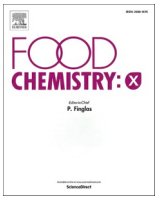
Food Chemistry: X 19 (2023) 100802

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Synthesis of lipoic acid ferulate and evaluation of its ability to preserve fish oil from oxidation during accelerated storage

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ARTICLE INFO

Keywords:
Synthesis
Fish oil
Antioxidant activity
Oxidative stability
Ferulic acid
Lipoic acid

ABSTRACT

Lipoic acid ferulate (LAF) was synthesized and its anti-free radical ability in vitro was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) (ABTS) assays. Protective effects of LAF in stabilizing fish oil were tested, compared to antioxidants such as lipoic acid, ferulic acid and *tert*-butylhydroxyquinone (TBHQ) by measuring peroxide values, thiobarbituric acid reactants, *p*-anisidine values, nuclear magnetic resonance (NMR) spectra and gas chromatography–mass spectrometry (GC–MS) spectra of fish oil during accelerated storage (12 days, 80 °C). The inhibitory effect of these antioxidants on fish oil oxidation followed the order TBHQ ≧ LAF > ferulic acid > lipoic acid. In addition, the omega-3 polyunsaturated fatty acids were the first to be oxidized. The formation of oxidation products followed a first-order kinetic model, and the addition of LAF effectively reduced the reaction rate constants. Therefore, LAF can effectively slow down the formation of oxidative products and prolong the shelf life of fish oil.

1. Introduction

Fish oil usually refers to the oil derived from marine pelagic fish species, such as swordfish, tuna, mackerel, salmon, and so on (Lv et al., 2020). Fish oil is very rich in polyunsaturated fatty acids (PUFAs) such as eicosa pentaenoic acid (EPA) and docosa hexaenoic acid (DHA), which belong to the omega-3 PUFAs. These PUFAs have a range of beneficial effects on human body, such as boosting retinal and brain development in babies and reducing symptoms in patients with cardiovascular disease and Alzheimer's. Furthermore, fish oil is an indispensable source of lipids in animal culture, especially in Marine animals, and plays an important role in maintaining the growth and health of aquatic animals (Albert, Derraik, Cameron-Smith, Hofman, Tumanov, Villas-Boas, et al., 2015; Yu, Ren, Wei, Xing, Xu, Li, et al., 2022). However, it is because of the presence of these PUFAs that fish oil is extremely prone to oxidation and rancidity. The oxidation of long-chain PUFAs involves a number of complex chemical reactions, including the breakdown of fatty acid chains and the formation of various smaller molecules (Liang et al., 2018). The oxidation products, such as peroxides, alcohols, aldehydes, carboxylic acids and their corresponding esterification products, can cause rancid odors and unpleasant flavours, color changes, and reduced nutritional value (Agregan et al., 2017; Bondoc & Şindilar, 2002). Hence, oxidation is closely related to the nutritional value and safety of fish oil, and preserve fish oil is still a problem to be solved (Bondoc, 2016). Adding antioxidants is an important means of preserving lipids. Antioxidants can delay or inhibit the occurrence of lipid oxidation by efficiently chelating metal ions, trapping and neutralizing free radicals, quenching singlet oxygen, removing oxygen, etc (Agregan et al., 2017; Huang et al., 2017). A variety of effective synthetic antioxidants have been widely used such as TBHQ, butyl hydroxy anisd (BHA), 2, 6-di-*tert*-butyl-4-methylphenol (BHT) and gallic acid propyl ester (PG), and they can effectively protect PUFAs from oxidative deterioration.

Ferulic acid is a hydroxycinnamic acid originally isolated from *Ferula foetida*, which is present in large quantities in agricultural waste (Dulong et al., 2018). Ferulic acid owns strong antioxidant, antibacterial, antithrombosis, and anti-cancer activities and is widely used as an antioxidant for fats in food industries (Amic et al., 2020). As a multipotent

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https://doi.org/10.1016/j.fochx.2023.100802
Received 7 April 2023; Received in revised form 2 July 2023; Accepted 17 July 2023
Available online 26 July 2023

antioxidant, ferulic acid can act as a free radical scavenger due to its large conjugated structure and multiple unsaturated bonds (Singh et al., 2021). However, the poor lipophilicity of ferulic acid is the main limiting factor for its wide application (Nicks et al., 2012). Recently, improving the lipophilicity by modifying the functional groups of ferulic acid has aroused great interest of researchers (Wu et al., 2020). Furthermore, it has been observed that modified ferulic acid derivatives such as ferulic paeonol ester, triterpene alcohol monoesters and alkyl ferulate have higher antioxidant activity than the acid itself (Chigorimbo-Murefu, Riva, & Burton, 2009; Pellerito et al., 2020; Yu, et al., 2021).

As a natural compound that can be derived from plants or animals, lipoic acid is also called alpha thioctic acid or 1,2-dithiolane-3-pentanoicacid, which was discovered by Snell in 1937 and isolated from potato's extract by Reed in 1951 (Moeinian et al., 2019). Because of the disulfide five membered ring structure with high electron density, lipoic acid can directly react with free radicals, thereby having high antioxidant capacity (Xiang et al., 2019; Ma et al., 2020; Moeinian et al., 2019). Moreover, lipoic acid is hydrophobic and soluble in organic solvents such as chloroform, ethanol, petroleum ether and acetonitrile, however, the instability against oxidation and thermal process hinder its application (Saliq et al., 2020). Interestingly, the drawbacks could be overcome by molecular combinations obtained by joining two biologically active molecules (Kaki, Grey, & Adlercreutz, 2012). In fact, in order to produce new molecules with a wide range of applications, a larger number of studies related to modifications of natural compounds emerges (Kaki, Grey, & Adlercreutz, 2012). Besides, it has been reported that the combination of two natural biologically active molecules is highly likely to produce hybrid molecules with enhanced biologically activities (Melagraki et al., 2009).

Inspired by the studies described above, we synthesized LAF (Fig. 1). The lipophilicity of LAF was significantly higher than that of ferulic acid, and it was chemically more stable than both ferulic acid and lipoic acid, thus, LAF may have higher antioxidant activity and a wider range of applications. To evaluate the antioxidant capacity of LAF in vitro, the free radical resistance of TBHQ, ferulic acid, lipoic acid and LAF were evaluated by DPPH and ABTS assay. Further, the oxidative stability of fish oil before and after addition of these compounds during accelerated storage up to 12 days at 80 °C under dark were also investigated. The oxidation of fish oil was monitored by peroxide values (POV), thiobarbituric acid reactive substance (TBARS), *para*-anisidine values (*p*AV), GC–MS and NMR spectra assay.

2. Materials and methods

2.1. Materials

Fish oil was purchased from Zhejiang Xinglong Ma Industry Co., Ltd. (Zhejiang, China). Ferulic acid and lipoic acid were purchased from Shanghai Maclin Biochemical Technology Co., Ltd. (Shanghai). DPPH and ABTS with purity not <98% were obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). All other analytical or spectroscopical pure chemicals are readily available commercially. The synthesized compounds were purified by silica gel columns (Merck, Kieselgel 60, 70–230 mesh) with ethyl acetate/petroleum ether as an eluent. The synthesized compounds' NMR spectra operating at 300 MHz were acquired on a Bruker spectrometer (DPX 300, USA) using $CDCl_3$ as solvent. Infrared spectrum (IR) spectra measured by a potassium bromide method were recorded on a BIORAD Tensor 27 spectrometer with 0.5 cm^{-1} resolution (Brucker Optics, Germany).

2.2. Synthesis of LAF

LAF synthesis was carried out as follows: in a round-bottomed flask, ferulic acid (3.0 g, 0.016 mol), lipoic acid (3.18 g, 0.016 mol), EDC·HCl (7.11 g, 0.036 mol) and DMAP (4-dimethylaminopyridine) (0.30 g, 0.0025 mol) were mixed with 100 mL dichloromethane. The resulting reaction mixture was a bright yellow color and stirred at room temperature for 4 h, and then a lot of ice-water was added when the reaction was completed. The resulting mixture was extracted with ether (3 × 500 mL), and the ether layers obtained by extraction were combined and immediately washed with water (2 × 200 mL) before drying with anhydrous $MgSO_4$ for 24 h. After solvent recovery using a rotary evaporator, the resulting residue was carefully purified by silica gel column chromatography with a mixture of ethyl acetate and petroleum as eluent to obtain LAF (3.96 g, yield 67%, content > 98%).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 16 Hz, 1H), 7.18–7.15 (m, 2H), 7.08 (d, J = 8 Hz, 1H), 6.42 (d, J = 16 Hz, 1H), 3.89 (s, 3H), 3.63–3.61 (m, 1H), 3.21–3.15 (m, 2H), 2.64–2.53 (m, 2H), 2.52–2.48 (m, 1H), 1.98–1.93 (m, 1H), 1.85–1.74 (m, 4H), 1.66–1.57 (m, 2H); ^{13}CNMR ($CDCl_3$, 100 MHz) δ 172.04, 171.27, 151.47, 146.35, 141.86, 132.93, 123.34, 121.64, 117.64, 111.43, 56.38, 55.96, 40.27, 38.51, 34.62, 33.77, 28.66, 24.70; IR (cm^{-1}) 1679 (C═C), 1755 (O═C-O), 1421 (Ph), 1506 (Ph), 1620 (Ph), 2931 (C—C).

2.3. DPPH and ABTS radical scavenging activity assay

TBHQ, ferulic acid, lipoic acid and LAF were separately dissolved in ethanol/N,*N*-dimethylformamide (DMF) and diluted to 25, 50, 75, 100 and 125 μM. DPPH radical scavenging capacity of the resulting solutions with different concentrations of antioxidants was measured according to the method described in the previous literature (Liu et al., 2018). Briefly, different concentrations of samples (2 mL) were added to 2 mL of DPPH• ethanol solution (500 μM). The centrifuge tubes containing the mixtures were shaken by hand for one minute and kept at room temperature (20 °C) for 30 min in the dark. The absorbance of the yellow mixture was then immediately determined at 517 nm.

The ABTS assay was conducted by using the method described previously (Liu et al., 2018; Zheng et al., 2016). Briefly, the ABTS aqueous solution (7 mM, 100 mL) was mixed with potassium persulfate (2.45 mM, 100 mL) to generate ABTS radical cation. The mixture was immediately placed in darkness at room temperature for 12–16 h to obtain a dark green solution, which was then diluted with anhydrous ethanol to a light green color with an absorbance of 0.7 ± 0.02 at 734 nm. Then, 600 μL of different concentrations of sample solutions were allowed to react with 5 mL of ABTS solution for 10 min at 37 °C in the

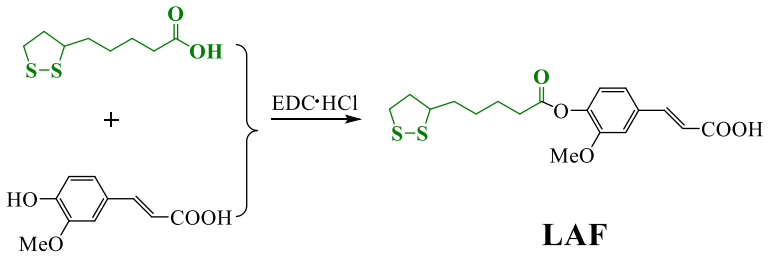

Fig. 1. Synthetic route of LAF.

dark. After the reaction, the absorbance of the resulting mixture at 734 nm was measured. All determinations were carried out in triplicate.

2.4. Evaluation of oxidative stability

2.4.1. Sample preparation

TBHQ (0.0499 g), ferulic acid (0.0583 g), lipoic acid (0.0619 g) and LAF (0.1147 g) were separately mixed with 5 mL of DMF. The resulting solutions and 5 mL of DMF without any antioxidant addition (for control) were separately added to the fresh fish oil (300 g). The final concentration of the four compounds was 1.0 mmol kg^{-1} fish oil, respectively. After 30 min of ultrasonic mixing at room temperature, 10 mL of each mixture was placed in polypropylene centrifuge tubes (sealed) and then stored in an oven at 80 °C for 12 days in dark, with sampling taken every 2 days. All the fish oil mixture were prepared in triplicate.

2.4.2. POV

POV assay of all samples was measured according to GB/T 5538-2005/ISO 3960:2001 with a slight modification (Zhang et al., 2010). Briefly, the fish oil sample (0.5 g) was dissolved in 50 mL of acetic acid: isooctane (3:2, v/v), and then 0.5 mL saturated solution of KI and 0.5 mL of starch indicator (0.05%) solution were added by a pipette. The resulting solution was quickly shaken in one direction by hand for 1 min. 30 mL distilled water was then poured into the dark liquid, and the resulting mixture was shaken again and titrated against sodium thiosulfate (0.01 M) until the bule color just disappeared. POV is expressed as mg equivalent of active oxygen per kg of fish oil.

2.4.3. TBARS

TBARS assay was performed according to the previous methods with slight modifications (Agregan et al., 2017; Huber et al., 2009). In brief, 0.375 g of thiobarbituric acid and 15 g of trichloroacetic acid were weighed into a beaker, followed by hydrochloric acid (0.25 M, 1000 mL), and the mixture was dissolved ultrasonic to obtain TBA working liquid. The fish oil sample (0.1 g) was added to 5 mL TBA solution and the mixture was heated in a water bath (100 °C) for 30 min for pink color development. The pink reaction mixture was cooled and centrifuged to obtain the supernatant, whose absorbance was then detected at 532 nm using a spectrophotometer (V-1100, Shanghai, China). The TBARS content was calculated from a standard curve prepared by using malonaldehyde and expressed as mg of MAD equivalents/kg sample.

2.4.4. pAV

*p*AV was determined to follow the formation of secondary oxidation products according to the previous reports (Liu et al., 2020; Umeda & Jorge, 2021). Previously weighed samples (0.1 g) were dissolved in 25 mL of isooctane, and their initial absorbance measurements were subsequently carried out in a spectrophotometer at 350 nm. Then, 1 mL of *p*-anisidine solution (0.25%, w/v) was added into 5 mL of isooctane liquid under test. The evenly mixed mixture was stored away from light for 10 min at ambient temperature, after which the final absorbance was subsequently measured and read.

2.4.5. NMR spectra

A 35 mg LAF-containing fish oil sample was mixed in a 5 mm diameter tube with 500 μL $CDCl_3$, which contained a small proportion (0.2%) of non-deuterated chloroform, and 0.03% of tetramethylsilane (TMS) generally used as a reference compound to calibrate chemical shift at 0.0 ppm (Aladdin, Shanghai, China). The ^{1}H NMR and ^{13}C NMR spectra of the fish oil samples with $CDCl_3$ as solvent were recorded on a Bruker DPX 300 spectrometer operating at the frequency of 300 MHz and 100 MHz. The NMR spectra given in the study were processed using MestreNova programme (Mestrelab Research, Santiago de Compostela, Spain).

2.4.6. Fatty acid composition

The fatty acid compositions of fish oil samples to be tested were determined by GC–MS after derivating fatty acids into fatty acid methyl esters (FAMEs) according to the previous reports (Albert et al., 2015; Lepage & Roy, 1986; Yu et al., 2021).

A Thermo Trace1300 gas chromatograph-ISQ7000 mass spectrometer (GC–MS, New York, USA) equipped with a fused silica DB-5 column (60 m long, 0.25 mm i.d. and 0.25 μm film thickness) was used for the separation and quantitative detection of the FAMEs. Helium with a flow rate of 1.5 mL min^{-1} was used as the carrier gas in GC–MS. The column temperature program was as follows: 140 °C for 5 min, heated up to 180 °C at 10 °C min^{-1}, followed by heating up to 210 °C at 2 °C min^{-1}, and finally ramped up to 260 °C at 10 °C min^{-1} and held for 10 min. The detector temperature was 260 °C, the sample size was 1 μL, and the separation ratio was 30:1. The mass spectrum was acquired at 70 eV in the electron impact (EI) mode at m/z 33–550. Chromeleon7.0 software was employed to analyze mass spectra and chromatograms. The data is first sorted using the NIST 17 database, summarized and then edited in the Excel 2016 software. The concentrations of all FAMEs were calculated based on the retention time, concentration and peak area of the inner target.

2.5. Statistical analysis

All the analytical tests were carried out in triplicate and data (except NMR pictures in the test) were reported as a mean value with its standard deviation (mean ± SD). An ANOVA test was used to compare the mean values and significant differences of parameters determined using Tukey's multiple comparison test ($P < 0.05$). All statistical analyses were performed by SPSS 18 statistical software (Chicago, IL, USA) software.

3. Results and discussion

3.1. DPPH and ABTS radical scavenging activity assay

The scavenging activities of TBHQ, ferulic acid, lipoic acid and LAF against DPPH and ABTS free radicals are displayed in Fig. 2. In detail, TBHQ, ferulic acid and lipoic acid showed considerable scavenging abilities of DPPH and ABTS free radical in a concentration-dependent manner (Fig. 2a). Besides, TBHQ owned the highest anti-DPPH and anti-ABTS activity, followed by ferulic acid, lipoic acid and LAF. Both methods clearly indicated that TBHQ, ferulic acid and lipoic acid possessed considerable antioxidant activity. However, LAF exhibited very poor quenching activity against the DPPH and ABTS free radicals.

Generally speaking, (poly) phenolic compounds can get rid of free radicals through HAT (H atom transfer), SET-PT (single electron transfer-proton transfer), and SPLET (sequential proton loss electron transfer) mechanisms (Thuy et al., 2020). The number and location of the hydroxyl and electron donating groups have an effect on the H atom transfer, electron transfer, or deprotonation ability of –OH, which in turn affects the ability of compounds to scavenge free radical (Agregan et al., 2017). Two hydroxyl groups in TBHQ may reduce the O—H bond dissociation enthalpies (HAT mechanism) to a greater extent, and as a result, the process of direct hydrogen transfer to a radical was further facilitated. Therefore, TBHQ exerted stronger DPPH and ABTS radical scavenging activity than ferulic acid. Ferulic acid exhibited stronger radical scavenging activities than lipoic acid, which is mainly due to the presence of phenol structure. Moreover, the esterification of lipoic acid with ferulic acid may reduce the hydrogen-donating or electron transfer ability, resulting in LAF owning the weakest scavenging activity. The esterification results in the loss of the hydrogen atom from the hydroxyl group, which reduces the hydrogen-donating ability. Alternatively, the esterification may increase ionization potential, resulting in a decreased electron transfer capacity.

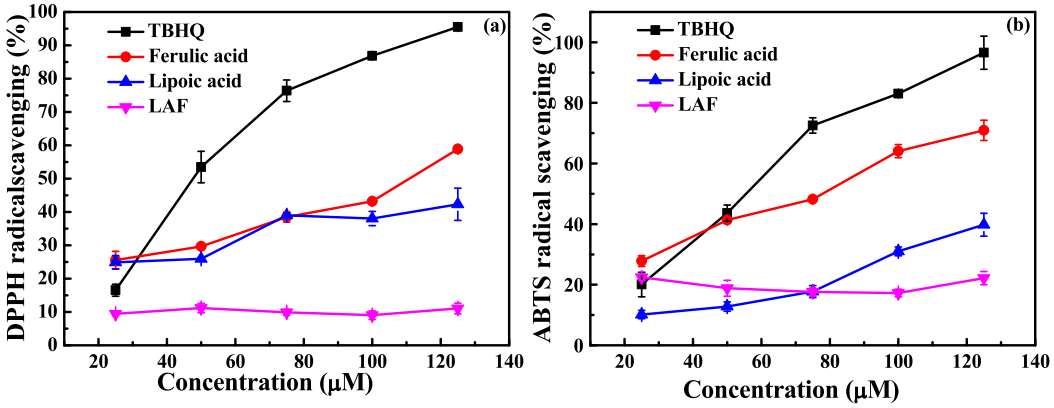

Fig. 2. Scavenging effects on DPPH (a) and ABTS (b) radical of TBHQ, ferulic acid, lipoic acid and LAF.

3.2. Stability evaluation of the test compounds on fish oil during accelerated storage

In general, POV is a quantitative index of primary oxidation products, while TBARS and *p*AV reflect the amount of secondary oxidation products (Zhang et al., 2019). The POV, TBARS and *p*AV values of the fish oil samples with and without antioxidants during the accelerated storage at 80 °C were shown in Fig. 3. The POV, TBARS and *p*AV values were well correlated with storage time, and they increased rapidly with the increase of heat treatment time ($P < 0.05$). In particular, the increase of these values accelerated after the fourth day. During the entire period of the accelerated storage, the POV, TBARS and *p*AV values of the samples reduced in the following order: control > lipoic acid > ferulic acid > LAF ≥ TBHQ, which demonstrated that the four antioxidants were effective in inhibiting the degradation of fish oil to produce primary and secondary oxidation products. In other words, TBHQ and LAF

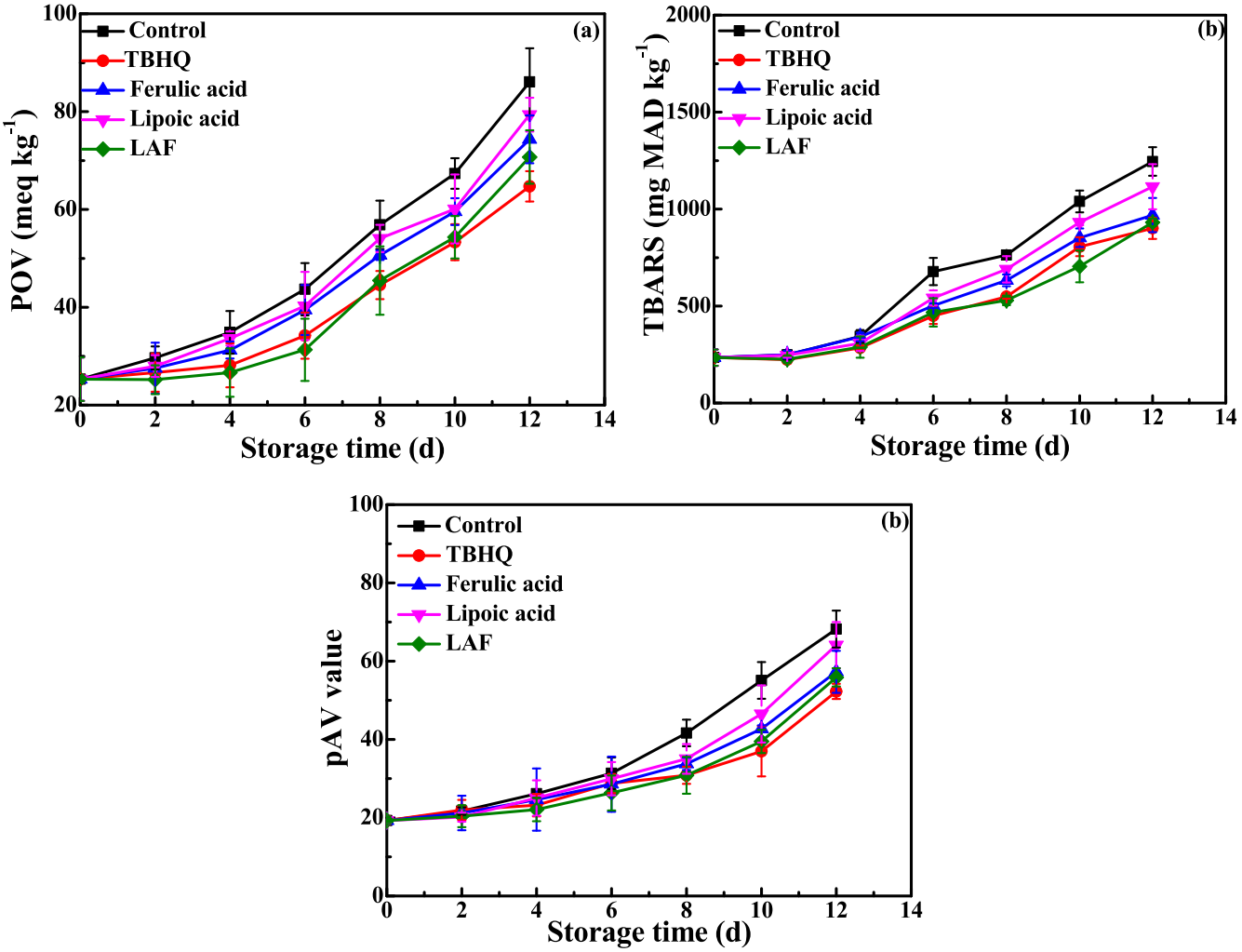

Fig. 3. The influence of TBHQ, ferulic acid, lipoic acid and LAF on POV (a), TBARS (b) and *p*AV (c) of fish oil samples during the accelerated storage at 80 °C for 12 days.

owned the strongest antioxidant capacity, followed by ferulic acid and lipoic acid. As we know, the antioxidant activity of phenolic compounds is proportional to the number of phenolic hydroxyl groups when the number is less than four (Chen et al., 2020). Therefore, the antioxidant capacity of TBHQ was stronger than that of ferulic acid. The weakest antioxidant capacity of lipoic acid might be due to the higher dissociation enthalpy and ionization potential of S—S or S—H bond. However, LAF showed comparable antioxidant activity to TBHQ, which was primarily ascribed to the increased lipophilicity and stability aroused by esterified phenolic hydroxyl in LAF. Similarly, it was also reported that phenolic compounds with modified functional groups had stronger antioxidant effect than themselves (Higgins et al., 2020; Yu et al., 2021). The results suggest that LAF provided effective protection against lipid oxidation, and LAF, like TBHQ, was an excellent lipid antioxidant.

Kinetic studies of lipid oxidation play an important role in understanding the degradation mechanism of the fish oil samples. Reactions rates of lipid oxidations are usually described by zero-order or first-order reaction (Rodrigues et al., 2017; Yeşilsu & Özyurt, 2019). Kinetic lines of zero-order reaction were drawn by plotting the changes of POV, TBARS or *p*AV versus time, while those of first-order reaction were drawn by plotting the logarithm of the changes of POV, TBARS or *p*AV versus time. Reaction rate constants (*k*) were determined from the slope of the kinetic lines. Table 1 lists *k* values together with the quality of the fitting. As depicted in Table 1, the addition of antioxidants to fish oil effectively reduced the reaction rate constants, and the smallest rate constants were observed in fish oil samples with TBHQ and LAF, which confirmed the good antioxidant capacity of TBHQ and LAF. Besides, the better fitting for the natural logarithm of the concentration of POV, TBARS or *p*AV was obtained for a first-order reaction. From the *k* values, it can be observed that malonaldehyde (TBARS values) was formed faster than 2-alkenals and 2,4-dienals (*p*AV values). Large amounts of malonaldehyde were mainly formed from PUFAs containing three or more double bonds (Solaesa et al., 2018). Therefore, the formation of oxidation products in fish oil followed a first-order kinetic model, and malonaldehyde was formed more rapidly during fish oil oxidation.

Table 1
Kinetic rate constant (*k*) and R^2 of fish oil samples.

n (order of reaction)	Samples	POV		TBARS		*p*AV	
		k[a]	R^2	k[a]	R^2	k[a]	R^2
Zero-order	Control	4.72 ± 0.38	0.963	84.34 ± 7.59	0.953	2.91 ± 0.55	0.818
	TBHQ	3.50 ± 0.42	0.921	59.22 ± 6.98	0.922	2.36 ± 0.34	0.887
	Ferulic acid	4.31 ± 0.41	0.949	63.99 ± 6.64	0.946	2.32 ± 0.18	0.965
	Lipoic acid	4.79 ± 0.47	0.946	83.50 ± 4.83	0.980	2.45 ± 0.54	0.764
	LAF	3.68 ± 0.56	0.876	65.92 ± 6.12	0.951	2.59 ± 0.38	0.886
First-order	Control	0.105 ± 0.003	0.996	0.160 ± 0.014	0.954	0.110 ± 0.008	0.973
	TBHQ	0.087 ± 0.006	0.978	0.130 ± 0.014	0.942	0.083 ± 0.006	0.965
	Ferulic acid	0.103 ± 0.006	0.978	0.140 ± 0.010	0.971	0.082 ± 0.005	0.980
	Lipoic acid	0.103 ± 0.005	0.984	0.152 ± 0.012	0.965	0.095 ± 0.009	0.952
	LAF	0.091 ± 0.011	0.912	0.128 ± 0.011	0.961	0.078 ± 0.007	0.952

[a] Units of k for POV (meq·kg^{-1}·h^{-1}); for TBARS ((mg MAD·kg^{-1}·h^{-1}) and for *p*AV (h^{-1}).

3.3. NMR spectra of fish oil with LAF

In terms of fish oil analysis, ^{1}H NMR and ^{13}C NMR spectroscopy are often utilized by numerous researchers for determining fatty acid profiles. In this study, the oxidation process of the fish oil containing LAF in accelerated storage condition was monitored at day 0 and day 12 by ^{1}H NMR and ^{13}C NMR spectroscopy. As shown in Fig. 4 and Fig. S1, the ^{1}H NMR spectra of samples at different storage times are similar to the spectrum of the initial fish oil. Since the LAF content was much lower than the fish oil content, the characteristic peaks of LAF were covered by those of the fish oil (Tan et al., 2017). The peaks at δ 0.83–1.03 ppm, δ 1.14–1.43 ppm, δ 1.54–1.74 ppm, δ 1.82–2.12 ppm, δ 2.25–2.36 ppm, δ 2.36–2.45 ppm, δ 2.75–2.90 ppm, δ 4.10–4.39 ppm, δ 5.21–5.31 ppm, δ 5.31–5.51 ppm, are respectively related to -CH_3, -$(CH_2)_n$-, -OCO-CH_2-CH_2-, -CH_2-CHCH-, -OCO-CH_2-, -OCO-CH_2-CH_2-, ═HC-CH_2-CH═, -CH_2-OCOR, -CHOCOR- and -CH═CH-. Accurately, the triplet (A) at δ 0.83–0.95 ppm is classified as terminal methyl group from SA (saturated fatty acid), MUFA (monounsaturated fatty acids) and omega-6 PUFA; the triplet (B) at δ 0.95–1.00 ppm corresponds to the terminal methyl groups characteristic of omega-3 PUFA; the doublet (C) at δ 1.14–1.43 ppm belongs to methylene group from SA, MUFA and omega-6 PUFA; the multiplet (J) at δ 2.75–2.90 ppm corresponds to the bis-allylic hydrogens, and the multiplet (L) at δ 5.20–5.30 ppm belongs to the *sn*-2 hydrogen from the glycerol backbone of triacylglycerols. It had been reported that the oxidation of omega-3 PUFA resulted in the reduced intensities of peaks B and J, whereas the intensity of peak L was not affected (Tan et al., 2017). According to Fig. 4., the peak intensities A, B, C and J (IA, IB, IC, and IJ) of the sample on day 12 significantly decreased compared to those on day 0, but the peak intensity L (IL) showed no change. Exactly, the peak intensity ratio IA/IL, IB/IL, IC/IL, and IJ/IL on day 12 decreased 4.29%, 8.80%, 1.22% and 3.53%, respectively. The results demonstrated that PUFAs, especially omega-3 PUFAs, were reduced in fish oil after 12 days of high temperature treatment.

As shown in Fig. 4, Fig. S2 and Table S1, the reduction of the peak intensity at position *sn*-2 was significantly larger than that at positions *sn*-1,3 in the carbonyl spectral region. The result reveals that fatty acids located at position *sn*-2 were less resistant to thermal oxidation, which is consistent with previous findings (Medina et al., 1998; Medina, Sacchi, & Aubourg, 1995). The peak intensities in the olefinic regions of the spectra of the sample on day 12 were significantly decreased than those on day 0. In particular, the intensity of C_4 and C_{19} peak of DHA was decreased by 10.99% and 8.37%, respectively, while that of C_6 of EPA was reduced by 6.42%, indicating that the double bonds nearest to the carbonyl group were oxidized first, followed by the unsaturation closest to the methyl terminus. Similar to the olefinic regions, the peak intensities in methylenic region also showed significant decrease, and the peaks with the most significant reduction in relative peak intensity belonged to C_6 of 22:5n-3 (32.56%), C_4 of EPA (16.67%) and ω2 of all n-3 PUFA (9.52%). This result confirmed that double bonds closest to the carbonyl group positions degraded faster during high temperature oxidation. The changes of relative peak intensities in the methyl region suggested that omega-3 PUFAs were more sensitive to heat than omega-6 fatty acids and all other fatty acids. Considering the relative decrease of the carbonyl, olefinic, methylenic and methyl resonances, fatty acids located at position *sn*-2 and unsaturation nearest to the carbonyl group were first oxidized in heat treatment. Further, omega-3 PUFAs were more susceptible to oxidation than others, which was consistent with the ^{1}H NMR results.

3.4. GC–MS analysis

Based on the above analysis, LAF and TBHQ have similar antioxidant activities, which are stronger than lipoic acid and ferulic acid. The fish oil samples stored at 80 °C on days 0 and 12 showed the greatest differences in POV, TBARS, *p*AV and NMR spectra. Thus, a total of 34 fatty

Fig. 4. The influence of LAF on ^{1}H NMR (top) and ^{13}C NMR (bottom) of fish oil samples during the accelerated storage at 80 °C for 0 day (1) and 12 days (2).

acids were identified by GC–MS analysis in Control (0) (the initial fish oil), Control (12) (the control fish oil stored at 80 °C for 12 days) as well as LAF (12) (the fish oil with LAF stored at 80 °C for 12 days). As presented in Table 2, the total fatty acid composition of the three samples showed significant differences. The contents of PUFAs (C22:6, C22:5, C22:4, C22:2, C20:5, C20:4, C20:3, C20:2, C18:3 and C18:2), MUFAs (C22:1, C20:1, C19:1, C18:1, C17:1, C16:1, C15:1 and C14:1) and SFAs (C24:0, C23:0, C22:0, C21:0, C20:0, C18:0, C17:0, C16:0, C15:0, C14:0, C13:0, C12:0, C11:0, C10:0 and C8:0) in Control (12) were significant lower than those in Control (0). Fortunately, the addition of LAF

Table 2
Total fatty acid composition of the fish oil samples.

Fatty acid (mg mL^{-1})	Fish oil samples (storage days)		
	Control (0)	Control (12)	LAF (12)
C8:0	0.026 ± 0.003^{b}	0.019 ± 0.002^{a}	0.023 ± 0.002^{ab}
C10:0	0.076 ± 0.004^{c}	0.054 ± 0.001^{a}	0.055 ± 0.003^{a}
C11:0	0.030 ± 0.002^{b}	0.025 ± 0.002^{a}	0.028 ± 0.003^{ab}
C12:0	1.296 ± 0.115^{c}	0.875 ± 0.081^{a}	1.081 ± 0.050^{b}
C13:0	0.926 ± 0.039^{c}	0.626 ± 0.016^{a}	0.684 ± 0.061^{ab}
C14:0	2.070 ± 1.254^{c}	1.401 ± 1.185^{a}	1.745 ± 1.112^{b}
C14:1	0.662 ± 0.045^{c}	0.431 ± 0.026^{a}	0.485 ± 0.049^{ab}
C15:0	10.769 ± 1.043^{c}	7.508 ± 0.584^{a}	8.654 ± 0.818^{ab}
C15:1	0.045 ± 0.004^{b}	0.034 ± 0.004^{a}	0.037 ± 0.003^{ab}
C16:0	137.965 ± 11.996^{c}	81.775 ± 4.921^{a}	109.464 ± 8.178^{b}
C16:1	18.177 ± 1.024^{c}	10.399 ± 0.576^{a}	14.128 ± 1.203^{b}
C17:0	8.884 ± 0.369^{b}	7.560 ± 0.223^{a}	8.393 ± 0.791^{ab}
C17:1	1.902 ± 0.113^{c}	1.578 ± 0.084^{a}	1.687 ± 0.069^{ab}
C18:0	1.657 ± 0.161^{c}	0.847 ± 0.042^{a}	1.247 ± 0.124^{b}
C18:1	236.417 ± 19.672^{c}	137.897 ± 10.571^{a}	198.765 ± 12.907^{b}
C18:2	38.718 ± 1.484^{c}	19.738 ± 1.486^{a}	33.302 ± 2.844^{b}
C18:3	8.466 ± 0.413^{c}	6.364 ± 0.173^{a}	7.471 ± 0.450^{b}
C19:1	0.525 ± 0.028^{c}	0.295 ± 0.015^{a}	0.369 ± 0.014^{b}
C20:0	7.716 ± 0.554^{b}	6.162 ± 0.143^{a}	6.903 ± 0.462^{ab}
C20:1	44.737 ± 3.651^{c}	31.870 ± 2.796^{a}	37.779 ± 3.326^{ab}
C20:2	3.135 ± 0.115^{b}	2.565 ± 0.014^{a}	2.902 ± 0.170^{b}
C20:3	4.453 ± 0.407^{c}	2.031 ± 0.163^{a}	3.196 ± 0.297^{b}
C20:4	20.170 ± 1.402^{b}	12.517 ± 1.052^{a}	18.403 ± 1.880^{b}
C20:5	133.477 ± 12.236^{c}	69.780 ± 5.479^{a}	106.490 ± 6.660^{b}
C21:0	1.399 ± 0.137^{b}	0.914 ± 0.054^{a}	1.075 ± 0.103^{b}
C22:0	3.357 ± 0.220^{b}	2.788 ± 0.107^{a}	3.173 ± 0.248^{ab}
C22:1	5.590 ± 0.262^{c}	3.246 ± 0.116^{a}	4.476 ± 0.288^{b}
C22:2	15.856 ± 1.408^{c}	5.919 ± 0.527^{a}	9.863 ± 0.893^{b}
C22:4	19.774 ± 0.615^{b}	16.155 ± 0.569^{a}	18.000 ± 1.475^{ab}
C22:5	11.315 ± 0.775^{c}	5.616 ± 0.414^{a}	8.680 ± 0.583^{b}
C22:6	199.458 ± 11.780^{c}	87.230 ± 6.706^{a}	172.248 ± 9.426^{b}
C23:0	0.556 ± 0.027^{b}	0.470 ± 0.017^{a}	0.564 ± 0.051^{b}
C24:0	7.177 ± 0.694	6.142 ± 0.327	6.931 ± 0.387
C24:1	1.412 ± 0.137^{b}	1.080 ± 0.112^{a}	1.229 ± 0.100^{ab}
ΣPUFA	454.824 ± 18.840^{c}	227.915 ± 8.065^{a}	380.554 ± 14.729^{b}
ΣMUFA	309.467 ± 23.438^{c}	186.830 ± 12.277^{a}	258.955 ± 11.612^{b}
ΣSFA	202.129 ± 14.838^{c}	129.777 ± 5.229^{a}	165.721 ± 5.919^{b}

Mean values in the same line with different superscript letters are significantly different ($P < 0.05$). Control (0) means the initial fish oil sample; Control (12) means the control sample stored at 80 °C for 12 days; LAF (12) means the control sample with LAF stored at 80 °C for 12 days.

effectively suppressed the reduction of all fatty acid contents, especially the omega-3 PUFA contents. Similar changes occurred in the content of ΣPUFA, ΣMUFA and ΣSFA in Control (0), Control (12) and LAF (12). Under accelerated storage conditions, the cleavage of C═C in long-chain fatty acids led to the release of low-boiling fatty acids and the formation of primary and secondary oxidation products, reducing the PUFA, MUFA, and SFA content per unit volume of fish oil (Miyashita, Uemura, & Hosokawa, 2018). Similar results were reported in previous studies on fish oil oxidation under high temperature treatments (Wang et al., 2011; Yu et al., 2021). Therefore, it is so easy to understand why the POV, TBARS and *p*AV values increased significantly with the prolongation of storage time in the previous section. Further, LAF may effectively reduce chain breakages and increase chain terminations, thereby playing an important role in the prevention and termination of lipid oxidation processes (Olmedo, Ribotta, & Grosso, 2019).

4. Conclusions

In summary, new compound LAF was synthesized and characterized, and its antioxidant capacity was evaluated by free radical scavenging and fish oil thermal stability test. In fish oil, the formation of oxidation products followed a first-order kinetic model, and malonaldehyde was formed more rapidly. Omega-3 PUFAs were more susceptible to oxidation than others, fatty acids located at position *sn*-2 and unsaturation nearest to the carbonyl group were first oxidized. The inclusion of LAF effectively suppressed the reduction of the omega-3 PUFA contents. The results demonstrated that similar to TBHQ, LAF can effectively inhibit the oxidative rancidity of fish oil during the whole process of high temperature storage. The inhibitory effect on oxidation followed the order TBHQ ≧ LAF > ferulic acid > lipoic acid. The synthesized LAF could be used as an efficient antioxidant to delay oxidation reactions, although further studies are necessary to evaluate its other biological activities except for antioxidant activities.

CRediT authorship contribution statement

Zhiyong Xue: Methodology, Investigation, Writing – review & editing. **Juan Liu:** Formal analysis. **Qing Li:** Writing – original draft. **Yuanyuan Yao:** Conceptualization. **Yalin Yang:** Writing – original draft. **Chao Ran:** Formal analysis, Resources. **Zhen Zhang:** Conceptualization, Visualization. **Zhigang Zhou:** Conceptualization, Writing – review & editing.

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.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (NSFC 32061133004, 31925038, 21602163 and U21A20267) and Hubei Key Laboratory of Biomass Fibers & Eco-Dyeing & Finishing (Wuhan Textile University), No. STRZ201907.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100802.

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