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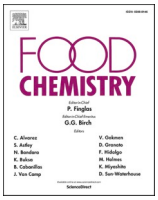

# Concomitant oxidation of fatty acids other than DHA and EPA plays a role in the characteristic off-odor of fish oil

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ABSTRACT

The aim of the present research was to explore the development of off-odors in fish oil from the perspective of fatty acid oxidation. It was found that the off-odors elicited by the two major ω-3 PUFAs in fish oil, i.e. DHA and EPA, were different from those by fish oil. Results showed that simultaneous oxidation of fatty acids other than DHA and EPA can be involved. The off-odors of fish oil was successfully simulated by combining oxidized samples of DHA, EPA and sunflower oil. Therefore, oxidation of oleic and linoleic acids also contributed to the off-odors in fish oil. A novel analytical approach that consisted in the combination of gas chromatography-ion mobility spectrometry (GC-IMS) and orthogonal partial least squares discriminant analysis (OPLS-DA) was applied to identify differences in the volatile components between the recombinant oil and the fish oil.

## 1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3) are important constituents of fish oils and other marine lipids like algae oils. Generally, LC-PUFAs are readily degraded by lipid oxidation reactions that give rise to hydroperoxides that in turn decompose into a myriad of secondary oxidation products such as hydrocarbons, vinyl alcohols, oxyesters, alkenals, alkadienals, vinyl ketones and others. Short-chain saturated and unsaturated carbonyl compounds, including both aldehydes and ketones, are recognized to be the major contributors to flavor deterioration in fish oil, imparting off-flavors such as fishy, metallic and rancidity primarily (de Oliveira, Minozzo, Licodiedoff, & Waszczynskyj, 2016; Miyashita, Uemura & Hosokawa, 2018; Song et al., 2020). Their high susceptibility to oxidative degradation makes it difficult to use fish oils as ingredients in foods and food supplements (Chen et al., 2016; Güner, Yilmaz, & Yüceer, 2019; Chang & Lee, 2020).

The refining process has limited capability to remove the unpleasant odor from fish oil. Seven deodorization methods were adopted to remove the volatile components in crude fish oil, while the deodorized oil contained different degrees of fishy smell (Song et al., 2018). High concentrations of 2,4-heptadienal and 2,4-decadienal were found to be involved in the unpleasant odor of refined sardine oil (Soldo et al., 2019).

Volatile lipid oxidation products are normally measured by different sampling techniques, such as static or dynamic procedures, followed by gas chromatography (GC) with different detectors, flame ionization detector (FID) or mass spectrometry (MS) detector. Gas chromatography-ion mobility spectrometry (GC-IMS) has recently drawn attention in the analysis of volatile compounds owing to its high sensitivity, simple operation and low cost. Based upon the different mobility of ionized molecules in an electrostatic field, it provides a fingerprint of the aroma that can be used to discriminate samples with different quality grades (Di Serio et al., 2021).

*Abbreviations:* ALA, alpha-linolenic acid; AO, algae oil; AV, *p*-anisidine value; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; FD, flavor dilution; FID, flame ionization detector; FO, fish oil; GC, gas chromatography; GC-IMS, gas chromatography ion mobility spectrometry; LC-PUFAs, long chain polyunsaturated fatty acids; MOS, metal oxide semiconductors; MS, mass spectrometry; OPLS-DA, orthogonal partial lest squares discriminant analysis; PCA, principal component analysis; PUFAs, polyunsaturated fatty acids; PV, peroxide value; RO, recombined oil; SHS-GC-IMS, static headspace gas-chromatography-ion mobility spectrometry.

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The volatile compounds responsible for fishy off-odors have been widely studied (Karahadian & Lindsay, 1989; Milo & Grosch, 1993; Venkateshwarlu, Let, Meyer & Jacobsen, 2004; Parlapani, Verdos, Haroutounian, & Boziaris, 2015; Li, Peng, Mei, & Xie, 2020; Miyashita et al., 2018). Some have been identified in fish oils and fish-oil containing foods. For instance, 1-penten-3-one, (*E*,*E*)-2,4-heptadienal, (*E*,*Z*)-2,6-nonanodienal and (*Z*)-4-heptenal were identified as potent odorants contributing to fishy off-flavor in a fish oil enriched milk (Venkateshwarlu, Let, Meyer, & Jacobsen, 2004). However, the sources and specific formation pathways of these compounds are not clear. Even though the odor substances in refined fish oil are mainly generated from lipid oxidation, the detailed relationship between the odor of fish oil and oxidized fatty acids is rarely reported. In this regard, different ω-3 PUFA standards, DHA, EPA and α-linolenic acid (ALA), subjected to different oxidation conditions produced the same odorants, but with different flavor dilution (FD) factors depending on the fatty acid and/or the type of oxidation applied, i.e. autoxidation or enzymatic oxidation with lipoxygenase (Hammer & Schieberle, 2013). *trans*-4,5-Epoxy-(*E*,*Z*)-2,7-decadienal, (*Z*)-1,5-octadien-3-one, (*Z*)-3-hexenal, (*Z*,*Z*)-2,5-octadienal, (*Z*,*Z*)-3,6-nonadienal and (*E*,*E*,*Z*)-2,4,6-nonatrienal were found to have the highest FD factors. Results suggested that a defined ratio of a few of these odorants was necessary to cause fishy off-flavor.

Given that DHA and EPA are the major ω-3 PUFAs found in fish oils (Kleiner, Cladis, & Santerre, 2015; Marsol-Vall, Aitta, Guo, & Yang, 2021), oxidation of both fatty acids should be the main cause of fishy off-flavor. However, the co-oxidation of fatty acids other than DHA and EPA may also contribute to the off-odors of fish oils (starting hypothesis). Exploring the development of off-flavors in fish oil from the perspective of fatty acid oxidation would help lay the groundwork for a systematic understanding of the mechanism of fish odor formation and adopt measures addressed to remove specific volatiles in fish oil in a targeted fashion. To the best of our knowledge, no study has been conducted to evaluate the contribution of fatty acids other than DHA and EPA to the sensory profile of fish oils.

The aim of this study was to determine the specific relationship between the odors of oxidized DHA and EPA and that of fish oil and whether fatty acids other than these two PUFAs make a significant contribution. Anchovy oil containing 19.0 % DHA and 9.9 % EPA was the oil selected as representative of fish oils. For comparative purposes, algae oil from *Schizochytrium sp*, comprising elevated contents of DHA (35.3 %) and negligible amounts of EPA (0.3 %), was also tested. The oils were purified by column chromatography to obtain their triacylglycerols (TAG) and thereby eliminate the influence of uncontrolled impurities. Off-odors developed under thermal oxidative conditions (60 °C) were evaluated and compared to those of DHA and EPA standards oxidized under the same conditions. The odor profile of the fish oil was successfully simulated from a combination of oxidized samples of DHA, EPA and sunflower oil. The sensory similarity between the recombinant oil and the oxidized fish oil were also verified by electronic nose (E-nose) analysis and triangle test. The sunflower oil was chosen to represent the fatty acids other than DHA and EPA in the fish oil. A novel analytical approach that consisted in the combination of GC-IMS and OPLS-DA was applied to identify differences in the volatile components between the recombinant oil and the fish oil.

## 2. Materials and methods

### 2.1. Standards and reagents

Anchovy oil was provided by Zhoushan Xinnuojia Bioengineering Co., Ltd. (Zhoushan, China). Algae oil and sunflower oil were purchased form Qingdao Mingyuan Chemical Co., Ltd.. Notit-8015 activated charcoal and activated clay were purchased from Jiejingclay Co., Ltd. (Leping, Jiangxi, China) and Zhongji Chemicals Import & Export Co., ltd. (Shanghai, China), respectively. All other reagents used in this study were of analytical grade and purchased from Sinopharm Chemical Reagent Co., ltd. (Qingdao, China). DHA standard (purity > 99 %), EPA standard (purity > 99 %) and a 37-component mixture of fatty acid methyl esters (FAME) were all purchased from ANPEL Laboratory Technologies Inc. (Shanghai, China).

### 2.2. Purification of oils

The anchovy, sunflower and algae oils were purified according to the method of Shimajiri, Shiota, Hosokawa & Miyashita (2013). A chromatography column (50 × 4 cm i.d.) was packed sequentially with an *n*-hexane slurry of activated clay (20 g) and activated carbon (30 g). An amount of 50 g of oil was passed through the column using 1 000 mL *n*-hexane. Then the solvent was removed in a rotary evaporator at 35 °C and the oil was further purified in another column (50 × 4 cm i.d.) packed with silica gel (200 g) and aluminum oxide (40 g), using *n*-hexane (500 mL) followed by a mixture of *n*-hexane/chloroform (1:4, v/v) (1 200 mL). The solvent was then removed in the rotary evaporator at 35 °C (1 h) followed by a stream of nitrogen that was bubbled into the oil at room temperature for 2 h. The purification process was applied in batches and the batches were blended between each other to obtain sufficient amounts of sample for the experiments.

### 2.3. Oxidation conditions

Approximately 120 g of purified fish oil was placed into a wide-mouth brown glass bottle (1 000 mL). The sample was then incubated in an oven at 60 °C. About 20 g of oil was taken out at 0.5, 1, 1.5, 2 and 3 days for subsequent analyses and these were respectively coded as FO followed by a number representing the sampling order (FO1-FO5). Approximately 350 mg of DHA and 350 mg of EPA standards were respectively weighed into 10-mL brown glass bottles and then incubated at 60 °C in the oven for 2 days. Purified algae oil (120 g) and purified sunflower oil (120 g) were also oxidized in 1000-mL brown glass bottles at 60 °C for 2 and 6 days, respectively. The samples of DHA and EPA used in the recombination assay were also oxidized at 60 °C for 2 days, whereas the sunflower oil was oxidized at the same temperature for 6 days. All the samples were protected with nitrogen and preserved at −20 °C for not >1 day until analyses.

### 2.4. Sensory assessment

A group of ten people (8 female/2 male; aged between 22 and 45 years) was recruited from the Ocean University of China (Qingdao, China) for sensory analysis. To recognize accurately the aroma of fish oil, they received intensive training for a week before the experiments. The odor vocabulary and attributes for the fish oil samples were those reported elsewhere (Serfert, Drusch, & Schwarz, 2010). Fishy, frying, metallic, rancid, grassy and painty were selected after a discussion among the panelists. The definition of each attribute and the references used are listed in Table 1S**.** These were those according to the standard practice for sensory evaluation of edible oils and fats (E18, 2012). The sensory evaluation was carried out in a sensory panel room kept at 21 ± 1 °C. An amount of 1 g oil sample was placed into a 50-mL glass vessel that was covered and given to each panelist. The entire samples of oxidized DHA or EPA (350 mg) were also transferred to the 50-mL glass vessels that were also covered and given to the panelists. The sensory evaluation process was the same for all samples, both oils and standards. The intensity of each odor attribute was evaluated using a 0–10 point scale with 0.5 steps. The odor intensity represented by the score was shown as follows: 0-not perceptible, 2-slightly perceptible, 4-perceptible, 6-considerably perceptible, 8-strongly perceptible and 10-very strongly perceptible. In this regard, a value within 0.1–2 would be slightly perceptible, between 2.1 and 4 perceptible, and so on. Each sample was tested three times by each panelist and 1-min rests were taken between the tests for sensory recovery.

### 2.5. Analysis of peroxide value (PV), p-anisidine value (AV) and fatty acid composition

The PV and AV applied were those according to Wen et al (2019). The preparation of FAME in the fatty acid composition analysis was carried out according to Zhang et al. (2019) with some modification. About 20 mg of purified oil was weighed into a tube showing a good airtightness and then 2 mL of 10 % $H_2SO_4$ in methanol was added. Then, the tube was filled with nitrogen and incubated at 90 °C for 90 min applying shaking each 20 min. After cooling at room temperature, 1 mL of *n*-hexane was added to extract the FAME. The analysis of FAME was performed by GC–MS as reported elsewhere (Menegazzo, Petenuci, & Fonseca, 2014). A 7890a GC–MS instrument (Agilent Technologies, Santa Clara, CA, USA) was used. This was equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.20 μm) (Agilent Technologies, Santa Clara, CA, USA). The initial temperature was set at 80 °C, then it was increased to 200 °C at 20 °C/min, and then to 280 °C at 5 °C/min. Finally, the temperature was increased to 300 °C at 10 °C/min and hold at 300 °C for 5 min. The temperature of the ion source was set at 250 °C and the mass spectrometer was operated in EI mode at 70 eV with a filament current of 25 μA.

The absolute losses of unsaturated fatty acids were calculated as reported elsewhere (Feitosa et al., 2019). Accordingly, the absolute contents of unsaturated fatty acids were determined from the fatty acid composition of the fresh sample and considering that no oxidative changes of saturated fatty acids took place compared to unsaturated fatty acids. This quantitative approach has shown comparable results to those obtained with an internal standard. In order to reduce the error associated with the calculation, the total amount of saturated fatty acids in the fresh sample was considered unchanged during the thermal treatment and this was used for the calculation.

### 2.6. Analysis of volatile compounds

Volatile compounds were analyzed by static headspace-gas chromatography-ion mobility spectrometry (SHS-GC-IMS) according to Guo et al (2018) with some modifications. A GC-IMS system (G.A.S., Dortmund, Germany) equipped with a headspace sampling unit (Gerstel GmbH, Mülheim, Germany), an autosampler (CTC Analytics AG, Zwingen, Switzerland), and an FS-SE-54-CB capillary column (0.25 μm, 15 m × 0.53 mm, Agilent Technologies, CA, USA) was used. An amount of 1 g of sample was weighed into a 20-mL headspace vial that was encapsulated and then incubated at 40 °C for 30 min. After incubation, 0.5 mL of gas from the headspace was taken with a syringe that had been heated to 50 °C and then this was injected into the injection port of the chromatograph, heated at 80 °C and working in splitless mode. The volatile compounds were carried through the capillary column by nitrogen (99.99 % purity) at isothermal conditions (45 °C) and the following flow program was applied: 2 mL/min for 5 min, 5 mL/min for 5 min, 10 mL/min for 5 min, 20 mL/min for 5 min, 50 mL/min for 5 min, 100 mL/min for 5 min. The volatile compounds were first separated in the capillary column, and then ionized at the IMS ionization chamber by a 3H ionization source (300 MBq activity) in a positive ion mode. The second separation occurred in the drift tube (9.8 cm) working at a constant voltage (5 kV) at 45 °C with a nitrogen flow of 150 mL/min. To avoid cross contamination, the syringe was automatically flushed with nitrogen at 150 mL/min for 0.5 min before and 3 min after each analysis.

### 2.7. Recombination of oxidized samples of DHA, EPA and sunflower oil

Oxidized samples of DHA and EPA standards were recombined with oxidized sunflower oil. Specifically, 100 mg of oxidized DHA and 50 mg of oxidized EPA were weighed into a brown glass bottle. Oxidized sunflower oil was gradually added into the bottle until the odor of the recombined oil approached that of FO3 (the fish oil sample incubated at 60 °C during 1.5 days). Finally, a total of 0.6 g of sunflower oil was used to obtain the recombined oil (also referred to as DHA&EPA&SO).

The odor profiles of the recombinant oil and FO3 were obtained by sensory evaluation as described above. Both the odor vocabulary and scoring criteria of odor intensity were those described above for the FO samples. Similarly, volatile components were also analyzed by SHS-GC-IMS and the parameters used were the same indicated above.

A multi-purpose sensory test of three samples, referred to as triangle test, was applied for the selection of differential sample. Two samples were identical and one was different. The three samples were coded with individual and random three-digit numbers, and then presented to the panelists at one time. The panelist was requested to identify the code representing the odd sample. For the present study, analytical discriminative sensory analysis was performed at the Sensory Lab located at the Department of Food Science and Technology of the Ocean University of China. A total of 16 untrained participants were recruited and they were given randomly coded samples along with the recombined oil and FO3. The detailed code of each sample, the serving orders, results and the *p*-value were according to ISO:4120 (*Available online: https://www.iso.org/standard/76666.html*) and are listed in Table 2S.

An E-nose instrument (ISENSO INTELLIGENT., China) was employed to analyze the volatile compounds produced by the recombinant oil and FO3. Fourteen metal oxide semiconductors (MOS) with different chemical composition and thickness together with pattern recognition algorithms formed the intelligent bionic olfactory system (Kachele, Zhang, Gao, & Adhikari, 2017). The operating parameters of the e-nose instrument were as follows: cleaning time, 120 s; sampling time, 60 s; gas flow, 1 L/min; and the initial responding value of MOS was <1.0. The samples were placed into 10-mL headspace extraction vials at room temperature for sampling.

### 2.8. Statistical analysis

LAV software (version 2.2.1) was used for the collection of SHS-GC-IMS data and fingerprint drawing. The information on concentrations of volatile compounds was obtained based upon the generation of GC-IMS fingerprints. Then these data were treated using SIMCA multivariate data analysis software (Version 16.0, Umetrics AB, Umeå, Sweden). The grouping information and the concentration of volatile compounds were set as primary and secondary variable, respectively. Then, the data were normalized and scaled using the type of Par. An unsupervised principal component analysis (PCA) was adopted to discriminate the samples. Supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was applied to identify the volatile compounds causing the subtle difference in odor profile between the recombinant oil and FO3. A total of 97 compounds were detected in these oil samples. The area values of the chromatographic peaks of the volatile compounds were processed by OPLS-DA and the data dimensions were reduced from 97 to 2. Both PCA and OPLS-DA were implemented with the SIMCA software. Unless indicated, all experiments were carried out in triplicate. The number of parallel tests was increased to six when the OPLS-DA was applied. Results were shown as mean ± standard deviation (n = 3). One-way ANOVA through Tukey's test was also used for comparisons between mean values in SPSS 20.0 software (SPSS Inc, Chicago, IL, USA). Origin 2017 (Northampton, MA, USA) was used for figure drawing.

## 3. Results and discussion

### 3.1. Development of off-odors in fish oil under thermal conditions (60 °C)

#### 3.1.1. Sensory analysis

Sensory analysis showed different profiles of the attributes evaluated along oxidative degradation of fish oil, i.e. fishy, painty, grassy, rancid, metallic and frying (Fig. 1). With the exception of frying and grassy, with scores of 1.8 and 2.3, respectively, all the attributes presented scores lower than 1.0 in the fresh or non-heated sample. Therefore, the predominant odors in the non-heated sample were grassy and frying, but

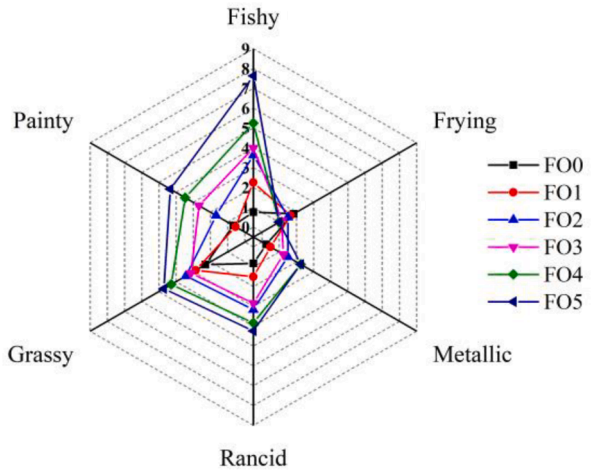


**Fig. 1.** Sensory odor profiles of fish oil along oxidation at 60 °C for 0, 0.5, 1, 1.5, 2 and 2.5 days. The intensity values for each odor attribute are indicated next. 0-not perceptible, 2-slightly perceptible, 4-perceptible, 6-considerably perceptible, 8-strongly perceptible and 10-very strongly perceptible.

not fishy. Rancid, painty and grassy increased progressively and in a similar way during the oil incubation, reaching values close to 5.0 at the end of the assay (FO5), i.e. considerably perceptible. As expected, the fishy attribute also increased progressively but significantly faster than the rest of the attributes evaluated, showing scores as high as 7.6 at the end of the assay (FO5), i.e. strongly perceptible. No significant increases were observed for frying, and metallic only increased to 2.3.

#### 3.1.2. Analysis of non-volatile oxidation products

The analyses of primary and secondary non-volatile oxidation products, as determined by the PV and AV, respectively, showed coherent results with the sensory analysis. Relatively high oxidation levels were detected early in the fish oil. With a fishy score of 2.3 (perceptible), the first oxidized sample analyzed (FO1), heated for 12 h, presented a Totox value of 112, obtained from high PV (52 meq/kg oil) and relatively high AV (8.9) (Table 3S). Concerning the most oxidized sample (FO5), with a fishy score close to 8.0 (strongly perceptible), the Totox value was as high as 685.

#### 3.1.3. Analysis of volatile oxidation products

SHS-GC-IMS analysis of volatile lipid oxidation products also showed significant changes along fish oil oxidation. With regard to the fingerprint of volatile substances, each point represents a volatile compound and the color provides information on the content of the volatile. When this increases the color changes in the order blue, white, yellow and red. A total of 92 compounds were detected. Some only presented slight changes in concentration along the incubation time, such as those with reference numbers 1 and 89, whereas others presented substantial enhancements, as it was the case for compounds 55–57 or 77–84, or reductions, as it was for compounds 5–7 or 24–26 (Fig. 2**A**). An unsupervised PCA model was applied to the set of SHS-GC-IMS data to evaluate differences between the samples (Xu et al., 2020). As shown in the score plot of the PCA, the heated samples differed a lot from the fresh oil (FO0) and, as expected, the longer the incubation time the larger were the differences (Fig. 2**B**). The loading plot showed that the compounds whose concentrations were increased were located at the zone of positive values of the first component (x-axis), whereas those that decreased were at the zone of negative values (Fig. 2**C**). Therefore, being consistent with the changes observed in the sensory profile, the volatile compounds showed large changes in the type and concentrations during the thermal treatment of the oil.

#### 3.1.4. Analysis of fatty acid composition

The oxidation levels of the samples were so high that significant changes in the fatty acid composition were observed. As expected, losses of DHA and EPA were predominant, but oleic (C18:1ω9) and linoleic (C18:2ω6) acid also decreased substantially (Table 1). The absolute losses of DHA and EPA obtained were as high as 2.7 and 1.6 g/100 g oil, respectively, for the sample with lower oxidation (FO1). Similarly, losses of oleic and linoleic acids were also as high as 1.7 and 1.1 g/100 g oil, respectively, for the same sample. The total loss of unsaturated fatty acids, i.e. MUFAs plus PUFAs, was 8.4 g/100 g oil for the FO1 sample. Therefore 8.4 wt% of fatty acids were degraded in this sample.

At the end of the assay the total loss of unsaturated fatty acids was as high as 19.3 g/100 g oil and the losses for DHA, EPA, oleic and linoleic acids were respectively 6.5, 3.4, 3.3 and 2.3 g/100 g oil. Therefore, the oxidation of fatty acids was dominated by DHA and EPA, however, concomitant oxidation of other unsaturated fatty acids, especially oleic and linoleic acid also occurred to a significant extent along the thermal treatment. Even though differences in the oxidation rates between fatty acids are considerably high when tested separately, their relative oxidation in blends depends on their relative proportions, especially in the absence of antioxidants as it was the case of the purified oil of the present study. Oxidation of those more stable can be influenced by the oxidation products of the more reactive fatty acids (Morales, Marmesat, Dobarganes, Márquez-Ruiz, & Velasco, 2012).

### 3.2. Off-odors of fish oil compared to those of DHA and EPA

As outlined above, DHA and EPA were the main fatty acids degraded during the thermal treatment of fish oil, but substantial oxidation was also observed for oleic and linoleic acids. Accordingly, the relationship between the odor of oxidized fish oil and oxidized DHA and EPA was explored.

SHS-GC-IMS analysis exhibited great differences in the type and concentration of volatiles between the oxidized fatty acid standards and the fish oil samples, as observed from the different colors in the fingerprint (Fig. 3**A**). In fact, PCA of the data showed great differences between them (Fig. 3**B**). It can be observed in the PCA loading plot that those volatiles that were detected in the fish oils but not in the fatty acid standards (Fig. 3A) were located at the zone of high negative values of the first component (x-axis) (Fig. 3**C**).

Sensory analysis also showed differences (Fig. 3**C**). The fishy and grassy attributes were predominant in both DHA and EPA. When compared to the FO samples with similar fishy scores, DHA showed a markedly high grassy score. Thus, DHA with a fishy score of 6.3, presented a grassy score of 7.0, whereas in FO5 with a fishy of 7.0 the grassy score was significantly lower, i.e. 4.7, and it was also lower (4.3) for FO4 presenting a fishy score of 5.3. This fact was not observed for EPA, which showed comparable attributes to those found in the FO sample with similar fishy score, except for the painty attribute, which was significantly lower. In this regard, while in the EPA with a fishy score of 4.7 the intensity of the painty attribute was 1.5, in the FO4 sample, with a fishy score of 5.3, the painty score was 3.5.

These results suggest that although oxidation of DHA and EPA was a key factor in the odor profile of fish oil, neither the standard DHA nor EPA were able to form the characteristic off-odor of fish oil. Given the results obtained for the losses of unsaturated fatty acids, the complex development of off-odors in fish oil can not only be attributed to the oxidation of DHA and EPA.

### 3.3. Off-odors of fish oil compared to those of low-EPA marine oil

Considering that different off-odor profiles were provided by DHA and EPA standards, different proportions of these fatty acids may have a role in the development of off-flavors in fish oils, which depends, among other factors, on the species they come from. To confirm this hypothesis, the anchovy oil containing 19.0 % DHA and 9.9 % EPA, was compared to

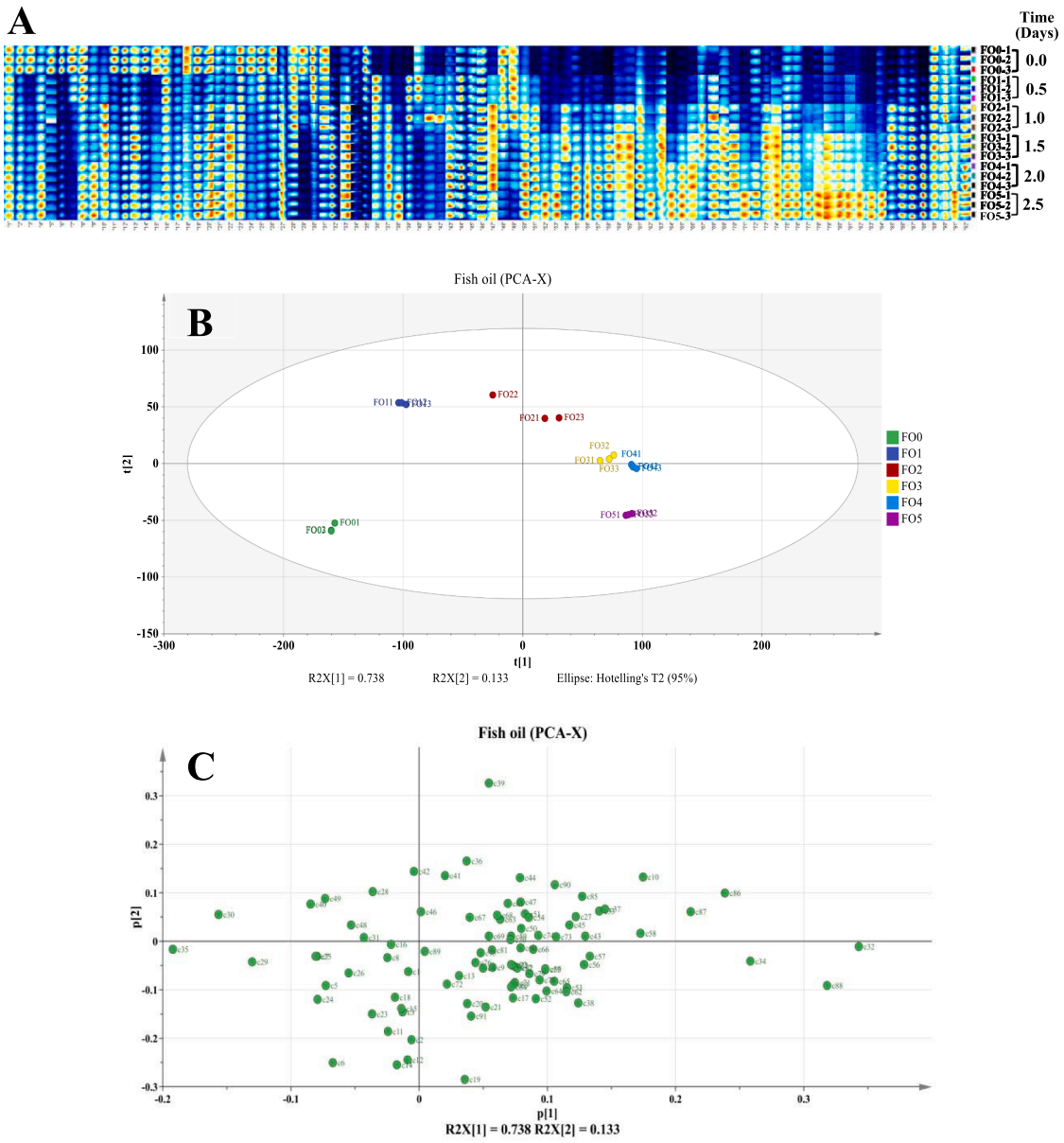


**Fig. 2.** SHS-GC-IMS fingerprints (**A**) and PCA score plot of the data for volatile substances (**B**) in fish oil along oxidation at 60 °C for 0, 0.5, 1, 1.5, 2 and 2.5 days. Color in **A** provides information on the content of the volatile compounds. When this increases the color changes in the order blue, white, yellow and red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a marine oil, namely, algae oil (AO) from *Schizochytrium sp*, comprising elevated contents of DHA (35.3 %) and negligible amounts of EPA (0.3 %). Both oils were purified and oxidized at the same experimental conditions.

A total of 112 volatile compounds were detected by SHS-GC-IMS analysis and identified using the IMS library of the instrument (Table 5S). As observed in the fingerprints (Fig. 1**S-A**), both oils presented similar concentrations for a number of these compounds, such as those with reference numbers 21, 24, 29, 38 and 74. However, clear differences were also observed for other volatiles. PCA of the data confirmed these differences. As shown in the PCA score plot (Fig. 1**S-B**), the algae oil samples were clearly separated from the FO samples, showing clear differences in the volatile compounds between them. Comparing the IMS fingerprints (Fig. 1S-A) with the PCA loading plot, it can be observed that the volatile compounds detected in the FO but not in the AO were located at the zone of high positive values of the first component (x-axis), whereas the opposite was found for those present in the AO and not detected in the FO (Fig. 1**S-C**).

Sensory analysis also showed differences between the fish and algae oil samples. While the odor intensities of frying, metallic, rancid and grassy were similar for the two oils, the painty and fishy intensities were significantly higher for the algae oil (Fig. 2**S**). Therefore, apart from the contribution of fatty acids other than LC-PUFAs to the development of off-odors in FO, different proportions of the two major LC-PUFAs can also have an effect.

### *3.4. Reconstruction of fish oil off-odors by recombination of oxidized samples of DHA, EPA and purified sunflower oil*

To demonstrate the contribution of fatty acids other than DHA and EPA to fish oil off-odors, an attempt was made to reconstruct FO malodors by recombining oxidized samples of DHA and EPA with an oxidized oil containing oleic and linoleic as the only major fatty acids susceptible to oxidative degradation. Sunflower oil (Table 4S) was the oil selected and this was blended at different proportions with a blend of oxidized samples of DHA and EPA 2:1 (w/w), i.e. at the proportions

**Table 1**
Fatty acid compositions (g/100 g oil) of the fish oil samples stored at 60 °C for different periods.

| | FO0 | FO1 | FO2 | FO3 | FO4 | FO5 |
|---|---|---|---|---|---|---|
| C10:0 | 0.11 ± 0.01a | 0.08 ± 0.00bc | 0.09 ± 0.00b | 0.07 ± 0.00cd | 0.09 ± 0.00b | 0.06 ± 0.00d |
| C12:0 | 0.68 ± 0.02a | 0.77 ± 0.06a | 0.71 ± 0.05a | 0.67 ± 0.02a | 0.70 ± 0.02a | 0.75 ± 0.05a |
| C14:0 | 5.69 ± 0.14a | 4.88 ± 0.07b | 5.06 ± 0.36b | 4.88 ± 0.14b | 4.80 ± 0.07b | 4.23 ± 0.18c |
| C15:0 | 1.99 ± 0.03a | 1.83 ± 0.06b | 1.89 ± 0.09ab | 1.81 ± 0.04b | 1.79 ± 0.02b | 1.56 ± 0.06c |
| C16:0 | 10.02 ± 0.14b | 10.52 ± 0.23ab | 10.11 ± 0.34b | 10.4 ± 0.29b | 10.55 ± 0.15ab | 11.27 ± 0.51a |
| C16:1 | 6.41 ± 0.24a | 5.49 ± 0.10b | 5.59 ± 0.49b | 5.21 ± 0.17bc | 5.29 ± 0.07bc | 4.77 ± 0.18c |
| C17:0 | 1.96 ± 0.06b | 2.14 ± 0.08ab | 2.22 ± 0.11a | 2.25 ± 0.07a | 2.21 ± 0.03a | 2.13 ± 0.08ab |
| C18:0 | 6.06 ± 0.2a | 5.20 ± 0.08b | 5.45 ± 0.35b | 5.10 ± 0.13b | 5.07 ± 0.10b | 4.99 ± 0.19b |
| C18:1ω9 | 14.49 ± 0.39a | 12.84 ± 0.31b | 13.07 ± 0.41b | 12.89 ± 0.37b | 12.57 ± 0.26b | 11.23 ± 0.47c |
| C18:2ω6 | 7.32 ± 0.03a | 6.22 ± 0.18b | 6.37 ± 0.12b | 6.16 ± 0.19b | 5.98 ± 0.12b | 5.00 ± 0.21c |
| C18:3ω3 | 0.73 ± 0.01a | 0.49 ± 0.01b | 0.49 ± 0.02b | 0.48 ± 0.03b | 0.47 ± 0.02b | 0.33 ± 0.02c |
| C20:0 | 1.22 ± 0.10a | 1.73 ± 0.10ab | 1.67 ± 0.11ab | 1.85 ± 0.05b | 1.82 ± 0.03b | 1.86 ± 0.07b |
| C20:1 | 2.74 ± 0.15bc | 2.99 ± 0.05ab | 3.13 ± 0.06a | 2.99 ± 0.08ab | 3.01 ± 0.08ab | 2.67 ± 0.14c |
| C20:2ω6 | 0.62 ± 0.03c | 0.85 ± 0.03ab | 0.83 ± 0.08b | 0.93 ± 0.05ab | 0.92 ± 0.02ab | 1.00 ± 0.08a |
| C20:4ω6 | 7.85 ± 0.11a | 7.05 ± 0.16b | 6.94 ± 0.18 | 6.97 ± 0.18b | 6.62 ± 0.14b | 5.69 ± 0.32c |
| C20:5ω3 | 9.88 ± 0.59a | 8.24 ± 0.66b | 8.33 ± 0.53b | 8.39 ± 0.42b | 8.02 ± 0.25b | 6.52 ± 0.53c |
| C22:0 | 0.58 ± 0.03c | 0.85 ± 0.02ab | 0.83 ± 0.04b | 0.88 ± 0.02ab | 0.87 ± 0.01b | 0.95 ± 0.04a |
| C22:1 | 0.51 ± 0.01b | 0.53 ± 0.05b | 0.57 ± 0.05b | 1.00 ± 0.08a | 0.52 ± 0.02b | 0.56 ± 0.03b |
| C24:0 | 0.52 ± 0.05d | 0.82 ± 0.03bc | 0.79 ± 0.05c | 0.92 ± 0.03ab | 0.93 ± 0.03a | 1.02 ± 0.04a |
| C24:1 | 1.63 ± 0.15ab | 1.69 ± 0.04ab | 1.63 ± 0.03ab | 1.79 ± 0.05a | 1.77 ± 0.07a | 1.52 ± 0.06b |
| C22:6ω3 | 19.01 ± 0.83a | 16.35 ± 0.68b | 16.40 ± 0.85b | 16.1 ± 0.55b | 15.71 ± 0.39b | 12.56 ± 0.62c |
| SFA | 28.83 ± 0.25a | 28.83 ± 0.34a | 28.83 ± 0.65a | 28.83 ± 1.04a | 28.83 ± 0.51a | 28.83 ± 1.85a |
| MUFA | 25.77 ± 0.65a | 23.54 ± 0.45ab | 23.40 ± 0.50ab | 23.88 ± 0.84a | 23.17 ± 0.58ab | 20.74 ± 2.12b |
| PUFA | 45.40 ± 1.05a | 39.21 ± 0.88b | 39.36 ± 0.98b | 39.03 ± 1.31b | 37.72 ± 1.01b | 31.10 ± 1.93c |

Data express mean values and deviation standard of three determinations. Data for FO1-FO5 were calculated from the fatty acid composition of the fresh sample (FO0) and considering that no oxidative changes of saturated fatty acids took place compared to unsaturated fatty acids, according to Feitosa et al. (2019). Different letters show significant differences ($p < 0.05$) according to Tukey's test. FO0, FO1, FO2, FO3, FO4, FO5 represent fish oil stored for 0, 0.5, 1, 1.5, 2, and 3 days, respectively. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

present in the FO.

The recombined oil showed a sensory profile similar to that of the FO3 sample (Fig. 4**A**). Compared to the oxidized samples of the individual standards, the fishy odor intensity of the recombined oil was similar to that of EPA, but lower than that of DHA, whereas the grassy attribute was lower compared to both DHA and EPA. Therefore, even though the three individual components did not provide the characteristic off-odor of fish oil when tested separately, they did when they were combined, showing clearly synergistic effects of odorants.

Related studies on different food products have also allowed for the reconstruction of flavors from recombination of odorants. For instance, Utz et al. (2021) successfully constructed the odor of dairy model systems using eight flavor-active compounds. Similarly, recombination of ten key aroma-active compounds allowed the simulation of the aroma profile of black garlic (Yang, Song, Wang, & Jing, 2019). Xu et al. (2021) were also successful in reconstructing the odor of French fries and frying soybean oil with key aroma-active compounds using aroma recombination experiments. Heptanal and (*E,Z*)-3,5-octadien-2-one were recombined and results showed that odorant synergistic effects of these two components were responsible for the fishy malodors in algae marine oils (Marsili & Laskonis, 2014). In addition, Venkateshwarlu et al. (2004) were also able to simulate fishy and metallic off-odors in milk from the combination of (*E,Z*)-2,6-nonadienal, 1-penten-3-one, (*Z*)-4-heptenal, and (*E,E*)-2,4-heptadienal. They found main effects of (*E,Z*)-2,6-nonadienal and 1-penten-3-one and that two-factor interactions of these four volatiles were essential in the development of off-flavors.

For further verifying the odor similarities between the recombined oil and the FO3 sample, a triangle test and an electronic nose analysis were conducted. Results showed that only 4 out of 16 panelists provided correct answers ($p > 0.05$) in the triangle test, indicating that the recombined oil did not have an aroma significantly different from that of FO3. Therefore, differences between the recombined oil and FO3 were not detected in the test. The electronic nose analysis also exhibited similarities. Fourteen metal oxide semiconductors (MOS) that provide selectivity towards 14 kinds of volatile compounds presented identical response to the recombined oil and FO3 sample (Fig. 4**B**). Overall, these results also suggest that synergistic effects occurred between odorants of the oxidized samples of DHA, EPA and sunflower oil to produce a similar odor profile to that of fish oil. Thus, the odors of fish oil were generated by the co-oxidation of DHA, EPA and other fatty acids. As a result, when studying the formation mechanism of fish oil odor, it is necessary to consider the oxidation of multiple fatty acids. According to the fatty acid loss of fish oil during oxidation, sunflower oil with appropriate oxidation extent can be used to simulate the oxidation of oleic and linoleic acids in fish oil.

### 3.5. Identification of differential volatile compounds between the recombined oil and oxidized fish oil

Although the odor of the recombined oil (RO) was similar to that of fish oil (FO3), differences in the volatile compounds were observed (Fig. 3**S-A**). Accordingly, the differential compounds between RO and FO3 were explored. An orthogonal partial least squares discriminant analysis (OPLS-DA), a supervised chemometric method for data mining, was applied (Xu, et al., 2020). The score plot of the OPLS-DA model shown in Fig. 3**S-B** provided a clear separation between the RC and FO3 samples. These were divided into two columns on the abscissa axis, which implies that the OPLS-DA model could distinguish well these two kinds of oils (Jiang et al., 2021; Xu et al., 2020). $R^2X$, $R^2Y$ and $Q^2$ values were 0.929, 0.996, and 0.996, respectively, representing high fit goodness and prediction ability. The robustness of the OPLS-DA model was evaluated using permutation tests (Fig. 3**S-C**). The $R^2$ and $Q^2$ of 200 times permutation tests were 0.215 and −0.614, respectively, indicating sufficient ruggedness of the OPLS-DA model. Differential volatile compounds were identified from results of Fig. 3**S-D-F**. Variables for VIP > 1.0 and for |p(corr)| > 0.8 in the S-plot were selected. In addition, those samples with jack-knifed confidence intervals including zero were removed. Finally, statistical significance was characterized by a fold change of 2.5 (ratio > 2.5 or < 0.4) and $p < 0.05$ (Jiang et al., 2021). With this selected parameter, 8 volatile compounds were finally filtered out. The information on the identified differential volatile compounds between RO and FO3 is shown in Table 2. Furaneol, β-pinene, ethyl-propanoate and 2,5-dimethylhexane were detected in RO but not in FO3, while 1-octen-3-ol, hexanal, pentanal and pentan-2-one were detected in both samples, although their concentrations in FO3 were extremely much lower (Figure 3S-A). The concentration of β-pinene showed a fold change of 0.285. However, the odor threshold of this compound has been reported to be high in mineral oil (430 mg/kg) and consequently β-pinene is expected to have a small influence on the odor.

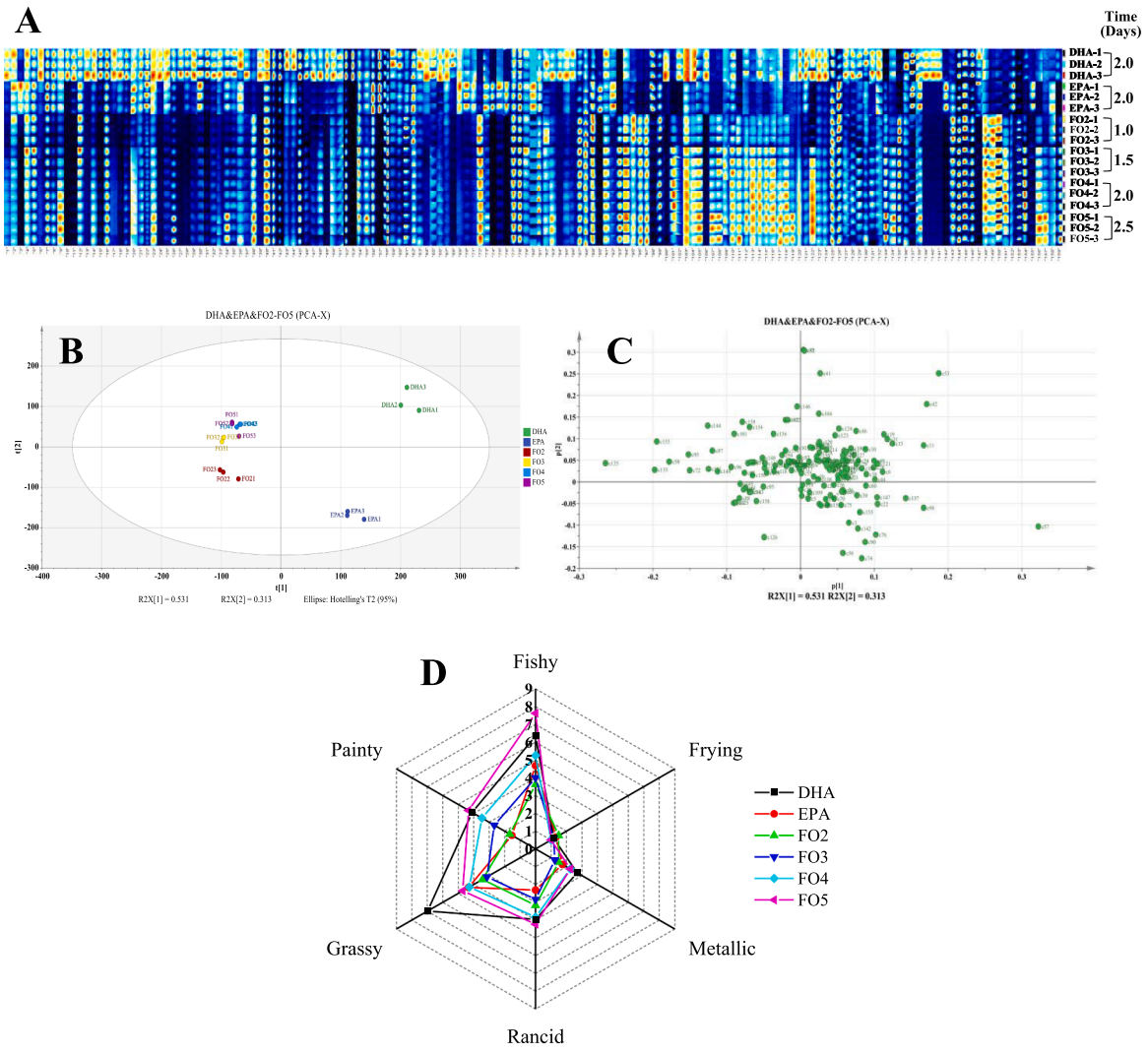


**Fig. 3.** HS-GC-IMS fingerprints (**A**), PCA score plot of the data for volatile substances (**B**) and sensory odor profiles (**C**) of fish oil along oxidation at 60 °C for 1, 1.5, 2 and 2.5 days compared to DHA and EPA samples oxidized at the same conditions for 2 days. Color in **A** provides information on the content of the volatile compounds. When this increases the color changes in the order blue, white, yellow and red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

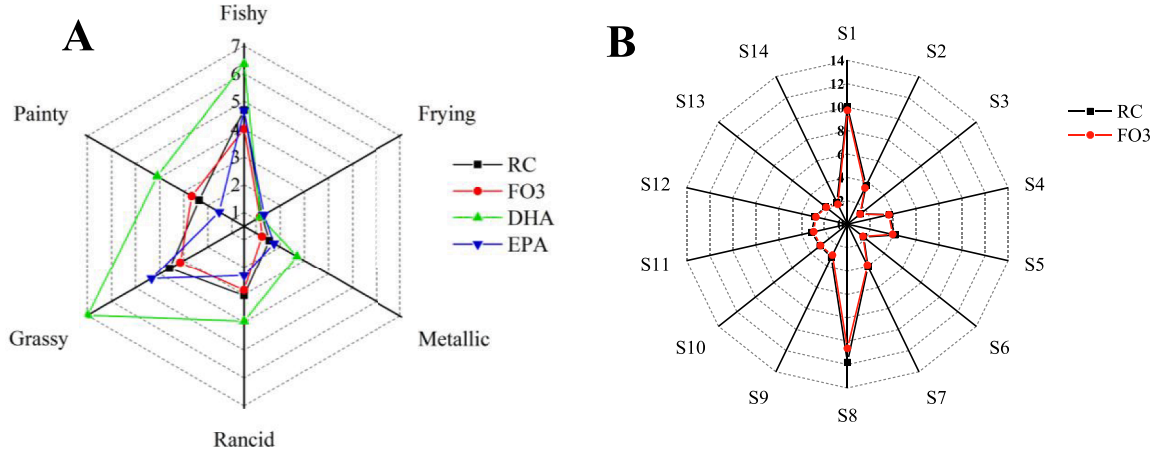


**Fig. 4.** Sensory odor profiles (**A**) and electronic nose analysis (**B**) of the recombination oil (RC, DHA&EPA&SO) and fish oil oxidized at 60 °C for 1.5 days (FO3). Analyses for DHA and EPA have also been included in **A** for comparative purposes.

**Table 2**
Information on the identified differential volatile compounds between the recombinant oil and FO3.

| NO. | Ref [1] | Compound | CAS | Formula | MW [2] | RI [3] | Rt [4] | Dt [5] | Odor type | Odor threshold(mg/kg) (medium) [6] | Fold change [7] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | C4 | Furaneol | C3658-77-3 | $C_6H_8O_3$ | 128.1 | 1082.6 | 812.9 | 1.1953 | Caramel | 0.025–0.05 (sunflower oil) | 0.299 |
| 2 | C10 | 1-Octen-3-ol | C3391-86-4 | $C_8H_{16}O$ | 128.2 | 985.9 | 579.422 | 1.1567 | Earthy | 0.9 (cottonseed oil) | 0.299 |
| 3 | C19 | β-Pinene | C127-91-3 | $C_{10}H_{16}$ | 136.2 | 980.4 | 567.966 | 1.5606 | Dry woody, resinous pine, hay, green | 430 (mineral oil) | 0.285 |
| 4 | C41 | Hexanal | C66-25-1 | $C_6H_{12}O$ | 100.2 | 790.8 | 260.587 | 1.5624 | Grassy | 0.3 (sunflower oil) | 0.276 |
| 5 | C51 | Pentanal | C110-62-3 | $C_5H_{10}O$ | 86.1 | 732.8 | 198.928 | 1.4171 | Fermented bread, fruity, nutty, berry | 0.24 (deodorized olive oil) | 0.311 |
| 6 | C52 | Ethyl propanoate | C105-37-3 | $C_5H_{10}O_2$ | 102.1 | 710.9 | 181.65 | 1.4542 | Fruity | 0.1 (deodorized olive oil) | 0.059 |
| 7 | C91 | Pentan-2-one | C107-87-9 | $C_5H_{10}O$ | 86.1 | 699.6 | 173.998 | 1.3827 | Sweet, fruity, ethereal, wine, woody | 288 (cheese 45 % fat dry matter) | 0.370 |
| 8 | C94 | 2,5-Dimethylhexane | C592-13-2 | $C_8H_{18}$ | 114.2 | 727.7 | 194.522 | 1.3966 | – | – | 0.270 |

[1] Compound reference in the GC-IMS analysis.
[2] Molecular weight.
[3] Retention index.
[4] Retention time from GC-IMS.
[5] drift time from GC-IMS.
[6] Odor thresholds obtained from van Gemert (2011).
[7] Data obtained from the OPLS-DA analysis.

Pentan-2-one also presented a high odor threshold of 288 mg/kg and a fold change value of 0.370 and therefore its impact on the odor was also expected to be limited. Another compound that also showed a high value of fold change was 2,5-dimethylhexane; however, its odor threshold has not been reported. As shown in Fig. 4**A**, the grassy odor of the recombined oil was slightly higher than that of FO3, which might be caused by hexanal with a fold change of 0.276 and odor threshold of 0.3 mg/kg. Hexanal is well accepted to come from ω-6 fatty acids, specifically from linoleic acid oxidation (Frankel, 2005). Therefore, this was generated during the oxidation of sunflower oil (Cao et al., 2014). Furaneol has a low odor threshold of 0.025–0.05 mg/kg and as a consequence it could be easily perceived. Ethyl propanoate showed a small value of fold change, which was indicative of remarkable differences in ethyl propanoate concentration between the two oils. In fact, it was not detected in the FO3 sample. Thus, these two compounds may be an important factor causing the small difference of odor observed between the recombined oil and FO3. 1-Octen-3-ol and pentanal could also be related to the small odor differences between the oils as their fold change values were 0.299 and 0.311, respectively, and their odor thresholds were as low as 0.9 and 0.24, respectively. Therefore, hexanal, furaneol, ethyl propanoate, 1-octen-3-ol and pentanal appeared to be involved in the odor differences found between the recombinant oil and the fish oil sample.

## 4. Conclusions

This study explicitly confirmed the formation mechanism of fish oil malodor from the perspective of fatty acid oxidation. From the results obtained it can be drawn that the complex development of off-odors in fish oil not only depends on the degradation of DHA and EPA, but also on the concomitant oxidation of other fatty acids, such as oleic and linoleic acids, also degraded to a significant extent. Different proportions of EPA and DHA also play a role in the sensory profile. Recombination of oxidized samples of DHA, EPA and sunflower oil can simulate the odor of fish oil, including fishy smell, by synergistic effects of their oxidation products.

## CRediT authorship contribution statement

**Yun-Qi Wen:** Conceptualization, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Chang-Hu Xue:** Funding acquisition. **Hong-Wei Zhang:** Formal analysis. **Li-Li Xu:** Visualization. **Xiao-Han Wang:** Visualization. **Shi-Jie Bi:** Data curation. **Qian-Qian Xue:** Data curation. **Yong Xue:** Conceptualization. **Zhao-Jie Li:** Conceptualization. **Joaquín Velasco:** Writing – review & editing, Visualization, Supervision. **Xiao-Ming Jiang:** Conceptualization, Supervision, Project administration.

## 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.

## Data availability

Data will be made available on request.

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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.2022.134724.

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