (BHT), sodium carbonate, sodium hydroxide, triphenylphosphine (TPP), ethylenediaminetetraacetic acid (EDTA), and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St. Louis, MO). Seventy six unlabeled oxylipin standards and nine deuterated surrogate standards (Table S1) were obtained from Cayman Chemicals (Ann Arbor, MI, USA) or Larodan (Monroe, MI, USA). Fatty acid standards (Table S2) were purchased from NuCheck Prep (Elysian, MN, USA). Fish and algae oil supplements were purchased from a local CVS Pharmacy store in Davis, CA, USA.

**Study Design.** In a series of experiments, we examined the optimal fish oil volumes that can be hydrolyzed with sodium carbonate base (Experiment 1); the effect of drying fish oil lipid extracts under nitrogen (a step used to reconstitute the lipids) on potential lipid due to residual air in the nitrogen tank (Experiment 2); the use of extraction solvent (methanol containing 0.1% acetic acid and 0.1% BHT) alongside sodium carbonate to increase hydrolysis efficiency by improving lipid solubility (Experiment 3); the effect of increasing base volume on hydrolysis efficiency (Experiment 4); and the efficiency of sodium carbonate versus sodium hydroxide on esterified oxylipins hydrolysis (Experiment 5). Cod liver oil supplements were used for optimizing the oxylipin extraction procedure.

In Experiment 6, we utilized the optimal procedure derived from Experiments 1 to 5 to measure oxylipins in various fish and algae oil supplements.

**Extraction of Oxylipins from Fish Oil Supplements.** *Experiment 1. Effect of Fish Oil Volume on Oxylipin Hydrolysis Efficiency.* The goal of Experiment 1 was to determine the minimum amount of oil volume needed for the base hydrolysis reaction to go to completion. Previously, when we hydrolyzed 10 μL of plant oil in 200 μL of extraction solvent (methanol containing 0.1% acetic acid and 0.1% BHT) and 200 μL of sodium carbonate, we observed significant ion suppression.[25] We therefore questioned whether this was due to inefficiencies in the hydrolysis reaction, leading to interferences with nonhydrolyzed compounds coeluting with the oxylipins and causing ion suppression.

A stock solution containing 500 μL of cod liver oil in 1500 μL of chloroform:methanol (2:1) was freshly prepared. Volumes of 8, 40, 80, and 120 μL of stock solution corresponding to 2, 10, 20, and 30 μL of fish oil, respectively, were aliquoted into 2 mL microcentrifuge tubes (SealRite, Germany) containing 10 μL of 2 μM surrogate standard mix and 10 μL of an antioxidant mix consisting of BHT, EDTA, and TPP at a concentration of 0.2 mg/mL each. The surrogate standard solution contained 2 μM d-11-11(12)- epoxyeicosatrienoic acid (d-11-11(12)-EpETrE), d11-14,15-dihydroxyeicosatrienoic acid (d11-14,15-DiHETrE), d4-6-keto-prostaglandin F1 alpha (d4-6-keto-PGF1a), d4-9-hydroxyoctadecadienoic acid (d4-9-HODE), d4-leukotriene B4 (d4-LTB4), d4-prostaglandin E2 (d4-PGE2), d4-thromboxane B2 (d4-TXB2), d6-20-hydroxyeicosatetraenoic acid (d6-20-HETE), and d8-5-HETE. Each sample was spiked with 20 pmol of each individual surrogate. The sample size was 3 aliquots per oil volume.

Plastic centrifuge tubes (2 mL) containing the fish oil stock solution, surrogate standard mix, and antioxidants were dried under nitrogen and reconstituted in 190 μL of extraction solvent containing 0.1% acetic acid and 0.1% BHT in methanol. The samples were subjected to base hydrolysis by adding 200 μL of 0.25 M sodium carbonate solution (1:1 methanol:water), vortexing, and heating at 60 °C for 30 min. The samples were acidified to pH 4−6 by adding 25 μL of acetic acid. Ultrapure water (1575 μL) was then added to adjust the methanol concentration of the sample to ∼15%.

Oxylipins were separated on Waters Oasis HLB (60 mg, 3 cm cartridge; Waters, Milford, MA) solid-phase extraction (SPE) columns. The columns were first washed with 3 mL of ethyl acetate and 6 mL of methanol and conditioned with 6 mL of an SPE buffer containing 0.1% acetic acid and 5% methanol in ultrapure water. The samples were then loaded onto the columns and washed twice with 3 mL of SPE buffer. The columns were dried under vacuum (15−20 psi) for 20 min. Oxylipins were eluted with 0.5 mL of methanol and 1.5 mL of ethyl acetate. The oxylipin extracts were dried under nitrogen, reconstituted in 100 μL of methanol, centrifuged at 15871*g* (13,000 rpm on an Eppendorf AG 5424R centrifuge, Germany) in 2 mL centrifuge tubes containing 0.1 μm filter (UFC30VV00 Ultrafree-MC VV Centrifugal Filter). The filtered oxylipin extracts were transferred to amber vials containing 150 μL of inserts and kept at −80 °C until they were analyzed by UPLC-MS/MS as described below. All samples were analyzed within 1 week following extraction.

*Experiment 2. Effect of Drying Time on Oxylipin Concentrations.* As shown in the Results section, in Experiment 1, the highest concentration of oxylipins and least matrix effects were found with 2 μL of fish oil, compared to 10, 20, and 30 μL. Here, we hypothesized that the low oxylipin yield in the higher-volume samples was due to possible degradation associated with the longer drying time of the high-volume oil aliquots than of the low-volume aliquots. It took 10 min to dry the 2 μL fish oil in 8 μL of solvent with nitrogen and up to 20 min to dry the 10, 20, and 30 μL fish oil in 40, 80, and 120 μL of solvent, respectively. Although samples were dried under 99.998% pure nitrogen (PRAXAIR, USA), it is possible that oxygen impurities in the tank might have altered oxylipin concentrations.

Thus, in this experiment, 2 μL of fish oil dissolved in 8 μL of solvent was dried for 10 or 20 min ($n$ = 3 per time-point), and 30 μL oil in 120 μL of solvent was dried for 20 min ($n$ = 3). If drying time was associated with oxylipin degradation, we would expect to see reductions in oxylipins in both the 2 and 30 μL oils dried for 20 min, compared to the 2 μL dried for 10 min. The oils were dried in Eppendorf tubes containing 10 μL of antioxidant solution and 10 μL of surrogate standard mix for oxylipin quantitation. The 2 μL oil samples that were dried for 10 min were placed on ice while the other samples were drying (for 20 min). After all samples were dried under nitrogen, 190 μL of extraction solvent and 200 μL of 0.25 M sodium carbonate solution were added to all tubes, which were then heated at 60 °C for 30 min. The samples were acidified with 25 μL of acetic acid and diluted with 1575 μL of ultrapure water as described above. Oxylipins were then separated with SPE, filtered, and stored at −80 °C until UPLC-MS/MS analysis.

*Experiment 3. Effect of Adding Extraction Solvent with Hydrolysis Reagent on Oxylipin Concentrations.* Fish oil is rich in triacylglycerols, which may not completely dissolve in the sodium carbonate base containing methanol/water (1:1). To test whether adding methanol extraction solvent containing 0.1% acetic acid and 0.1% BHT to the sample improves oil solubility and extraction efficiency, two volumes of fish oil (2 and 10 μL) were hydrolyzed in base with or without adding extraction solvent before hydrolysis.

8 and 40 μL of freshly prepared stock solution of 500 μL of cod liver oil in 1500 mL of 2:1 chloroform:methanol (corresponding to 2 and 10 μL of oil) were added to test tubes containing 10 μL of surrogate standard mix and 10 μL of antioxidant mix ($n$ = 3 per volume per method). The samples were dried under nitrogen. 190 μL of extraction solvent was added to 3 samples per volume (total of 6) followed by 200 μL of 0.25 M sodium carbonate solution (i.e., the "Extraction Solvent


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Group"). Sodium carbonate solution was added directly to another set of 3 samples per volume to test the effect of direct base hydrolysis (i.e., "No Extraction Solvent Group"). The samples were hydrolyzed at 60 °C for 30 min. After hydrolysis, 25 μL of acetic acid and 1575 μL of ultrapure water were added to both groups. Oxylipins were then separated with SPE as described above and kept at −80 °C until analysis by UPLC-MS/MS.

*Experiment 4. Effect of Increasing Hydrolysis Reagent Volume on Hydrolysis Efficiency.* In this experiment, we tested whether the volume of the base used for hydrolysis influences oxylipin concentrations. We hypothesized that increasing the base to oil ratio would improve the hydrolysis efficiency of bound oxylipins (i.e., more base relative to oil would facilitate more chemical interactions and improve hydrolysis efficiency). Two different volumes of sodium carbonate solution (0.25 M) were used: 200 (low-volume group (LV)) and 1000 μL (high-volume group (HV)) on four fish oil volumes including 1, 2, 5, and 10 μL.

A stock solution of 1000 μL of cod liver oil in 7000 μL of chloroform:methanol (2:1) was prepared, and volumes of 8, 16, 40, and 80 μL (corresponding to 1, 2, 5, and 10 μL of fish oil, respectively) were aliquoted into test tubes. For the LV group, 10 μL of surrogate standard mix and 10 μL of antioxidant mix were added. For the HV group, 10 μL of surrogate standard mix and 50 μL of antioxidant mix were added to maintain antioxidant concentrations following a 5-fold increase in solvent and base volume. The samples were dried under nitrogen. For the LV group, 190 μL of extraction solvent was added followed by 200 μL of 0.25 M sodium carbonate, while for the HV group, 950 μL of extraction solvent followed by 1000 μL of 0.25 M sodium carbonate was added. The samples were vortexed and hydrolyzed at 60 °C for 30 min. After cooling for 5 min at room temperature, the pH of the samples was lowered by adding 25 and 125 μL of acetic acid into LV and HV groups, respectively. The pH of a representative sample of each of the LV and HV samples was verified to be 4−6 by litmus paper. Millipore pure water, at 1575 and 7875 μL, was added to the LV and HV samples, respectively, to lower the methanol content to 15% before loading onto the SPE column. Free oxylipins were then extracted with Oasis HLB SPE columns, dried, reconstituted in 100 μL of methanol, filtered (0.1 μm filter) with an ultracentrifuge at 15871*g*, and kept at −80 °C until analysis by UPLC-MS/MS.

*Experiment 5. Effect of Sodium Carbonate Versus Sodium Hydroxide on Hydrolysis Efficiency.* The goal of this experiment was to compare the efficiency of hydrolysis with sodium carbonate to sodium hydroxide, in view of a recent study showing that potassium hydroxide efficiently hydrolyzes bound oxylipins .[27] A stock solution of 500 μL of cod liver oil in 7500 μL of chloroform:methanol (2:1) was prepared, and 32 μL (corresponding to 2 μL of oil) was added to test tubes containing 10 μL of surrogate standard mix and 10 μL of antioxidant mix. The samples were dried under nitrogen and reconstituted in 190 μL of extraction solvent. Then, 200 μL of 0.25 M sodium carbonate ($n$ = 5) or sodium hydroxide ($n$ = 5) was added. After vortexing, the samples were hydrolyzed at 60 °C for 30 min. The samples were then cooled for 5 min at room temperature, and the pH was adjusted to 4−6 by adding 25 μL of acetic acid. The samples were diluted by adding 1575 μL of Millipore pure water. Oxylipins were extracted with SPE, reconstituted in 100 μL of methanol, filtered, and kept at −80 °C until analysis by UPLC-MS/MS.

*Experiment 6. Measuring Oxylipins in Fish and Algae Oils.* To estimate the average intake of oxylipins derived from omega-3 oil supplements, we measured oxylipin composition of four different fish oil supplements and one algae oil supplement obtained from a CVS Pharmacy in Davis, CA. The oils analyzed were fish oil 1 (Nature's Bounty), fish oil 2 (CVS Health), fish oil 3 (Nature Made), fish oil 4 (cod liver oil, CVS Health), and algae oil (CVS Health) (Table 1).

A stock solution of 500 μL of oil in 7500 μL of chloroform:methanol (2:1) was prepared for each supplement, and 32 μL of stock solution (corresponding to 2 μL of oil; $n$ = 3) was added to centrifuge tubes containing 10 μL of surrogate standard mix and 10 μL of antioxidant mix. After drying under nitrogen, samples were reconstituted in 190 μL of extraction solvent (containing 0.1% acetic acid and 0.1% BHT) and hydrolyzed at 60 °C for 30 min after adding 200 μL of 0.25 M sodium hydroxide. This hydrolysis procedure was shown to be the most effective based on Experiments 1 to 5 above. Samples were cooled for approximately 5 min at room temperature, and 25 μL of acetic acid and 1575 μL of Millipore pure water were added. Oxylipins were then extracted with SPE as described above and reconstituted in 100 μL of methanol. Oxylipin extracts were filtered and kept at −80 °C until analysis by UPLC-MS/MS.

**Table 1. Oil Sources of the Omega-3 Containing Supplements Used in this Study**

| | oil source | other sources contributing to oil |
|---|---|---|
| fish oil 1 | oil from fish including anchovy, herring, mackerel, sardine | |
| fish oil 2 | oil from fish including mackerel, anchovy, menhaden, herring, sardine, jack | soy |
| fish oil 3 | oil from fish including mackerel, anchovy, menhaden, herring, sardine | |
| fish oil 4 (cod liver oil) | cod liver | |
| algae oil | algal DHA (*Schizochytrium* sp.) | sunflower oil, canola oil |

**Analysis of Oxylipins by UPLC-MS/MS.** Oxylipins were analyzed on an Agilent 1290 UPLC system coupled to an Agilent 6460 triple quadrupole mass spectrometer (Agilent Corporation, Palo Alto, CA, USA) equipped with an Agilent jetstream electrospray ion source (ESI). The hydrolyzed oxylipins were separated on an Agilent Eclipse Plus C18 reversed-phase column (2.1 × 150 mm, 1.8 μm particle size) maintained at 45 °C using a binary solvent system composed of Solvent A (Millipore water containing 0.1% acetic acid) and Solvent B (acetonitrile:methanol (80:15 v/v) with 0.1% acetic acid).[28] The solvent flow rate and gradient conditions are shown in Table S3. Oxylipin detection was carried out with an Agilent jetstream ESI operating in negative mode and using dynamic multiple reaction monitoring (MRM) conditions. The retention time, MRM conditions, and collision energy are presented in Table S1.

**Surrogate Standard Recovery.** The surrogate standard recovery, determined after hydrolysis and SPE, was calculated as follows

$$\text{surrogate recovery} = [A_{\text{surrogate}} \text{ in oxylipin extract of fish oil sample after hydrolysis and SPE}/A_{\text{surrogate}} \text{ in standard mix in methanol solvent}] \times 100$$

where $A_{\text{surrogate}}$ is the peak area of 2 pmol of each surrogate standard.

**Interexperimental Variability (Reproducibility) of Oxylipin Concentrations.** Interexperimental variability was calculated for the 2 and 10 μL oil volumes as the coefficient of variation (CV) between experiments. The CV was calculated as follows

$$\text{CV} = [\text{standard deviation of the means}/\text{overall mean}] \times 100$$

**Matrix Effects.** Matrix effects were determined by spiking 10 μL of standard mix containing 72 unlabeled oxylipin standards (0.25−5 μM) and nine deuterated oxylipins (0.2 μM) to (i) oxylipins hydrolyzed and extracted with SPE from fish oil (40 μL of oxylipin extract) and (ii) a blank methanol sample (40 μL). Another 40 μL of oxylipin extract from the fish oil samples was spiked with 10 μL of methanol (iii). 10 μL of each of samples i, ii, and iii was injected into a UPLC-MS/MS instrument, and the matrix effect was calculated according to the following equation

$$\text{matrix effect} = ([A_{\text{standard}} \text{ in i}]/([A_{\text{standard}} \text{ in ii}] + [A_{\text{standard}} \text{ in iii}])) \times 100$$

The matrix effect was calculated for both surrogate standards and unlabeled oxylipin standards. A range of 70−130% was considered acceptable.

**Analysis of Fatty Acids by Gas Chromatography (GC).** To confirm the fatty acid composition of the omega-3 oil pills, the cod liver oil used for method development in Experiments 1 to 5 and the fish and algae supplements used to estimate oxylipin exposure in Experiment 6


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**Table 2a. Effect of Fish Oil Volume on Oxylipin Concentrations (pmol/μL Oil), Determined in 2, 10, 20, and 30 μL of Fish Oil in Experiment 1**[a]

| | fish oil volume | | | |
|---|---|---|---|---|
| oxylipins | 2 μL | 10 μL | 20 μL | 30 μL |
| | | ALA-derived metabolites | | |
| 9-HOTrE | 0.69 ± 0.09[a] | 0.13 ± 0.07[b] | 0.08 ± 0.01[b] | 0.22 ± 0.13[b] |
| 13-HOTrE | 0.73 ± 0.11[a] | 0.28 ± 0.27[b] | 0.13 ± 0.04[b] | 0.26 ± 0.13[b] |
| | | EPA-derived metabolites | | |
| 5-HEPE | 50.68 ± 5.26[a] | 12.96 ± 5.72[b] | 9.12 ± 0.32[b] | 28.05 ± 19.15[ab] |
| 8-HEPE | 4.78 ± 0.78[a] | 0.84 ± 0.44[b] | 0.50 ± 0.05[b] | 1.32 ± 0.80[b] |
| 12-HEPE | 7.61 ± 0.64[a] | 1.19 ± 0.64[b] | 0.72 ± 0.09[b] | 1.98 ± 1.21[b] |
| 15-HEPE | 7.43 ± 0.96[a] | 1.23 ± 0.63[b] | 0.68 ± 0.08[b] | 1.65 ± 1.02[b] |
| 8(9)-EpETE | 5.34 ± 1.26[a] | 0.66 ± 0.38[b] | 0.50 ± 0.20[b] | 1.09 ± 0.55[b] |
| 11(12)-EpETE | 4.80 ± 1.36[a] | 0.64 ± 0.39[b] | 0.48 ± 0.22[b] | 1.16 ± 0.60[b] |
| 14(15)-EpETE | 15.77 ± 4.50[a] | 2.35 ± 1.16[b] | 1.45 ± 0.68[b] | 3.95 ± 2.41[b] |
| 17(18)-EpETE | 185.13 ± 50.05[a] | 28.46 ± 16.20[b] | 18.81 ± 10.80[b] | 47.44 ± 26.39[b] |
| 14,15-DiHETE | 15.03 ± 2.88[a] | 4.01 ± 1.96[b] | 2.39 ± 0.42[b] | 6.84 ± 3.95[b] |
| 17,18-DiHETE | 22.59 ± 3.65[a] | 6.49 ± 3.08[b] | 3.71 ± 0.60[b] | 10.68 ± 6.53[b] |
| | | DHA-derived metabolites | | |
| 17-HDoHE | 21.99 ± 4.73[a] | 3.93 ± 1.83[b] | 2.31 ± 0.33[b] | 5.33 ± 2.69[b] |
| 7(8)-EpDPE | 3.01 ± 0.82[a] | 0.41 ± 0.20[b] | 0.32 ± 0.14[b] | 0.77 ± 0.43[b] |
| 10(11)-EpDPE | 4.59 ± 0.82[a] | 0.68 ± 0.33[b] | 0.50 ± 0.15[b] | 1.33 ± 0.75[b] |
| 13(14)-EpDPE | 5.08 ± 1.09[a] | 0.75 ± 0.36[b] | 0.46 ± 0.17[b] | 1.26 ± 0.78[b] |
| 16(17)-EpDPE | 6.25 ± 1.89[a] | 1.06 ± 0.58[b] | 0.78 ± 0.34[b] | 2.09 ± 1.21[b] |
| 19(20)-EpDPE | 23.45 ± 7.32 | 7.25 ± 3.74 | 6.80 ± 1.17 | 19.21 ± 10.22 |
| | | LA-derived metabolites | | |
| 9-HODE | 2.08 ± 0.40[a] | 0.45 ± 0.24[b] | 0.29 ± 0.04[b] | 0.81 ± 0.45[b] |
| 13-HODE | 3.76 ± 0.81[a] | 0.95 ± 0.46[b] | 0.68 ± 0.07[b] | 2.10 ± 1.23[ab] |
| 9-oxo-ODE | 0.71 ± 0.19[a] | 0.14 ± 0.06[b] | 0.08 ± 0.01[b] | 0.19 ± 0.11[b] |
| 13-oxo-ODE | 1.93 ± 0.66[a] | 0.48 ± 0.22[b] | 0.26 ± 0.04[b] | 0.61 ± 0.28[b] |
| 9(10)-EpOME | 0.53 ± 0.07[a] | 0.06 ± 0.03[b] | 0.04 ± 0.01[b] | 0.08 ± 0.05[b] |
| 12(13)-EpOME | 1.75 ± 0.29[a] | 0.21 ± 0.11[b] | 0.14 ± 0.06[b] | 0.31 ± 0.17[b] |
| 9,10-DiHOME | 0.68 ± 0.13[a] | 0.18 ± 0.08[b] | 0.09 ± 0.02[b] | 0.26 ± 0.16[b] |
| 12,13-DiHOME | 0.27 ± 0.06[a] | 0.07 ± 0.04[b] | 0.04 ± 0.01[b] | 0.11 ± 0.07[b] |
| | | DGLA-derived metabolites | | |
| 15(S)-HETrE | 0.48 ± 0.12[a] | 0.13 ± 0.07[b] | 0.08 ± 0.01[b] | 0.21 ± 0.11[b] |
| | | AA-derived metabolites | | |
| 5-HETE | 1.86 ± 0.37[a] | 0.57 ± 0.26[b] | 0.48 ± 0.03[b] | 1.39 ± 0.82[ab] |
| 8-HETE | 0.43 ± 0.13[a] | 0.08 ± 0.04[b] | 0.04 ± 0.01[b] | 0.12 ± 0.07[b] |
| 9-HETE | 0.30 ± 0.05[a] | 0.06 ± 0.03[b] | 0.04 ± 0.01[b] | 0.09 ± 0.04[b] |
| 11-HETE | 0.39 ± 0.09[a] | 0.07 ± 0.04[b] | 0.04 ± 0.01[b] | 0.12 ± 0.06[b] |
| 12-HETE | 0.27 ± 0.01[a] | 0.05 ± 0.02[b] | 0.027 ± 0.004[b] | 0.07 ± 0.04[b] |
| 15-HETE | 0.49 ± 0.07[a] | 0.09 ± 0.05[b] | 0.06 ± 0.01[b] | 0.16 ± 0.08[b] |
| 20-HETE | 2.51 ± 0.68[a] | 0.54 ± 0.27[b] | 0.33 ± 0.03[b] | 0.94 ± 0.45[b] |
| 15-oxo-ETE | 0.39 ± 0.04[a] | 0.10 ± 0.05[b] | 0.07 ± 0.01[b] | 0.17 ± 0.11[b] |
| 5(6)-EpETrE | 0.22 ± 0.06[a] | 0.03 ± 0.02[b] | 0.02 ± 0.01[b] | 0.03 ± 0.02[b] |
| 8(9)-EpETrE | 0.18 ± 0.06[a] | 0.04 ± 0.02[b] | 0.03 ± 0.01[b] | 0.09 ± 0.05[ab] |
| 11(12)-EpETrE | 0.31 ± 0.08[a] | 0.05 ± 0.03[b] | 0.04 ± 0.01[b] | 0.11 ± 0.07[b] |
| 14(15)-EpETrE | 0.72 ± 0.15[a] | 0.12 ± 0.05[b] | 0.07 ± 0.04[b] | 0.19 ± 0.11[b] |
| 5,6-DiHETrE | 0.22 ± 0.07 | 0.09 ± 0.04 | 0.07 ± 0.004 | 0.23 ± 0.15 |
| 8,9-DiHETrE | 0.09 ± 0.02[a] | 0.02 ± 0.01[b] | 0.01 ± 0.003[b] | 0.03 ± 0.02[b] |
| 11,12-DiHETrE | 0.05 ± 0.01[a] | 0.01 ± 0.004[b] | 0.007 ± 0.001[b] | 0.02 ± 0.01[b] |
| 14,15-DiHETrE | 0.07 ± 0.02[a] | 0.02 ± 0.01[b] | 0.01 ± 0.002[b] | 0.04 ± 0.02[ab] |
| LXA4 | 1.86 ± 0.51 | 0.70 ± 0.31 | 0.57 ± 0.04 | 1.63 ± 0.96 |

[a]Data are reported as mean ± SD ($n$ = 3) and analyzed by one-way ANOVA followed by Tukey's post-hoc test. Different letters represent significant differences between the means ($p$ < 0.05).

were subjected to analysis with gas chromatography (GC). The algae oil purchased belonged to the *Schizochytrium* sp., which are part of the Thraustochytriaceae family known to produce large amounts of PUFAs.[29] For each oil, 160 μL of stock solution (500 μL oil in 7500 μL chloroform:methanol (2:1) corresponding to 10 μL oil) and 145 μL of 8.6 mg/mL triheptadecanoic acid in 2:1 v/v chloroform:methanol (internal standard) were added to 8 mL glass tubes and dried under nitrogen ($n$ = 3). Then, 400 μL of toluene was added followed by 3 mL of methanol and 600 μL of 8% HCl (37% ACS reagent, Sigma-Aldrich) in methanol. The samples were transesterified at 90 °C for 60 min and cooled to room temperature for approximately 5 min. 1 mL of hexane and 1 mL of water were added, and the samples were allowed to sit for


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**Table 2b. Effect of Fish Oil Volume on Extraction Recovery (%) of Labeled Surrogates, Determined in 2, 10, 20, and 30 μL of Fish Oil in Experiment 1[a]**

| | | fish oil volume | | | |
|---|---|---|---|---|---|
| extraction recovery (%) | blank | 2 μL | 10 μL | 20 μL | 30 μL |
| d-11-11(12)-EpETrE | 56 | 60 ± 12[a] | 77 ± 23[a] | 68 ± 8[a] | 22 ± 10[b] |
| d11-14,15-DiHETrE | 56 | 56 ± 6[a] | 88 ± 31[ab] | 133 ± 21[b] | 48 ± 23[a] |
| d4-6-keto-PGF1a | 120 | 69 ± 9[ab] | 101 ± 32[a] | 121 ± 10[a] | 43 ± 20[b] |
| d4-9-HODE | 68 | 63 ± 10[ab] | 89 ± 27[a] | 105 ± 16[a] | 35 ± 17[b] |
| d4-LTB4 | 78 | 60 ± 11[ab] | 88 ± 24[a] | 98 ± 14[a] | 33 ± 16[b] |
| d4-TXB2 | 88 | 50 ± 8[a] | 73 ± 22[ab] | 94 ± 14[b] | 34 ± 15[a] |
| d6-20-HETE | 44 | 55 ± 8[ab] | 82 ± 23[bc] | 105 ± 17[c] | 37 ± 16[a] |
| d8-5-HETE | 46 | 57 ± 8[a] | 87 ± 30[ab] | 112 ± 13[b] | 39 ± 18[a] |

[a]Data are reported as mean ± SD ($n$ = 3 for all samples except for the blank sample ($n$ = 1)) and analyzed by one-way ANOVA followed by Tukey's post-hoc test (blank not included in statistical analysis). Different letters represent significant differences between the means ($p < 0.05$).

**Table 3. Effect of Drying Time on the Concentrations of Oxylipins (pmol/μL Oil) and Extraction Recovery (%) of Labeled Surrogates, Determined in 2 μL of Fish Oil Dried for 10 and 20 min and 30 μL of Fish Oil Dried for 20 min (Experiment 2)[a]**

| oxylipins | 2 μL oil - 10 min drying | 2 μL oil - 20 min drying | 30 μL oil - 20 min drying |
|---|---|---|---|
| ALA-derived metabolites | | | |
| 9-HOTrE | 0.30 ± 0.02[a] | 0.40 ± 0.05[a] | 0.13 ± 0.01[b] |
| 13-HOTrE | 0.42 ± 0.05[a] | 0.50 ± 0.05[a] | 0.19 ± 0.03[b] |
| EPA-derived metabolites | | | |
| 5-HEPE | 46.24 ± 6.70[a] | 45.25 ± 2.94[a] | 33.40 ± 0.47[b] |
| 8-HEPE | 4.07 ± 0.57[a] | 5.19 ± 0.05[b] | 1.44 ± 0.06[c] |
| 12-HEPE | 3.30 ± 1.20[a] | 3.74 ± 0.55[a] | 1.19 ± 0.05[b] |
| 15-HEPE | 4.34 ± 0.54[a] | 4.73 ± 0.52[a] | 1.19 ± 0.04[b] |
| 8(9)-EpETE | 1.45 ± 0.48 | 2.92 ± 2.31 | 0.57 ± 0.11 |
| 11(12)-EpETE | 1.54 ± 0.27 | 2.50 ± 1.80 | 0.42 ± 0.08 |
| 14(15)-EpETE | 7.40 ± 1.90 | 9.14 ± 4.59 | 1.15 ± 0.40 |
| 17(18)-EpETE | 88.82 ± 15.06[ab] | 102.68 ± 52.59[a] | 11.40 ± 3.52[b] |
| 5,6-DiHETE | ND | ND | 0.06 ± 0.02 |
| 8,15-DiHETE | ND | ND | ND |
| 14,15-DiHETE | 3.68 ± 0.23[a] | 5.25 ± 0.62[b] | 1.43 ± 0.14[c] |
| 17,18-DiHETE | 6.48 ± 0.32[a] | 8.21 ± 0.64[b] | 2.18 ± 0.31[c] |
| DHA-derived metabolites | | | |
| 17-HDoHE | 7.63 ± 0.05[a] | 9.85 ± 0.54[b] | 2.38 ± 0.26[c] |
| 7(8)-EpDPE | 1.16 ± 0.08 | 2.08 ± 1.62 | 0.40 ± 0.07 |
| 10(11)-EpDPE | 1.56 ± 0.08 | 3.07 ± 2.01 | 0.55 ± 0.07 |
| 13(14)-EpDPE | 2.58 ± 0.21 | 3.91 ± 2.11 | 0.51 ± 0.11 |
| 16(17)-EpDPE | 3.78 ± 0.35[ab] | 4.51 ± 2.13[a] | 0.77 ± 0.20[b] |
| 19(20)-EpDPE | 12.36 ± 0.33 | 13.94 ± 5.68 | 6.74 ± 0.78 |
| LA-derived metabolites | | | |
| 9-HODE | 0.7 ± 0.005[a] | 0.83 ± 0.14[a] | 0.31 ± 0.02[b] |
| 13-HODE | 1.45 ± 0.27[a] | 1.68 ± 0.15[a] | 0.81 ± 0.06[b] |
| 9-oxo-ODE | 0.28 ± 0.05[a] | 0.33 ± 0.02[a] | 0.07 ± 0.005[b] |
| 13-oxo-ODE | 0.79 ± 0.12[a] | 1.18 ± 0.01[b] | 0.22 ± 0.03[c] |
| 9(10)-EpOME | 0.21 ± 0.07 | 0.38 ± 0.26 | 0.04 ± 0.01 |
| 12(13)-EpOME | 0.67 ± 0.05 | 1.25 ± 0.83 | 0.16 ± 0.02 |
| 9,10-DiHOME | 0.20 ± 0.02[a] | 0.23 ± 0.01[a] | 0.06 ± 0.004[b] |
| 12,13-DiHOME | 0.10 ± 0.01[a] | 0.12 ± 0.01[a] | 0.03 ± 0.002[b] |
| DGLA-derived metabolites | | | |
| 15(S)-HETrE | 0.24 ± 0.03[a] | 0.31 ± 0.05[a] | 0.11 ± 0.01[b] |
| AA-derived metabolites | | | |
| 5-HETE | 1.48 ± 0.35 | 1.46 ± 0.17 | 1.1 ± 0.004 |
| 8-HETE | ND | ND | 0.07 ± 0.01 |
| 9-HETE | 0.11 ± 0.16 | 0.07 ± 0.12 | 0.06 ± 0.01 |
| 11-HETE | 0.27 ± 0.01[a] | 0.30 ± 0.05[a] | 0.1 ± 0.005[b] |
| 12-HETE | 0.15 ± 0.01 | 0.14 ± 0.12 | 0.04 ± 0.001 |
| 15-HETE | 0.20 ± 0.04[ab] | 0.29 ± 0.06[a] | 0.11 ± 0.01[b] |
| 20-HETE | 0.94 ± 0.22[a] | 1.26 ± 0.17[a] | 0.29 ± 0.02[b] |

| oxylipins | 2 μL oil - 10 min drying | 2 μL oil - 20 min drying | 30 μL oil - 20 min drying |
|---|---|---|---|
| AA-derived metabolites | | | |
| 5-oxo-ETE | ND | ND | 0.05 ± 0.05 |
| 12-oxo-ETE | ND | ND | ND |
| 15-oxo-ETE | 0.38 ± 0.01[a] | 0.53 ± 0.09[a] | 0.13 ± 0.01[b] |
| 5(6)-EpETrE | 0.06 ± 0.02 | 0.15 ± 0.11 | 0.02 ± 0.004 |
| 8(9)-EpETrE | 0.1 ± 0.001 | 0.19 ± 0.10 | 0.04 ± 0.01 |
| 11(12)-EpETrE | 0.1 ± 0.002 | 0.19 ± 0.10 | 0.04 ± 0.01 |
| 14(15)-EpETrE | 0.57 ± 0.10[a] | 0.64 ± 0.23[a] | 0.05 ± 0.02[b] |
| 5,6-DiHETrE | 0.14 ± 0.04[ab] | 0.10 ± 0.02[a] | 0.06 ± 0.01[b] |
| 5,15-DiHETE | ND | ND | ND |
| 8,9-DiHETrE | ND | ND | 0.009 ± 0.001 |
| 11,12-DiHETrE | 0.02 ± 0.01[a] | 0.02 ± 0.002[a] | 0.005 ± 0.001[b] |
| 14,15-DiHETrE | 0.03 ± 0.001[a] | 0.04 ± 0.004[b] | 0.01 ± 0.0002[c] |
| 6-keto-PGF1a | ND | ND | ND |
| 20-COOH-LTB4 | ND | ND | ND |
| 20-OH-LTB4 | ND | ND | ND |
| 6-trans-LTB4 | ND | ND | ND |
| LTB3 | ND | 0.001 ± 0.002 | 0.01 ± 0.01 |
| LTB4 | ND | ND | ND |
| LTC4 | ND | ND | ND |
| LTD4 | ND | ND | ND |
| LTE4 | ND | ND | ND |
| LXA4 | 1.01 ± 0.14[a] | 1.08 ± 0.04[a] | 0.52 ± 0.04[b] |
| TXB2 | ND | ND | ND |
| extraction recovery (%) | | | |
| d-11-11(12)-EpETrE | 119 ± 4[a] | 127 ± 5[a] | 79 ± 5[b] |
| d11-14,15-DiHETrE | 129 ± 8[a] | 124 ± 9[a] | 177 ± 9[b] |
| d4-6-keto-PGF1a | 121 ± 4[ab] | 125 ± 7[a] | 111 ± 1[b] |
| d4-9-HODE | 93 ± 4 | 85 ± 15 | 86 ± 4 |
| d4-LTB4 | 104 ± 5[ab] | 107 ± 2[a] | 96 ± 3[b] |
| d4-TXB2 | 100 ± 5 | 98 ± 2 | 98 ± 3 |
| d6-20-HETE | 101 ± 2 | 99 ± 18 | 114 ± 5 |
| d8-5-HETE | 55 ± 3 | 53 ± 5 | 58 ± 0.4 |

[a]Data are represented as mean ± SD ($n$ = 3 for samples with 2 μL fish oil-20 min drying and 30 μL fish oil-20 min drying, $n$ = 2 for samples with 2 μL fish oil-10 min drying). Data were statistically analyzed by one-way ANOVA followed by Tukey's post-hoc test. Different letters in each row represent significant differences between the means ($p < 0.05$). ND: not detected.



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**Table 4. Effect of Adding Extraction Solvent (Methanol with 0.1% Acetic Acid and 0.1% BHT) to Sodium Carbonate on the Concentrations of Oxylipins (pmol/μL Oil) and Extraction Recovery (%) of Labeled Surrogates in Cod Liver Oil (Experiment 3)[a]**

| | fish oil volume (2 μL) | | fish oil volume (10 μL) | |
|---|---|---|---|---|
| oxylipins | without extraction solvent | with extraction solvent | without extraction solvent | with extraction solvent |
| | | ALA-derived metabolites | | |
| 9-HOTrE | ND | 0.77 ± 0.15[a] | ND | 0.25 ± 0.12[b] |
| 13-HOTrE | ND | 0.65 ± 0.11[a] | ND | 0.25 ± 0.11[b] |
| | | EPA-derived metabolites | | |
| 5-HEPE | 39.08 ± 6.96[ab] | 62.39 ± 8.43[a] | 14.47 ± 2.97[b] | 45.86 ± 22.16[ab] |
| 8-HEPE | 0.44 ± 0.02[a] | 6.83 ± 1.37[b] | 0.09 ± 0.01[a] | 2.03 ± 0.89[a] |
| 12-HEPE | 0.32 ± 0.06[a] | 6.58 ± 1.64[b] | 0.07 ± 0.003[a] | 1.84 ± 0.94[a] |
| 15-HEPE | 0.32 ± 0.03[a] | 6.63 ± 1.81[b] | 0.08 ± 0.01[a] | 1.98 ± 0.85[a] |
| 8(9)-EpETE | ND | 1.50 ± 0.58 | ND | 0.56 ± 0.21 |
| 11(12)-EpETE | 0.99 ± 0.15 | 1.24 ± 0.62 | 0.21 ± 0.05 | 0.61 ± 0.27 |
| 14(15)-EpETE | 5.87 ± 0.63 | 4.99 ± 3.15 | 1.13 ± 0.22 | 3.02 ± 1.34 |
| 17(18)-EpETE | 80.55 ± 6.44[a] | 51.37 ± 34.63[ab] | 14.19 ± 3.27[b] | 32.46 ± 14.85[ab] |
| 17,18-DiHETE | 1.60 ± 0.17[ab] | 12.12 ± 1.74[c] | 0.41 ± 0.04[a] | 4.99 ± 1.32[b] |
| 14,15-DiHETE | 1.06 ± 0.16[a] | 5.97 ± 0.26[b] | 0.22 ± 0.03[a] | 2.55 ± 0.86[c] |
| | | DHA-derived metabolites | | |
| 17-HDoHE | ND | 17.19 ± 2.83[a] | 0.26 ± 0.03[b] | 4.96 ± 2.51[b] |
| 7(8)-EpDPE | 0.49 ± 0.05[ab] | 1.17 ± 0.45[a] | 0.14 ± 0.01[b] | 0.46 ± 0.16[ab] |
| 10(11)-EpDPE | 0.98 ± 0.10[ab] | 1.42 ± 0.55[a] | 0.31 ± 0.04[b] | 0.72 ± 0.25[ab] |
| 13(14)-EpDPE | 2.30 ± 0.25 | 2.02 ± 1.04 | 0.54 ± 0.13 | 1.03 ± 0.39 |
| 16(17)-EpDPE | 3.32 ± 0.33[a] | 2.17 ± 1.25[ab] | 0.73 ± 0.16[b] | 1.31 ± 0.50[ab] |
| 19(20)-EpDPE | 15.94 ± 1.75[a] | 12.14 ± 4.04[ab] | 3.36 ± 0.57[b] | 12.26 ± 5.49[ab] |
| | | LA-derived metabolites | | |
| 9-HODE | 0.34 ± 0.02[a] | 1.46 ± 0.30[b] | 0.13 ± 0.02[a] | 0.54 ± 0.24[a] |
| 13-HODE | 0.67 ± 0.05[a] | 2.32 ± 0.38[b] | 0.35 ± 0.04[a] | 1.09 ± 0.49[a] |
| 9-oxo-ODE | ND | 0.37 ± 0.03[a] | 0.04 ± 0.01[b] | 0.13 ± 0.05[c] |
| 13-oxo-ODE | ND | ND | ND | 0.34 ± 0.11 |
| 9(10)-EpOME | ND | 0.15 ± 0.05[a] | 0.02 ± 0.01[b] | 0.05 ± 0.02[b] |
| 12(13)-EpOME | 0.40 ± 0.07[ab] | 0.52 ± 0.23[a] | 0.07 ± 0.02[b] | 0.22 ± 0.10[ab] |
| 9,10-DiHOME | 0.07 ± 0.01[ab] | 0.36 ± 0.02[c] | 0.02 ± 0.004[a] | 0.12 ± 0.04[b] |
| 12,13-DiHOME | ND | 0.16 ± 0.02[a] | 0.01 ± 0.002[b] | 0.07 ± 0.02[b] |
| | | DGLA-derived metabolites | | |
| 15(S)-HETrE | ND | 0.27 ± 0.05[a] | ND | 0.11 ± 0.04[b] |
| | | AA-derived metabolites | | |
| 5-HETE | 1.66 ± 0.26[a] | 1.69 ± 0.27[a] | 0.44 ± 0.07[b] | 1.34 ± 0.66[ab] |
| 8-HETE | ND | 0.33 ± 0.06[a] | ND | 0.11 ± 0.05[b] |
| 9-HETE | ND | 0.23 ± 0.03[a] | ND | 0.07 ± 0.03[b] |
| 11-HETE | ND | 0.39 ± 0.08[a] | ND | 0.13 ± 0.06[b] |
| 12-HETE | ND | 0.26 ± 0.08[a] | ND | 0.07 ± 0.03[b] |
| 15-HETE | ND | 0.53 ± 0.12[a] | ND | 0.15 ± 0.08[b] |
| 20-HETE | ND | 1.41 ± 0.35[a] | ND | 0.52 ± 0.17[b] |
| 15-oxo-ETE | ND | 0.22 ± 0.03[a] | 0.01 ± 0.01[b] | 0.10 ± 0.03[c] |
| 5(6)-EpETrE | ND | 0.06 ± 0.03 | ND | 0.02 ± 0.01 |
| 8(9)-EpETrE | 0.14 ± 0.01[a] | 0.10 ± 0.04[ab] | 0.03 ± 0.01[b] | 0.06 ± 0.02[b] |
| 11(12)-EpETrE | 0.1 ± 0.002[a] | 0.12 ± 0.04[a] | 0.03 ± 0.01[b] | 0.06 ± 0.02[ab] |
| 14(15)-EpETrE | 0.47 ± 0.01[a] | 0.24 ± 0.14[ab] | 0.11 ± 0.02[b] | 0.16 ± 0.07[b] |
| 5,6-DiHETrE | 0.15 ± 0.02 | 0.10 ± 0.02 | 0.07 ± 0.004 | 0.12 ± 0.04 |
| 11,12-DiHETrE | ND | 0.03 ± 0.004[a] | ND | 0.011 ± 0.004[b] |
| 14,15-DiHETrE | ND | 0.05 ± 0.01[a] | ND | 0.02 ± 0.01[b] |
| LXA4 | 0.41 ± 0.07[ab] | 0.61 ± 0.05[a] | 0.18 ± 0.02[b] | 0.55 ± 0.19[a] |
| | | extraction recovery (%) | | |
| d-11-11(12)-EpETrE | 32 ± 2 [a] | 128 ± 22 [c] | 56 ± 8 [ab] | 89 ± 25[bc] |
| d11-14,15-DiHETrE | 35 ± 5[a] | 119 ± 19[b] | 74 ± 14[ab] | 108 ± 34[b] |
| d4-6-keto-PGF1a | 71 ± 6 | 98 ± 18 | 111 ± 14 | 104 ± 33 |
| d4-9-HODE | 50 ± 5[a] | 134 ± 18[b] | 83 ± 14[ab] | 106 ± 33[ab] |
| d4-LTB4 | 46 ± 6[a] | 120 ± 9[b] | 94 ± 17[ab] | 91 ± 27[ab] |
| d4-TXB2 | 43 ± 5[a] | 91 ± 2[b] | 68 ± 6[ab] | 81 ± 27[ab] |
| d6-20-HETE | 29 ± 2[a] | 108 ± 17[b] | 57 ± 8[ab] | 95 ± 28[ab] |


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**Table 4. continued**

| oxylipins | fish oil volume (2 μL) | | fish oil volume (10 μL) | |
|---|---|---|---|---|
| | without extraction solvent | with extraction solvent | without extraction solvent | with extraction solvent |
| | extraction recovery (%) | | | |
| d8-5-HETE | 34 ± 3[a] | 99 ± 18[b] | 62 ± 11[ab] | 83 ± 24[ab] |

[a]Data are presented as mean ± SD ($n$ = 3) and statistically analyzed by two-way ANOVA followed by Tukey's post-hoc test. Different letters in each row represent significant differences between the means ($p$ < 0.05). ND: not detected.

10 min at room temperature to allow phase separation. The top hexane layer containing the fatty acid methyl esters (FAMEs) was transferred to a new Eppendorf tube (1.5 mL) containing 450 μL of water, and the tubes were centrifuged at 13000 rpm for 2 min. The top hexane layer was transferred to another centrifuge tube, dried under nitrogen, and reconstituted in 1 mL of hexane. Samples were stored at −80 °C until analysis by GC.

FAMEs were analyzed on a PerkinElmer Clarus 500 GC system containing a Flame Ionization Detector (FID). FAMEs were separated using a DB-FFAP nitroterephthalic acid modified polyethylene glycol capillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness; Agilent Technologies, Santa Clara, CA, USA). Injector and detector temperatures were at 240 and 300 °C, respectively. The flow rate of the carrier gas (helium) was 1.3 mL/min. The oven temperature program was initially set at 50 °C for 2 min. It was increased to 180 °C at a rate of 10 °C/min, maintained at 180 °C for 5 min, ramped up to 240 °C by 5 °C/min, and held at 240 °C for 5 min. The injection volume was 1 μL. A mix of 31 FAME standards (NuChek Prep, Elysian, MN, USA) was used to identify fatty acids based on retention times. Retention times of FAME external standards are shown in Table S2. Concentrations of individual fatty acids were determined by comparing their peak areas to that of the internal standard.

**Statistical Analysis.** Statistical analysis was performed using GraphPad Prism v. 7.03 (La Jolla, CA, USA). For the oxylipin measurements from Experiment 1 (the effect of fish oil volume), Experiment 2 (effect of drying time), and Experiment 6 (comparison of oxylipins in various oil types), one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons was applied on oxylipins detected across all groups. No statistical test was used if there were nondetected metabolites in one or more experimental groups (this was a rare occurrence). For Experiment 3 (effect of extraction solvent) and Experiment 4 (effect of sodium carbonate base volume), two-way ANOVA followed by Tukey's post-hoc test was performed to capture the effects of extraction solvent and sodium carbonate volume on oil oxylipin concentrations. In Experiment 5 (comparing sodium carbonate to sodium hydroxide base), an unpaired $t$ test was used. Both oxylipin and fatty acid concentrations are reported as mean ± SD. Statistical significance was set at $p$ < 0.05.

## RESULTS

**Experiment 1. Effect of Fish Oil Volume on Oxylipin Hydrolysis Efficiency.** Oxylipin concentrations in 2, 10, 20, and 30 μL of cod liver oil hydrolyzed with 0.25 M sodium carbonate are presented in Table 2a. One-way ANOVA followed by Tukey's post-hoc test showed that concentrations of most oxylipins were significantly higher in the 2 μL cod liver oil amounting to ∼2 mg of oil ($p$ < 0.05) than in the 10, 20, and 30 μL volumes (∼10−30 mg of oil). As shown in Table 2a, almost all epoxide and hydroxy metabolites of ALA, EPA, DHA, LA, DGLA, and AA were significantly higher in the 2 μL low-volume oil than in the 10, 20, and 30 μL oils ($p$ < 0.05), which did not differ significantly from each other ($p$ > 0.05). AA-derived 8(9)-epoxyeicosatrienoic acid (8(9)-EpETrE), 14,15-dihydroxyeicosatrienoic acid (14,15-DiHETrE), and 5-hydroxyeicosatetraenoic acid (5-HETE), LA-derived 13-hydroxyoctadecadienoic acid (13-HODE), and EPA-derived 5-hydroxyeicosapentaenoic acid (5-HEPE) were significantly higher in the 2 and 30 μL oil volumes than in 10 and 20 μL ($p$ < 0.05). DHA-derived 19(20)-epoxydocosapentaenoic acid (19(20)-EpDPE) and AA-derived 5,6-DiHETrE and lipoxin A4 (LXA4) were not significantly different between the groups ($p$ > 0.05).

Surrogate standard recovery following hydrolysis of the 2, 10, 20, and 30 μL volumes ($n$ = 3/volume) and a blank sample (i.e., antioxidant and surrogate standards in methanol, $n$ = 1) is shown in Table 2b. The recovery of the surrogate standards ranged from 22−133% except for d4-PGE2, which was completely degraded during hydrolysis and therefore not detected. Higher standard recoveries were observed following hydrolysis of 10 and 20 μL compared to 2 or 30 μL oils ($p$ < 0.05). The standard recovery from the 2 and 30 μL oils was comparable to that from the blank sample with no oil.

Matrix effects were 73−141% across all oil volumes for the nine deuterated surrogates used to quantify oxylipins, suggesting minimal ion suppression/enhancement (Table S4). Matrix effects were 45 to 161% for the 2 μL oil volume and 49 to 194% for the 10 μL oil. Ion suppression was evident in 29 oxylipins with a response ratio of <70% in the 20 μL oil volume. Ion enhancement (response ratio > 130%) was seen in 21 oxylipins in the 30 μL oil volume. Three leukotrienes, i.e., leukotriene C4 (LTC4), leukotriene D4 (LTD4), and leukotriene E4 (LTE4), were completely ion-suppressed in all oil volumes.

**Experiment 2. Effect of Drying Time on Oxylipin Concentrations.** Low volumes of oil are dissolved in proportionally low volumes of methanol/chloroform and thus require less drying time than higher volumes. To test whether drying time affects oxylipin concentrations, 2 μL of oil in 8 μL of chloroform/methanol was dried under nitrogen for 10 or 20 min and compared to drying 30 μL of oil in 120 μL of chloroform/methanol for 20 min.

As shown in Table 3, drying 30 μL of oil in solvent under nitrogen for 20 min resulted in significantly lower oxylipin concentrations in 27 out of 44 metabolites compared to drying 2 μL of oil for 10 or 20 min. This means that the reduction in oxylipins in 30 μL oil was likely due reduced hydrolysis efficiency of the high oil volume rather than drying time, consistent with Experiment 1. The surrogate recoveries were comparable between the groups except for d11-11(12)-EpETrE, d4-6-keto-PGF1a and d4-LTB4, which were significantly lower in 30 μL oil vs 2 μL oil, and d11-14,15-DiHETrE, which was significantly higher in 30 μL oil vs 2 μL oil. These findings are consistent with the recoveries observed in Experiment 1.

**Experiment 3. Effect of Adding Extraction Solvent with Hydrolysis Reagent on Oxylipin Concentrations.** The effect of hydrolyzing in extraction solvent (methanol containing 0.1% acetic acid and 0.1% BHT) versus direct hydrolysis with 0.25 M sodium carbonate was tested at different oil volumes (2 and 10 μL). Two-way ANOVA showed a significant main effect of methanol extraction solvent, oil volume, and interaction between solvent and oil volume for 30, 39, and 26 out of 43 detected oxylipins, respectively. Tukey's post-hoc analysis showed that the addition of extraction solvent with the hydrolysis reagent significantly increased the


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**Table 5. Effect of Sodium Carbonate Volume (Experiment 4) on Oxylipin Concentrations (pmol/μL Oil) and Extraction Recovery (%) of Labeled Surrogates Determined in 1, 2, 5, and 10 μL of Cod Liver Oil Dissolved in 190 μL of Extraction Solvent and Hydrolyzed with 200 μL of 0.25 M Base (Low-Volume Base; LV Treatment) or Dissolved in 950 μL of Extraction Solvent and 1000 μL of 0.25 M Base (High-Volume Base; HV Treatment)[a]**

| | 1 μL oil | | 2 μL oil | | 5 μL oil | | 10 μL oil | |
|---|---|---|---|---|---|---|---|---|
| oxylipins | LV(128 mM base / 2.8 mM oil) | HV(128 mM base / 0.56 mM oil) | LV(128 mM base / 5.6 mM oil) | HV(128 mM base / 1.1 mM oil) | LV(128 mM base / 14 mM oil) | HV(128 mM base / 2.8 mM oil) | LV(128 mM base / 28 mM oil) | HV(128 mM base / 5.6 mM oil) |
| | | | | ALA-derived metabolites | | | | |
| 9-HOTrE | 1.12 ± 0.09$^{a}$ | 1.02 ± 0.18$^{a}$ | 0.80 ± 0.11$^{abc}$ | 0.92 ± 0.13$^{ab}$ | 0.40 ± 0.09$^{d}$ | 0.58 ± 0.05$^{bcd}$ | 0.30 ± 0.10$^{d}$ | 0.44 ± 0.04$^{cd}$ |
| 13-HOTrE | 1.28 ± 0.06$^{a}$ | 0.92 ± 0.14$^{ab}$ | 0.86 ± 0.22$^{ab}$ | 0.97 ± 0.14$^{ab}$ | 0.51 ± 0.12$^{bc}$ | 0.62 ± 0.03$^{bc}$ | 0.42 ± 0.16$^{c}$ | 0.56 ± 0.04$^{bc}$ |
| | | | | EPA-derived metabolites | | | | |
| 5-HEPE | 49.17 ± 7.49$^{abc}$ | 50.08 ± 10.37$^{ab}$ | 44.74 ± 4.21$^{abc}$ | 62.75 ± 2.45$^{a}$ | 29.28 ± 4.87$^{bc}$ | 43.88 ± 1.55$^{abc}$ | 28.37 ± 8.28$^{c}$ | 32.31 ± 1.49$^{bc}$ |
| 8-HEPE | 12.30 ± 0.71$^{ab}$ | 11.87 ± 2.57$^{ab}$ | 9.16 ± 1.42$^{bc}$ | 13.89 ± 1.45$^{a}$ | 3.98 ± 0.64$^{de}$ | 8.03 ± 0.30$^{bcd}$ | 2.89 ± 0.93$^{e}$ | 5.09 ± 0.69$^{cde}$ |
| 12-HEPE | 11.30 ± 0.46$^{a}$ | 9.02 ± 1.73$^{ab}$ | 8.09 ± 1.72$^{ab}$ | 11.59 ± 2.08$^{a}$ | 3.56 ± 0.83$^{c}$ | 5.82 ± 0.63$^{bc}$ | 2.44 ± 0.86$^{c}$ | 3.42 ± 0.27$^{c}$ |
| 15-HEPE | 11.53 ± 0.20$^{a}$ | 7.64 ± 1.42$^{b}$ | 8.10 ± 1.79$^{ab}$ | 7.74 ± 0.45$^{b}$ | 3.88 ± 0.82$^{c}$ | 4.94 ± 0.34$^{bc}$ | 2.65 ± 0.85$^{c}$ | 3.55 ± 0.17$^{c}$ |
| 8(9)-EpETE | 9.48 ± 4.71$^{a}$ | 2.76 ± 0.49$^{b}$ | 4.21 ± 0.63$^{ab}$ | 2.86 ± 0.31$^{b}$ | 1.65 ± 0.27$^{b}$ | 1.51 ± 0.26$^{b}$ | 1.62 ± 0.68$^{b}$ | 1.66 ± 0.10$^{b}$ |
| 11(12)-EpETE | 10.19 ± 5.45$^{a}$ | 2.01 ± 0.74$^{b}$ | 5.47 ± 1.60$^{ab}$ | 2.56 ± 0.28$^{b}$ | 2.10 ± 0.59$^{b}$ | 0.97 ± 0.23$^{b}$ | 1.73 ± 0.67$^{b}$ | 0.89 ± 0.02$^{b}$ |
| 14(15)-EpETE | 41.06 ± 24.70$^{a}$ | 7.96 ± 3.97$^{ab}$ | 25.41 ± 9.92$^{ab}$ | 12.28 ± 1.20$^{ab}$ | 9.59 ± 3.10$^{ab}$ | 3.26 ± 1.09$^{b}$ | 6.29 ± 2.51$^{b}$ | 2.32 ± 0.32$^{b}$ |
| 17(18)-EpETE | 484.11 ± 290.43$^{a}$ | 102.37 ± 41.17$^{ab}$ | 327.21 ± 143.58$^{ab}$ | 154.23 ± 14.37$^{ab}$ | 116.86 ± 41.17$^{ab}$ | 46.44 ± 18.07$^{b}$ | 74.14 ± 23.85$^{ab}$ | 33.93 ± 3.42$^{ab}$ |
| 14,15-DiHETE | 8.60 ± 0.94$^{a}$ | 4.97 ± 0.94$^{c}$ | 7.59 ± 0.97$^{ab}$ | 4.73 ± 0.51$^{c}$ | 4.21 ± 0.20$^{c}$ | 3.53 ± 0.30$^{c}$ | 3.46 ± 1.07$^{c}$ | 3.27 ± 0.56$^{c}$ |
| 17,18-DiHETE | 12.24 ± 1.54$^{a}$ | 9.27 ± 2.29$^{ab}$ | 11.97 ± 1.40$^{a}$ | 8.49 ± 1.43$^{ab}$ | 6.90 ± 0.90$^{ab}$ | 5.92 ± 0.43$^{b}$ | 6.23 ± 2.20$^{b}$ | 5.64 ± 0.56$^{b}$ |
| | | | | DHA-derived metabolites | | | | |
| 17-HDoHE | 30.26 ± 2.64$^{a}$ | 27.81 ± 3.71$^{a}$ | 24.10 ± 1.89$^{a}$ | 24.52 ± 1.93$^{a}$ | 9.61 ± 1.99$^{bc}$ | 15.24 ± 1.48$^{b}$ | 6.40 ± 1.75$^{c}$ | 11.74 ± 1.09$^{bc}$ |
| 7(8)-EpDPE | 7.09 ± 3.65$^{a}$ | 2.18 ± 0.46$^{b}$ | 3.39 ± 0.54$^{ab}$ | 2.35 ± 0.25$^{ab}$ | 1.32 ± 0.21$^{b}$ | 1.19 ± 0.16$^{b}$ | 1.29 ± 0.54$^{b}$ | 1.15 ± 0.02$^{b}$ |
| 10(11)-EpDPE | 8.94 ± 4.35$^{a}$ | 2.55 ± 0.62$^{b}$ | 4.51 ± 1.02$^{ab}$ | 2.87 ± 0.26$^{b}$ | 1.79 ± 0.28$^{b}$ | 1.21 ± 0.18$^{b}$ | 1.49 ± 0.62$^{b}$ | 0.97 ± 0.02$^{b}$ |
| 13(14)-EpDPE | 13.60 ± 7.56$^{a}$ | 3.36 ± 1.33$^{ab}$ | 8.35 ± 3.10$^{ab}$ | 4.48 ± 0.31$^{ab}$ | 3.08 ± 0.64$^{b}$ | 1.52 ± 0.35$^{b}$ | 2.22 ± 0.91$^{b}$ | 1.17 ± 0.06$^{b}$ |
| 16(17)-EpDPE | 18.77 ± 11.06$^{a}$ | 4.46 ± 1.89$^{ab}$ | 11.30 ± 4.03$^{ab}$ | 6.32 ± 0.58$^{ab}$ | 4.69 ± 1.19$^{ab}$ | 2.31 ± 0.71$^{b}$ | 3.45 ± 1.31$^{b}$ | 1.84 ± 0.07$^{b}$ |
| 19(20)-EpDPE | 52.38 ± 25.79$^{a}$ | 19.04 ± 5.92$^{ab}$ | 36.84 ± 9.66$^{ab}$ | 27.03 ± 2.50$^{ab}$ | 18.27 ± 2.77$^{ab}$ | 18.06 ± 2.25$^{ab}$ | 16.33 ± 4.75$^{b}$ | 19.08 ± 0.39$^{ab}$ |
| | | | | LA-derived metabolites | | | | |
| 9-HODE | 2.66 ± 0.03$^{a}$ | 2.68 ± 0.35$^{a}$ | 1.98 ± 0.46$^{abc}$ | 2.22 ± 0.25$^{ab}$ | 0.92 ± 0.19$^{d}$ | 1.38 ± 0.04$^{bcd}$ | 0.74 ± 0.27$^{d}$ | 1.03 ± 0.13$^{cd}$ |
| 13-HODE | 4.03 ± 0.08$^{a}$ | 3.92 ± 0.56$^{a}$ | 3.12 ± 0.73$^{abc}$ | 3.46 ± 0.33$^{ab}$ | 1.54 ± 0.26$^{d}$ | 2.22 ± 0.09$^{bcd}$ | 1.38 ± 0.50$^{d}$ | 1.78 ± 0.23$^{cd}$ |
| 9-oxo-ODE | 0.67 ± 0.11$^{a}$ | 0.60 ± 0.21$^{ab}$ | 0.63 ± 0.24$^{ab}$ | 0.43 ± 0.07$^{ab}$ | 0.24 ± 0.01$^{ab}$ | 0.26 ± 0.06$^{ab}$ | 0.18 ± 0.06$^{b}$ | 0.23 ± 0.03$^{ab}$ |
| 13-oxo-ODE | 2.38 ± 0.60$^{a}$ | 1.35 ± 0.36$^{abc}$ | 2.12 ± 0.35$^{ab}$ | 1.26 ± 0.19$^{bc}$ | 0.79 ± 0.07$^{c}$ | 0.81 ± 0.21$^{c}$ | 0.64 ± 0.22$^{c}$ | 0.90 ± 0.17$^{bc}$ |
| 9(10)-EpOME | 0.95 ± 0.33$^{a}$ | 0.38 ± 0.02$^{b}$ | 0.50 ± 0.14$^{ab}$ | 0.25 ± 0.02$^{b}$ | 0.13 ± 0.01$^{b}$ | 0.11 ± 0.02$^{b}$ | 0.11 ± 0.05$^{b}$ | 0.08 ± 0.005$^{b}$ |
| 12(13)-EpOME | 4.34 ± 2.02$^{a}$ | 1.46 ± 0.04$^{b}$ | 2.18 ± 0.36$^{ab}$ | 1.44 ± 0.10$^{b}$ | 0.68 ± 0.15$^{b}$ | 0.56 ± 0.06$^{b}$ | 0.59 ± 0.28$^{b}$ | 0.48 ± 0.04$^{b}$ |
| 9,10-DiHOME | 0.40 ± 0.03$^{a}$ | 0.26 ± 0.05$^{ab}$ | 0.38 ± 0.08$^{a}$ | 0.17 ± 0.02$^{bc}$ | 0.18 ± 0.01$^{bc}$ | 0.11 ± 0.01$^{c}$ | 0.15 ± 0.05$^{bc}$ | 0.1 ± 0.005$^{bc}$ |
| 12,13-DiHOME | 0.23 ± 0.03$^{a}$ | 0.22 ± 0.03$^{a}$ | 0.24 ± 0.05$^{a}$ | 0.15 ± 0.03$^{ab}$ | 0.10 ± 0.01$^{b}$ | 0.09 ± 0.01$^{b}$ | 0.08 ± 0.03$^{b}$ | 0.08 ± 0.01$^{b}$ |
| | | | | DGLA-derived metabolites | | | | |
| 15(S)-HETrE | 0.45 ± 0.06$^{a}$ | 0.34 ± 0.08$^{abc}$ | 0.35 ± 0.07$^{ab}$ | 0.32 ± 0.03$^{abc}$ | 0.19 ± 0.02$^{bc}$ | 0.23 ± 0.01$^{bc}$ | 0.17 ± 0.05$^{c}$ | 0.20 ± 0.03$^{bc}$ |
| | | | | AA-derived metabolites | | | | |
| 5-HETE | 1.57 ± 0.15$^{abc}$ | 1.66 ± 0.23$^{ab}$ | 1.58 ± 0.18$^{abc}$ | 1.90 ± 0.08$^{a}$ | 1.04 ± 0.22$^{c}$ | 1.49 ± 0.08$^{abc}$ | 1.00 ± 0.27$^{c}$ | 1.22 ± 0.05$^{bc}$ |
| 8-HETE | 0.59 ± 0.01$^{a}$ | 0.36 ± 0.08$^{b}$ | 0.45 ± 0.08$^{a}$ | 0.37 ± 0.01$^{b}$ | 0.19 ± 0.03$^{c}$ | 0.21 ± 0.01$^{c}$ | 0.13 ± 0.05$^{c}$ | 0.1 ± 0.002$^{c}$ |
| 9-HETE | 0.40 ± 0.04$^{a}$ | 0.32 ± 0.07$^{ab}$ | 0.24 ± 0.16$^{ab}$ | 0.30 ± 0.01$^{ab}$ | 0.12 ± 0.03$^{b}$ | 0.18 ± 0.01$^{ab}$ | 0.10 ± 0.03$^{b}$ | 0.13 ± 0.02$^{ab}$ |
| 11-HETE | 0.54 ± 0.03$^{a}$ | 0.39 ± 0.06$^{b}$ | 0.43 ± 0.09$^{ab}$ | 0.37 ± 0.02$^{b}$ | 0.19 ± 0.03$^{c}$ | 0.22 ± 0.02$^{c}$ | 0.13 ± 0.04$^{c}$ | 0.16 ± 0.02$^{c}$ |
| 12-HETE | 0.47 ± 0.02$^{a}$ | 0.29 ± 0.05$^{b}$ | 0.33 ± 0.07$^{b}$ | 0.29 ± 0.02$^{b}$ | 0.14 ± 0.02$^{c}$ | 0.16 ± 0.01$^{c}$ | 0.09 ± 0.03$^{c}$ | 0.11 ± 0.01$^{c}$ |
| 15-HETE | 0.88 ± 0.09$^{a}$ | 0.73 ± 0.12$^{a}$ | 0.64 ± 0.16$^{abc}$ | 0.70 ± 0.08$^{ab}$ | 0.28 ± 0.06$^{d}$ | 0.41 ± 0.05$^{bcd}$ | 0.21 ± 0.07$^{d}$ | 0.30 ± 0.01$^{cd}$ |



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**Table 5. continued**

| oxylipins | 1 μL oil | | 2 μL oil | | 5 μL oil | | 10 μL oil | |
|---|---|---|---|---|---|---|---|---|
| | LV(128 mM base / 2.8 mM oil) | HV(128 mM base / 0.56 mM oil) | LV(128 mM base / 5.6 mM oil) | HV(128 mM base / 1.1 mM oil) | LV(128 mM base / 14 mM oil) | HV(128 mM base / 2.8 mM oil) | LV(128 mM base / 28 mM oil) | HV(128 mM base / 5.6 mM oil) |
| | | | | AA-derived metabolites | | | | |
| 20-HETE | 2.36 ± 0.57$^{a}$ | 2.32 ± 0.39$^{a}$ | 1.80 ± 0.28$^{ab}$ | 1.82 ± 0.28$^{ab}$ | 0.89 ± 0.11$^{bc}$ | 0.85 ± 0.12$^{bc}$ | 0.68 ± 0.18$^{c}$ | 0.66 ± 0.08$^{bc}$ |
| 15-oxo-ETE | 0.38 ± 0.13$^{a}$ | 0.11 ± 0.03$^{c}$ | 0.35 ± 0.10$^{ab}$ | 0.13 ± 0.01$^{c}$ | 0.17 ± 0.01$^{abc}$ | 0.10 ± 0.03$^{c}$ | 0.14 ± 0.05$^{bc}$ | 0.13 ± 0.03$^{bc}$ |
| 5(6)-EpETrE | 0.46 ± 0.19$^{a}$ | 0.12 ± 0.03$^{b}$ | 0.21 ± 0.01$^{b}$ | 0.12 ± 0.02$^{b}$ | 0.08 ± 0.02$^{b}$ | 0.05 ± 0.01$^{b}$ | 0.07 ± 0.03$^{b}$ | 0.05 ± 0.004$^{b}$ |
| 8(9)-EpETrE | 0.45 ± 0.19$^{a}$ | 0.19 ± 0.04$^{ab}$ | 0.31 ± 0.02$^{ab}$ | 0.20 ± 0.02$^{ab}$ | 0.1 ± 0.003$^{b}$ | 0.10 ± 0.02$^{b}$ | 0.12 ± 0.05$^{b}$ | 0.11 ± 0.01$^{b}$ |
| 11(12)-EpETrE | 0.88 ± 0.45$^{a}$ | 0.24 ± 0.06$^{b}$ | 0.56 ± 0.19$^{ab}$ | 0.30 ± 0.03$^{ab}$ | 0.21 ± 0.05$^{b}$ | 0.11 ± 0.02$^{b}$ | 0.16 ± 0.06$^{b}$ | 0.09 ± 0.004$^{b}$ |
| 14(15)-EpETrE | 2.05 ± 1.25$^{a}$ | 0.42 ± 0.21$^{ab}$ | 1.48 ± 0.79$^{ab}$ | 0.58 ± 0.08$^{ab}$ | 0.46 ± 0.17$^{ab}$ | 0.14 ± 0.04$^{b}$ | 0.28 ± 0.10$^{ab}$ | 0.10 ± 0.02$^{b}$ |
| 5,6-DiHETrE | 0.13 ± 0.01 | 0.09 ± 0.02 | 0.12 ± 0.03 | 0.09 ± 0.01 | 0.08 ± 0.001 | 0.06 ± 0.002 | 0.08 ± 0.03 | 0.07 ± 0.002 |
| 8,9-DiHETrE | ND | ND | 0.02 ± 0.03 | 0.04 ± 0.03 | 0.02 ± 0.003 | 0.04 ± 0.005 | 0.02 ± 0.01 | 0.02 ± 0.003 |
| 11,12-DiHETrE | 0.04 ± 0.004$^{a}$ | 0.03 ± 0.004$^{ab}$ | 0.02 ± 0.02$^{ab}$ | 0.02 ± 0.002$^{ab}$ | 0.02 ± 0.001$^{b}$ | 0.02 ± 0.001$^{b}$ | 0.01 ± 0.01$^{b}$ | 0.01 ± 0.002$^{b}$ |
| 14,15-DiHETrE | 0.07 ± 0.01$^{a}$ | 0.04 ± 0.01$^{bc}$ | 0.06 ± 0.004$^{ab}$ | 0.05 ± 0.004$^{abc}$ | 0.03 ± 0.003$^{c}$ | 0.03 ± 0.003$^{c}$ | 0.03 ± 0.01$^{c}$ | 0.03 ± 0.003$^{c}$ |
| LTB4 | ND | 0.12 ± 0.02$^{a}$ | 0.03 ± 0.04$^{ab}$ | 0.06 ± 0.04$^{ab}$ | 0.02 ± 0.02$^{b}$ | 0.05 ± 0.01$^{ab}$ | 0.03 ± 0.03$^{ab}$ | 0.04 ± 0.01$^{ab}$ |
| LXA4 | 1.40 ± 0.44 | 0.86 ± 0.19 | 1.07 ± 0.16 | 0.89 ± 0.14 | 0.86 ± 0.03 | 0.87 ± 0.21 | 0.85 ± 0.28 | 1.13 ± 0.21 |
| | | | | extraction recovery (%) | | | | |
| d-11-11(12)EpETrE | 103 ± 9$^{a}$ | 109 ± 2$^{a}$ | 74 ± 24$^{ab}$ | 101 ± 3$^{a}$ | 89 ± 3$^{ab}$ | 79 ± 11$^{ab}$ | 69 ± 16$^{ab}$ | 52 ± 4$^{b}$ |
| d11-14,15-DiHETrE | 133 ± 5 | 150 ± 9 | 103 ± 50 | 166 ± 6 | 142 ± 2 | 183 ± 16 | 142 ± 39 | 172 ± 2 |
| d4-6-keto-PGF1a | 110 ± 8 | 87 ± 7 | 88 ± 35 | 93 ± 4 | 105 ± 6 | 96 ± 5 | 87 ± 22 | 91 ± 5 |
| d4-9-HODE | 129 ± 5 | 133 ± 8 | 98 ± 41 | 140 ± 6 | 125 ± 4 | 143 ± 10 | 110 ± 30 | 114 ± 3 |
| d4-LTB4 | 81 ± 4 | 84 ± 5 | 62 ± 24 | 85 ± 3 | 77 ± 1 | 84 ± 12 | 70 ± 19 | 72 ± 3 |
| d4-TXB2 | 89 ± 3 | 88 ± 4 | 68 ± 27 | 92 ± 4 | 82 ± 2 | 94 ± 8 | 74 ± 19 | 84 ± 3 |
| d6-20-HETE | 118 ± 2 | 99 ± 2 | 87 ± 39 | 98 ± 10 | 119 ± 6 | 112 ± 10 | 114 ± 28 | 94 ± 1 |
| d8-5-HETE | 99 ± 2 | 116 ± 8 | 75 ± 32 | 119 ± 5 | 101 ± 5 | 129 ± 8 | 97 ± 25 | 117 ± 3 |

[a]Data are represented as mean ± SD, $n = 3$ for all samples, except sample 10 μL-HV ($n = 2$) due to sample loss. The base-to-oil molar ratio is shown in the second row of the table; base molarity was a constant, whereas oil molarity increased with increasing oil volume. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test. Different letters in each row represent significant differences between the means ($p < 0.05$). ND: not detected.





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concentrations of 8 out of 43 oxylipins and 6 out of 43 oxylipins in the 2 μL and 10 μL oils, respectively, compared to direct hydrolysis of each oil volume ($p < 0.05$; Table 4). Notably, several low abundance compounds were not detected when the hydrolysis base reagent was used directly, without the extraction solvent. For instance, in the 2 or 10 μL oil volumes, 9-hydroxyoctadecatrienoic acid (9-HOTrE), 13-HOTrE, 8(9)-epoxyeicosatetraenoic acid (8(9)-EpETE), 17-hydroxydocosahexaenoic acid (17-HDoHE), 9-oxo-octadecadienoic acid (9-oxo-ODE), 13-oxo-ODE, 9(10)-epoxyoctadecamonoenoic acid (9(10)-EpOME), 12,13-dihydroxyoctadecamonoenoic acid (12,13-DiHOME), 15(S)-hydroxyeicosatrienoic acid (15(S)-HETrE), 8-HETE, 9-HETE, 11-HETE, 12-HETE, 15-HETE, 20-HETE, 15-oxo-eicosatetraenoic acid (15-oxo-ETE), 5(6)-EpETrE, 11,12-DiHETrE, and 14,15-DiHETrE were not detected in the groups without extraction solvent. Independent of extraction solvent, oxylipins were significantly higher when 2 μL of oil was subjected to hydrolysis than when 10 μL was used, consistent with the findings of Experiment 1.

Two-way ANOVA showed a significant effect of extraction solvent on the recovery of all surrogates except d4-6-keto-PGF1a, and a significant interaction between oil volume and extraction solvent for d11-11(12)-EpETrE and d4-LTB4 ($p < 0.05$; Table 4). Adding the extraction solvent significantly increased recoveries for all oxylipin surrogates in the 2 μL oil volume (except for d4-6-keto-PGF1a) compared to 2 μL samples without extraction solvent. No significant effect of extraction solvent on standard recovery was observed for 10 μL oil. For both oil volumes (2 and 10 μL), the percent recoveries ranged from 81−134% when extraction solvent was added, and 29−111% when no solvent was added. Overall, these results suggest greater surrogate standard recovery when the extraction solvent is added to the oil.

Both analyte and surrogate peak areas in the samples without extraction solvent were lower than those with extraction solvent (Figure S1), suggesting impaired recovery of oxylipins in the samples without extraction solvent.

**Experiment 4. Effect of Increasing Hydrolysis Reagent Volume on Hydrolysis Efficiency.** To test whether increasing the volume of the hydrolysis reagent improves hydrolysis efficiency by increasing the base/oil ratio, we hydrolyzed 1, 2, 5, and 10 μL of fish oil with 200 μL (low volume, LV) and 1000 μL (high volume, HV) of 0.25 M sodium carbonate in the presence of extraction solvent. We hypothesized that HV sodium carbonate will increase oxylipin concentrations by improving the hydrolysis efficiency, particularly when used on the >2 μL oil volumes. Our observations did not support the hypothesis.

As shown in Table 5, two-way ANOVA showed significant effects of hydrolysis reagent volume (on 43 out of 45 oxylipins), oil volume (on 28 out of 45 oxylipins), and interaction effects between hydrolysis reagent and oil volumes (on 10 out of 45 oxylipins). Tukey's post-hoc analysis showed that HV base hydrolysis resulted in significantly lower oxylipins than LV base hydrolysis, particularly for PUFA-epoxide metabolites in 1 μL oil volume. Consistent with our previous results (Experiments 1 and 3), increasing oil volume past 2 μL significantly decreased oxylipins in both LV and HV treated samples. Collectively, these findings suggest that adding more base does not improve oxylipin yield and in many cases degrades fatty acid epoxides.

Two-way ANOVA showed no significant main effects of oil volume, hydrolysis reagent volume, or an interaction of these two factors on surrogate standard recovery, indicating that high volumes of hydrolysis reagent did not degrade the deuterated surrogate standards.

**Experiment 5. Effect of Sodium Carbonate and Sodium Hydroxide on Hydrolysis Efficiency.** To examine whether a stronger base would improve oxylipin yield, the hydrolysis efficiencies of sodium carbonate and sodium hydroxide (stronger base) were compared ($n = 5$ per base added at 0.25 M each). As shown in Table 6a, unpaired $t$ test analysis showed that oxylipin concentrations were significantly higher for 33 out of 44 detected oxylipins in samples hydrolyzed with sodium hydroxide than those hydrolyzed with sodium carbonate. Only LXA4 was found to be significantly higher (by 27%) with sodium carbonate than with sodium hydroxide hydrolysis. No significant effect of hydrolysis base type was observed on 8-HEPE, 17,18-DiHETE, 17-HDoHE, 9-oxo-ODE, 13-oxo-ODE, 12,13-DiHOME, 5-HETE, 11,12-DiHETrE, and 14,15-DiHETrE ($p > 0.05$).

As shown in Table 6b, surrogate recovery was not significantly different between the two bases and comparable to the blanks ($n = 1$ per base) for all surrogates except d4-TXB2 and d6-20-HETE, which were significantly lower (19 vs 62%) and higher (238 vs 99%), respectively, with sodium hydroxide than with sodium carbonate.

The high percent recovery of d6-20-HETE in sodium hydroxide treated oil (238%) was due to ion enhancement (341%) as indicated in Table S5. A similar magnitude of ion enhancement (237%) was also seen for 20-HETE, the compound quantified by labeled d6-20-HETE. Ion enhancement was not seen for 13- and 9-oxo-ODE (83−97%), suggesting that a surrogate other than d6-20-HETE should be used in the future to quantify these fatty acid ketones following sodium hydroxide hydrolysis.

**Intra- and Interexperimental Variability of Oxylipin Concentrations.** Tables S6 and S7 show the reproducibility of oxylipin concentrations across the five experiments that used 2 μL oil volume and across the three experiments that used 10 μL volumes, respectively. The intraexperimental variance in most oxylipins was lower in the 2 μL oil than in the 10 μL oil, as evidenced by a low CV (i.e., below 20%) in all five experiments that used 2 μL of oil and a relatively higher (>30%) CV in the three experiments that used 10 μL of oil. Despite the high precision within each experiment for the 2 μL oil volume, mean oxylipin concentrations were generally lower in Experiments 2, 3, and 5 than in Experiments 1 and 4, leading to high interexperiment variability. This is likely due to technical error associated with handling low solvent oil volumes (e.g., pipetting low stock solvent volumes). In contrast, intraexperiment precision was low for the 10 μL oil samples, although the means were consistent across experiments. This is likely due to the high variance between experiments, which would result in a center mean value spanning a wide confidence interval.

**Experiment 6. Measuring Oxylipins in Fish and Algae Oils.** Oxylipins were quantified in 2 μL of oil from four fish oil soft gels and one algae oil soft gel obtained from the local pharmacy using low-volume (200 μL) sodium hydroxide base in the presence of extraction solvent (oil brands are in Table 1). Total oxylipin concentrations derived by summing individual oxylipins (Table S8) showed that fish oil 1 contained the highest amount of oxylipins (7765 pmol/μL) followed by fish oil 3 (1984 pmol/μL), fish oil 2 (1835 pmol/μL), fish oil 4 (1254 pmol/μL), and algae oil (916 pmol/μL).

Figure 1a−d shows DHA, EPA, AA, and LA/ALA/DGLA metabolites, respectively, in the fish and algae oils (these are


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**Table 6a. Effect of Sodium Carbonate vs Sodium Hydroxide on Hydrolysis Efficiency (Experiment 5)[a]**

| | hydrolysis reagent | |
|---|---|---|
| oxylipins | sodium carbonate (mean ± SD) | sodium hydroxide (mean ± SD) |
| ALA-derived metabolites | | |
| 9-HOTrE | 0.28 ± 0.06 | 0.48 ± 0.11* |
| 13-HOTrE | 0.33 ± 0.04 | 0.49 ± 0.11* |
| EPA-derived metabolites | | |
| 5-HEPE | 41.86 ± 2.88 | 64.12 ± 18.47* |
| 8-HEPE | 2.60 ± 0.64 | 3.18 ± 0.60 |
| 12-HEPE | 2.50 ± 0.50 | 4.52 ± 1.03* |
| 15-HEPE | 3.83 ± 0.65 | 10.63 ± 2.52* |
| 8(9)-EpETE | 2.91 ± 1.71 | 11.94 ± 4.46* |
| 11(12)-EpETE | 2.46 ± 1.71 | 7.95 ± 2.53* |
| 14(15)-EpETE | 8.94 ± 7.24 | 21.61 ± 9.62* |
| 17(18)-EpETE | 85.63 ± 69.09 | 199.29 ± 83.36* |
| 14,15-DiHETE | 5.99 ± 0.70 | 8.99 ± 2.04* |
| 17,18-DiHETE | 8.05 ± 1.04 | 9.37 ± 1.91 |
| DHA-derived metabolites | | |
| 17-HDoHE | 5.12 ± 0.81 | 6.73 ± 1.60 |
| 7(8)-EpDPE | 2.06 ± 1.11 | 5.55 ± 2.20* |
| 10(11)-EpDPE | 2.57 ± 1.39 | 6.63 ± 2.37* |
| 13(14)-EpDPE | 3.28 ± 2.25 | 7.42 ± 2.66* |
| 16(17)-EpDPE | 4.51 ± 3.17 | 10.46 ± 4.28* |
| 19(20)-EpDPE | 15.03 ± 6.47 | 27.14 ± 9.25* |
| LA-derived metabolites | | |
| 9-HODE | 0.98 ± 0.14 | 1.47 ± 0.36* |
| 13-HODE | 1.83 ± 0.26 | 2.64 ± 0.56* |
| 9-oxo-ODE | 0.34 ± 0.07 | 0.29 ± 0.04 |
| 13-oxo-ODE | 0.36 ± 0.03 | 0.32 ± 0.06 |
| 9(10)-EpOME | 0.34 ± 0.15 | 1.01 ± 0.28* |
| 12(13)EpOME | 0.97 ± 0.53 | 2.70 ± 0.90* |
| 9,10-DiHOME | 0.26 ± 0.03 | 0.33 ± 0.06* |
| 12,13-DiHOME | 0.14 ± 0.02 | 0.17 ± 0.03 |
| DGLA-derived metabolites | | |
| 15(S)-HETrE | 0.21 ± 0.04 | 0.33 ± 0.08* |
| AA-derived metabolites | | |
| 5-HETE | 0.79 ± 0.06 | 0.94 ± 0.23 |
| 8-HETE | 0.17 ± 0.02 | 0.40 ± 0.07* |
| 9-HETE | 0.12 ± 0.02 | 0.19 ± 0.05* |
| 11-HETE | 0.15 ± 0.02 | 0.22 ± 0.05* |
| 12-HETE | 0.11 ± 0.03 | 0.22 ± 0.05* |
| 15-HETE | 0.24 ± 0.06 | 0.40 ± 0.08* |
| 20-HETE | 1.35 ± 0.17 | 1.79 ± 0.37* |
| 5(6)-EpETrE | 0.14 ± 0.09 | 0.35 ± 0.12* |
| 8(9)-EpETrE | 0.16 ± 0.08 | 0.36 ± 0.10* |
| 11(12)-EpETrE | 0.21 ± 0.12 | 0.54 ± 0.17* |
| 14(15)-EpETrE | 0.43 ± 0.31 | 1.01 ± 0.40* |
| 5,6-DiHETrE | 0.12 ± 0.01 | 0.37 ± 0.08* |
| 11,12-DiHETrE | 0.02 ± 0.003 | 0.03 ± 0.01 |
| 14,15-DiHETrE | 0.05 ± 0.005 | 0.06 ± 0.02 |
| 20-COOH-LTB4 | ND | 0.23 ± 0.05 |
| 6-tans-LTB4 | ND | 0.19 ± 0.13 |
| LXA4 | 1.36 ± 0.18 | 1.00 ± 0.25* |

[a]Oxylipin concentrations (pmol/μL oil) were determined in 2 μL of fish oil reconstituted in extraction solvent and hydrolyzed with 200 μL of sodium carbonate vs sodium hydroxide. Data are reported as mean ± SD ($n$ = 5 for all samples) and analyzed by unpaired $t$ test. Asterisks represent significant differences between the means ($p$ < 0.05). ND: not detected.

**Table 6b. Effect of Sodium Carbonate vs Sodium Hydroxide on Extraction Recovery (%) of Labeled Oxylipins (Experiment 5)[a]**

| | hydrolysis reagent | | | |
|---|---|---|---|---|
| | sodium carbonate | | sodium hydroxide | |
| extraction recovery (%) | blank | oil | blank | oil |
| d-11-11(12)-EpETrE | 76 | 79 ± 6 | 57 | 70 ± 10 |
| d11-14,15-DiHETrE | 60 | 102 ± 6 | 82 | 125 ± 27 |
| d4-6-keto-PGF1a | 81 | 59 ± 22 | 55 | 79 ± 15 |
| d4-9-HODE | 95 | 129 ± 5 | 101 | 145 ± 27 |
| d4-LTB4 | 60 | 73 ± 3 | 58 | 74 ± 14 |
| d4-TXB2 | 58 | 62 ± 3 | 9 | 19 ± 5* |
| d6-20-HETE | 70 | 99 ± 3 | 146 | 238 ± 37* |
| d8-5-HETE | 59 | 92 ± 4 | 52 | 96 ± 19 |

[a]Data are represented as mean ± SD ($n$ = 5 for all samples except for blank sample ($n$ = 1)). Data were analyzed by the unpaired $t$ test. Asterisks represent significant differences between the means ($p$ < 0.05).

figures based on Table S8). Also, representative UPLC-MS/MS chromatograms of oxylipin standards in methanol and corresponding analytes detected in a fish oil sample are presented in Figures S2 and S3, respectively. Statistical analysis of individual oxylipins with one-way ANOVA showed significant differences ($p$ < 0.05) among the various oil types. Tukey's post-hoc test showed that fish oil 1 contained significantly higher concentrations of all EPA, AA, ALA, and DGLA metabolites than other oil types ($p$ < 0.05). DHA-derived metabolites were higher in fish oil 1 and algae oil than in other oil types for most of the compounds. LA metabolites were generally higher ($p$ < 0.05) in algae oil than in fish oils except for 12(13)-EpOME, which did not differ significantly between the various oils ($p$ > 0.05).

EPA metabolites were the most abundant oxylipins in all fish oils analyzed (84−87%) followed by DHA metabolites (10−14%), AA metabolites (1−3%), and LA metabolites (0.4−1%). 17(18)-EpETE was the major EPA metabolite detected in all fish oil samples, constituting 59−70% of total oxylipins followed by 14(15)-EpETE (6−9%), 19(20)-EpDPE (4−6%), 8(9)-EpETE (2−7%), and 11(12)-EpETE (2−6%).

In algae oil, DHA metabolites constituted 83% of total oxylipins followed by EPA- (11%), LA- (5%), and AA-metabolites (0.4%). DHA-derived 19(20)-EpDPE, 16(17)-EpDPE, and 13(14)-EpDPE constituted ∼54% of total oxylipins at similar proportions each (16−19%) followed by 7(8)-EpDPE (9%) and 17-HDoHE (7%). Two EPA-derived metabolites including 17(18)-EpETE and 15-HEPE were found in algae oil at 5% and 4% of total oxylipins, respectively.

The mean recovery of the deuterated surrogate standards (shown in Figure 1e) did not differ significantly between the groups, except for d-11-11(12)-EpETrE and d4-9-HODE, which were lower in fish oil 1 than in the other oils.

**Fatty Acid Composition.** The fatty acid percent composition of fish and algae oil supplements is presented in Table 7. Concentrations are presented in Table S9. A representative chromatogram of the fatty acid profile of the fish oil 4 (cod liver oil) supplement is shown in Figure S4.

Fish oil 1 contained mainly EPA (54%) followed by DHA (21%), gondonic acid (C20:1 n-9; 4%), docosapentaenoic acid (DPA, 22:5n-3; 3.5%), oleic acid (C18:1n-9 cis; 3%), and stearic acid (C18:0; 2.5%). Total omega-3 content on the label was 697 mg/g of oil (900 mg per 1290 mg oil capsule), which is close to our measured value of 650 mg/g oil.


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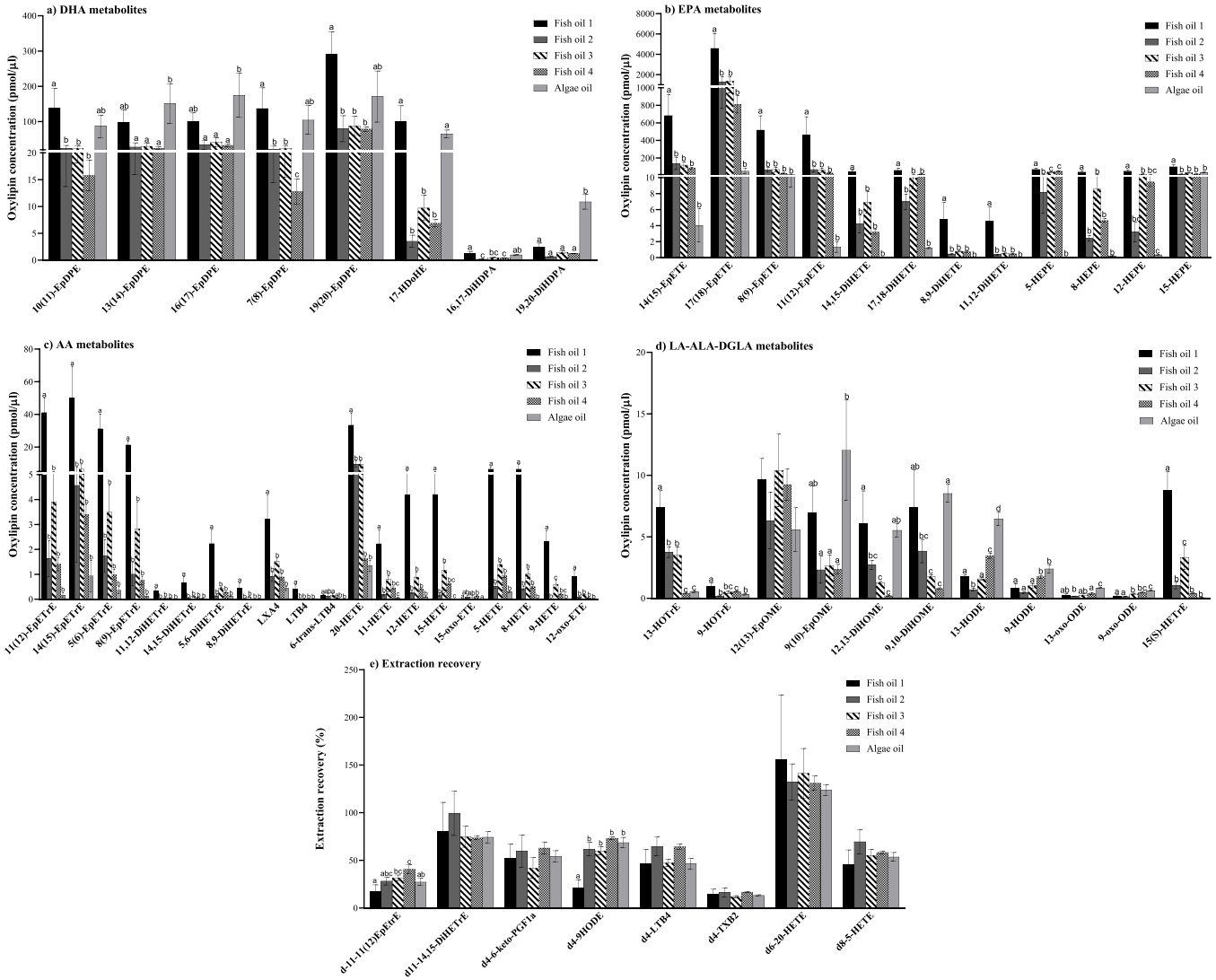


**Figure 1.** Analysis of oxylipins in fish and algae oils (Experiment 6). Oxylipin concentrations (pmol/$\mu$L oil) were determined in 2 $\mu$L of oil reconstituted in extraction solvent and hydrolyzed with 200 $\mu$L of 0.25 M sodium hydroxide. (a) DHA-derived oxylipins, (b) EPA-derived oxylipins, (c) AA-derived oxylipins, and (d) LA-, ALA-, and DGLA-derived oxylipins. (e) Extraction recoveries of labeled surrogates. Data are mean ± SD of $n$ = 3 for all samples except for the blank sample in (e), where $n$ = 1. Data are analyzed by one-way ANOVA followed by Tukey's post-hoc test. Different letters represent significant differences between the means ($p < 0.05$).

Fish oils 2 and 3 were similar in composition and contained ~21% EPA, ~18% palmitic acid, ~14% DHA, ~12% oleic acid, ~10% palmitoleic acid, ~8% myristic acid, ~3.5% stearic acid, ~3.5% vaccenic acid, and ~2% n-3 DPA. EPA + DHA content in the label was 250 mg per capsule (each capsule = 1 gram) for fish oil 3, which is in agreement with our measured value of 251.5 mg/g oil. Also, total omega-3 content of fish oil 2 in the label was 300 mg/g oil, which is in agreement with our measured value of 277.28 mg/g oil.

In fish oil 4 (cod liver oil), oleic acid was the most abundant fatty acid (17.1%) followed by palmitic acid (13.6%), DHA (12.6%), EPA (11.9%), gondonic acid (10.6%), palmitoleic acid (8.5%), and myristic acid (5.3%). Measured concentrations of EPA and DHA were 100.44 ± 1.19 and 106.76 ± 1.24 mg/g oil, respectively, consistent with the reported amount of ~90 mg of EPA and DHA, each, per gram of oil on the label and in agreement with literature values.[30]

Algae oil contained mainly DHA (46.3%) followed by myristic acid (27.2%), palmitic acid (12.9%), and n-6 DPA (8.7%). Total omega-3 content calculated for algae oil was 404 mg/g oil, and DHA content was 398 mg/g. The amount of DHA on the label was 300 mg per capsule, but the amount of oil per capsule was not specified.

## DISCUSSION

In the present study, a thorough assessment of the hydrolysis procedure showed that hydrolysis of 2 $\mu$L of fish oil in 200 $\mu$L of 0.25 M sodium hydroxide and 190 $\mu$L of extraction solvent (methanol containing 0.1% acetic acid and 0.1% BHT) increased oxylipin yields and surrogate standard recoveries. Using this optimized method, we found that EPA metabolites were the most abundant oxylipins in the fish oil supplements surveyed (~84−87%), whereas DHA-derived oxylipins were most abundant in algae oil (83%). In fish oils, EPA-derived 17(18)-EpETE was the most abundant metabolite (59−70% of total oxylipins), whereas in algae oil, DHA-derived 19(20)-EpDPE and 16(17)-EpDPE were most abundant (each accounting for ~19% of total oxylipins).


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Table 7. Fatty Acid Percent Composition of the Fish and Algae Oil Supplements ($n$ = 3)[a]

| fatty acids | fish oil 1 | fish oil 2 | fish oil 3 | fish oil 4[b] | algae oil |
|---|---|---|---|---|---|
| C8:0 | ND | ND | ND | ND | ND |
| C10:0 | ND | ND | ND | ND | ND |
| C11:0 | ND | ND | ND | ND | ND |
| C12:0 | ND | ND | ND | ND | 0.62 ± 0.01 |
| C14:0 | 0.11 ± 0.04 | 8.02 ± 0.01 | 7.42 ± 0.02 | 5.25 ± 0.05 | 27.24 ± 0.15 |
| C14:1 | ND | 0.3 ± 0.004 | 0.2 ± 0.002 | 0.2 ± 0.002 | ND |
| C15:0 | ND | 0.51 ± 0.01 | 0.7 ± 0.001 | 0.4 ± 0.001 | ND |
| C16:0 | 0.58 ± 0.02 | 18.64 ± 0.27 | 17.97 ± 0.01 | 13.55 ± 0.04 | 12.88 ± 0.04 |
| C16:1 | 0.29 ± 0.03 | 10.55 ± 0.06 | 9.99 ± 0.02 | 8.46 ± 0.02 | 0.18 ± 0.01 |
| C18:0 | 2.44 ± 0.03 | 3.40 ± 0.07 | 3.7 ± 0.001 | 2.74 ± 0.01 | 0.4 ± 0.001 |
| C18:1 *cis* | 2.90 ± 0.03 | 12.38 ± 0.10 | 11.83 ± 0.01 | 17.12 ± 0.02 | 0.66 ± 0.09 |
| C18:1 n-7 | 1.5 ± 0.004 | 3.45 ± 0.03 | 3.3 ± 0.004 | 4.3 ± 0.004 | 0.14 ± 0.05 |
| C18:2 n-6 | 0.5 ± 0.002 | 1.24 ± 0.01 | 1.54 ± 0.02 | 1.9 ± 0.001 | 1.4 ± 0.003 |
| C18:3 n-6 | 0.40 ± 0.02 | 0.47 ± 0.01 | 0.47 ± 0.01 | 0.3 ± 0.002 | 0.16 ± 0.01 |
| C18:3 n-3 | 0.28 ± 0.02 | 0.79 ± 0.01 | 0.9 ± 0.002 | 0.9 ± 0.001 | 0.4 ± 0.001 |
| C20:0 | 1.10 ± 0.03 | 0.29 ± 0.01 | 0.5 ± 0.0004 | 0.26 ± 0.01 | 0.09 ± 0.002 |
| C20:1 n-9 | 3.98 ± 0.02 | 1.00 ± 0.01 | 1.48 ± 0.03 | 10.58 ± 0.03 | ND |
| C20:2 n-6 | 0.45 ± 0.03 | 0.16 ± 0.02 | 0.2 ± 0.004 | 0.3 ± 0.001 | ND |
| C20:3 | 0.34 ± 0.01 | 0.13 ± 0.01 | 0.15 ± 0.02 | 0.09 ± 0.004 | 0.1 ± 0.003 |
| C20:4 n-6 | 3.48 ± 0.03 | 0.91 ± 0.01 | 1.6 ± 0.002 | 0.7 ± 0.002 | 0.4 ± 0.0002 |
| C20:3 n-3 | 0.19 ± 0.01 | 0.1 ± 0.003 | 0.1 ± 0.005 | 0.14 ± 0.01 | ND |
| C20:5 n-3 | 54.68 ± 0.36 | 21.08 ± 0.12 | 20.55 ± 0.04 | 11.88 ± 0.03 | 0.3 ± 0.003 |
| C22:0 | ND | ND | ND | ND | ND |
| C22:1 | 1.56 ± 0.06 | 0.2 ± 0.002 | 1.11 ± 0.01 | 6.80 ± 0.09 | ND |
| C22:2 | ND | ND | ND | ND | ND |
| C22:5 n-6 | 0.69 ± 0.01 | 0.3 ± 0.002 | 0.4 ± 0.002 | ND | 8.72 ± 0.02 |
| C22:5 n-3 | 3.42 ± 0.01 | 2.21 ± 0.01 | 2.1 ± 0.003 | 1.48 ± 0.01 | ND |
| C22:6 | 21.13 ± 0.07 | 13.89 ± 0.02 | 13.62 ± 0.07 | 12.63 ± 0.06 | 46.26 ± 0.07 |
| C24:1 | ND | ND | ND | ND | ND |
| ∑Saturated Fatty Acids | 4.24 | 30.86 | 30.31 | 22.23 | 41.27 |
| ∑Monounsaturated Fatty Acids | 10.25 | 27.87 | 27.98 | 47.50 | 0.98 |
| ∑PUFAs | 85.51 | 41.27 | 41.71 | 30.27 | 57.75 |
| ∑n-6 PUFAs | 5.81 | 3.22 | 4.41 | 3.23 | 10.79 |
| ∑n-3 PUFAs | 79.69 | 38.05 | 37.30 | 27.04 | 46.96 |

[a]ND: Not detected. [b]Cod liver oil. PUFA, polyunsaturated Fatty Acid.

Lower oil volume (1−2 μL) resulted in significantly higher concentrations of oxylipins (Tables 2a and 6a) due to improvement in the hydrolysis efficiency, which appeared to be impaired by volumes greater than 2 μL (Experiments 1 and 4). Notably, some analytes were not detected in 1 μL of oil, likely because sensitivity was lost at that level, suggesting that 2 μL is optimal. The percent recovery and matrix effects were generally comparable across all oil volumes and treatments (Experiments 1 and 4), suggesting minimal losses in the SPE column and minimal ion suppression/enhancement. Small but significant differences in standard recovery or matrix effects, when present, did not appear to impact the calculated oxylipin concentrations.

The addition of methanol extraction solvent containing 0.1% acetic acid and 0.1% BHT with hydrolysis reagent (sodium carbonate) increased oxylipin concentrations at both 2 and 10 μL compared to direct hydrolysis (Experiment 3). This is likely due to reduced recovery of oxylipins resulting from degradation during the hydrolysis or losses during SPE (Figure S1).There is also a possibility that the addition of extraction solvent enhanced the solubility of fish oil triglycerides containing oxylipins, thus improving hydrolysis efficiency.

Adding more sodium carbonate, in the presence of extraction solvent, did not further improve the hydrolysis efficiency of oil (Experiment 4). However, adding sodium increased the concentrations of epoxy (35−300%), monohydroxy (19−180%), and dihydroxy (16−200%) oxylipins compared to sodium carbonate (Experiment 5). This suggests that sodium hydroxide is a better hydrolysis reagent than sodium carbonate.

The high abundance of EPA- and DHA-derived oxylipins in fish and algae oils is likely related to precursor PUFA content. For instance, EPA was the most abundant PUFA in fish oils 1, 2, and 3, consistent with the high abundance of EPA-derived oxylipins in these oils (85−87%). DHA was the second most abundant PUFA in the fish oils analyzed, consistent with the observation that DHA-derived oxylipins were the second most abundant metabolites (10−11%). In cod liver oil (fish oil 4), EPA and DHA were present in equal proportions, but EPA metabolites were more abundant (84%) than DHA metabolites (14%), suggesting selective enzymatic processes in cod that may favor EPA metabolism. Algae oil contained mainly DHA (46%), consistent with the 83% DHA-metabolites (of total oxylipins) detected there.

It is likely that the high PUFA epoxide content of fish and algae oils originated from enzymatic synthesis rather than auto-oxidation. This is because the high abundance of 17(18)-EpETE (59−70% of total oxylipins) in all fish oils measured and the high abundance of EpDPE species in algae suggest selective enzymatic processes favoring these compounds over others.


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The formation of epoxides in fish is catalyzed by CYP450 enzymes.[12,31] In humans and rodents, various CYP450 isoforms including CYP2C, CYP1A1, CYP2E1, CYP2J2, and CYP4A1 are known to convert PUFAs into epoxides.[32,33] CYP2P2 and CYP2P3 isoforms have been identified in the killifish liver and intestine.[12] CYP2P isoforms have also been identified in the cod liver.[34] In algae, epoxygenase enzymes similar to CYP450 might be involved in the synthesis of epoxides.[20] However, to our knowledge, CYP enzyme characterization in *Schizochytrium* sp., the strain used to produce the algae oils assessed in this study, has not been reported.

Nonenzymatic oxidation might also be involved in oxylipin generation in fish and algae oils. The extraction of oil from fish, for instance, involves the removal of free fatty acids, free oxylipins, and pro-oxidants such as metals from crude oil during the refining process[35] but leaves behind esterified lipids, including esterified oxylipins captured in this study. It is possible that storage of the encapsulated oil resulted in auto-oxidation due to trace amounts of metals in the oil[35] and oxygen permeation into the oil capsules.[36]

Exposure to oxygen during sample preparation may also oxidize PUFAs present in oils. For instance, drying SPE cartridges for about 20 min has been shown to increase epoxy metabolites in plasma samples when silica C8 SPE cartridges were used for oxylipin extraction.[27] This increase was not observed when polymeric Oasis-MAX SPE cartridges were used.[27] In this study, we used Oasis-HLB columns, which are structurally similar to the Oasis-MAX cartridge. Therefore, it is unlikely that the high concentrations of epoxide metabolites in fish and algae oils analyzed in this study were an artifact of sample preparation.

The analysis of randomly selected store samples revealed specific enrichment of EPA-derived 17(18)-EpETE in fish oils and DHA-derived EpDPEs in algae oil, suggesting that over-the-counter omega-3 oil supplements may be a potential source of EPA and DHA metabolites shown to have anti-inflamatory effects in vivo.[14−16] However, the oils also contained other potentially harmful oxylipins such as LA-derived hydroxy and epoxide metabolites previously shown to facilitate inflammatory processes in vivo.[37,38] While LA-derived oxylipins are bioavailable and incorporate into tissues following oral intake, the bioavailability of EPA- and DHA-derived oxylipins found in fish or algae oil sources remains to be tested.[39,40]

Based on our measurements, consuming 2 fish oil pills (assuming each pill contains 1 mL oil) per person per day provides an estimated average intake of ∼6419 nmol of total oxylipins, ∼5490 nmol of EPA-derived oxylipins, ∼726 nmol of DHA-derived oxylipins, ∼140 nmol of AA-derived oxylipins, and ∼44 nmol of LA-derived oxylipins per person per day. Comparatively, two algae oil soft gels per day will provide an estimated intake of ∼1832 nmol of total oxylipins, ∼1530 nmol of DHA-derived oxylipins, ∼208 nmol of EPA-derived oxylipins, ∼84 nmol of LA-derived oxylipins, and ∼8 nmol of AA-derived oxylipins per person per day. EPA- and DHA-derived oxylipins and AA-derived epoxides are known to have anti-inflammatory and pro-resolving effects in vivo, where they are generated enzymatically from precursor PUFAs.[14−16]

In summary, a hydrolysis method was optimized to quantify oxylipins in fish and algae oils. The analysis revealed enrichment of EPA-derived oxylipins in fish oils and DHA-derived oxylipins in algae oil, as well as other oxylipins from LA, ALA, DGLA, and AA. While the bioavailability of these compounds from fish and algae oils requires further testing, our data suggest that over-the-counter omega-3 oil supplements are sources of EPA and DHA oxidized metabolites, as well as other oxylipins. Future studies should also validate the utility of oxylipin measurements as potential markers of PUFA oxidation in oil supplements.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c02461.

Table S1. List of 76 oxylipins measured by UPLC-MS/MS and nine deuterated internal standards, retention time, precursor ion, product ion, and collision energy. Table S2. Fatty acid methyl ester standard retention time. Table S3. Solvent gradient and flow rate in LC. Table S4. Matrix effects (ion suppression/enhancement) for nine surrogate standards and 72 oxylipins extracted from 2, 10, 20, or 30 μL of cod liver oil in Experiment 1 ($n$ = 1 per oil volume). Table S5. Matrix effects (ion suppression/enhancement) for nine surrogate standards and 72 oxylipins hydrolyzed using 0.25 M sodium carbonate or sodium hydroxide in Experiment 5 ($n$ = 1 per base). Table S6. Mean, SD, and CV of oxylipins measured (pmol/μL oil) across five different experiments where 2 μL of cod liver oil was hydrolyzed with 200 μL of 0.25 M sodium carbonate in the presence of extraction solvent. Interexperimental mean, SD, and CV of means from each experiment are presented in the last column. Table S7. Mean, SD, and CV of oxylipins measured (pmol/μL oil) across three different experiments where 10 μL of cod liver oil was hydrolyzed with 200 μL of 0.25 M sodium carbonate in the presence of extraction solvent. Interexperimental mean, SD, and CV of means from each experiment are presented in the last column. Table S8. Concentrations (pmol/μL oil) of oxylipins in fish and algae oils (Experiment 6). Oxylipin concentrations were determined in 2 μL of oil reconstituted in extraction solvent and hydrolyzed with 200 μL of sodium hydroxide (0.25 M). Table S9. Fatty acid concentration (mg/g oil) of oil supplements ($n$ = 3). Figure S1. Effect of adding extraction solvent (methanol with 0.1% acetic acid and 0.1% BHT) with hydrolysis reagent on oxylipin peak area (Experiment 3). Peak areas of oxylipins and their representative deuterated surrogates in 2 and 10 μL of fish oil with or without addition of extraction solvent before hydrolysis are shown for metabolites derived from (a) DHA, (b) EPA, (c) AA, (d) LA-ALA-DGLA, and (e) surrogate standards. Figure S2. Representative UPLC-MS/MS chromatogram of pure oxylipin standard mixtures (0.25−5 μM) dissolved in methanol. Figure S3. Representative UPLC-MS/MS chromatogram of 76 oxylipins and nine surrogates in a fish oil sample (fish oil 3, Experiment 6). Figure S4. Representative GC chromatogram of fatty acids detected in fish oil 4 (cod liver oil) (PDF)

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### Author Contributions

S.E. and A.Y.T. designed the experiments. S.E. performed the experiments, analyzed the data, and wrote the manuscript. Z.Z. contributed to the experiments and data analysis. A.Y.T. supervised the study. All authors reviewed the manuscript.


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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS USED

AA, arachidonic acid; ALA, alpha-linolenic acid; ANOVA, analysis of variance; CYP450, cytochrome P450; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; DiHDPA, dihydroxydocosapentaenoic acid; DiHETE, dihydroxyeicosatetraenoic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecamonoenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; EpDPE, epoxydocosapentaenoic acid; EpETE, epoxyeicosatetraenoic acid; EpETrE, epoxyeicosatrienoic acid; EpOME, epoxyoctadecamonoenoic acid; ESI, electrospray ionization; FAME, fatty acid methyl esters; GC-FID, gas chromatography-flame ionization detector; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; HODE, hydroxyoctadecadienoic acid; HOTrE, hydroxyoctadecatrienoic acid; HV, high-volume; LA, linoleic acid; LTB4, leukotriene B4; LTC4, leukotriene C4; LTD4, leukotriene D4; LTE4, leukotriene E4; LXA4, lipoxin A4; LV, low-volume; MRM, multiple reaction monitoring; oxo-ETE, oxo-eicosatetraenoic acid; oxo-ODE, oxo-octadecadienoic acid; PGE, prostaglandin E; PGF1a, prostaglandin F1 alpha; PUFA, polyunsaturated fatty acid; SPE, solid-phase extraction; TXB, thromboxane B; UPLC-MS/MS, ultrahigh-performance liquid chromatography coupled with tandem mass-spectrometry

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