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Lipase-catalyzed preparation, bioavailability and functional properties of a DHA-enriched tuna oil

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ABSTRACT

Docosahexaenoic acid (DHA) in the form of triacylglycerols (TAG) are widely recognized for their health benefits. As naturally occurring DHA in the form of TAG are limited, this work developed and optimized a *Candida rugosa* lipase-catalyzed selective hydrolysis process to enrich DHA in tuna oil. The DHA-enriched tuna oil produced at optimal conditions (lipase concentration of 4 %, ratio of water to tuna oil of 1:3, reaction temperature of 55°C) contained 46.12 % of DHA, in which 55.24 % of DHA was located in *sn*-2 position of the glycerides. *In-vitro* bioavailability test shows, the DHA-enriched tuna oil (7.89 %) had significantly higher cellular uptake of DHA as compared to unprocessed oil. The DHA-enriched oil demonstrated increased intracellular oxidation and mitochondrial damage of Caco-2 cell.

1. Introduction

Omega-3 polyunsaturated fatty acids (n-3 PUFA), such as docosahexaenoic acid (DHA), are widely recognized for their health benefits. It is a carboxylic acid with a 22 - carbon chain and six cis double bonds in the DHA hydrocarbon chain (Fig. 1(A))(Chen et al., 2019). Numerous epidemiological and clinical studies have shown that DHA can reduce the incidence of cardiovascular disease, Alzheimer's disease and type 2 diabetes (Patel et al., 2021). In addition, DHA supplementation has been shown to have pro-apoptotic behavior. DHA regulate the outer membrane potential of cytosolic mitochondria in breast cancer cells resulting in up - regulation of *caspase 9* (pro-apoptotic genes) and reduction of tumors' growth (Newell et al., 2019). In another research, DHA supplementation was found to increase malondialdehyde and reactive oxygen species in Caco-2 cells which lead to protein misfolding, endoplasmic reticulum stress, cellular autophagy and cell apoptosis (Pettersen et al., 2016).

As biosynthesis of DHA from alpha - linolenic acid (ALA, 18:3 ω - 3) in human body occurs at a slow rate (1.5 nmol g^{-1} d^{-1}) (Wen et al.), it is recommended that adult obtain intake of about 0.1 g of DHA per day (Efsa, 2010). DHA supplements usually present in three forms, namely triglycerides (TAG), free fatty acids (FFA), and ethyl esters (EE)(Zhang et al., 2022). DHA-EE is the most common commercially available supplement; nevertheless, low hydrolysis rates of DHA-EE by pancreatic lipase raises concerns about their metabolic efficiency and lymphatic transport in the body (Jiang et al., 2022; Jin et al., 2022). In contrast, TAG which are naturally occurring forms of DHA have been reported to be highly bioavailable (Yang et al., 2020).

Fish oil is a natural source of DHA in the form of TAG. Tuna oil contains 18.7–26.8 % of DHA (Sun et al., 2023; Xia et al., 2020; Zhang et al., 2017). Studies have been done to isolate, purify and/or enrich DHA in fish oil in order to transform it into functional food ingredient or pharmacologically active compound. Zhang et al. used low-temperature crystallisation to concentrate DHA in tuna oil (DHA >39.8 %). However, the crystallisation process took more than 12 h and involved the usage of organic solvents such as acetone, hexane and acetonitrile (Zhang et al., 2017). Separation and purification of EPA and DHA by high performance liquid chromatography has also been extensively reported but is not applicable to large-scale industrial production (Karrar et al., 2023). Urea complexation is another method used to concentrate DHA but

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challenges exist in terms of low recovery rate and residual solvents (Karrar et al., 2023). In contrast, lipase-catalyzed partial hydrolysis is a good way to produce DHA-enriched oil. This method produced high yield of DHA in the form of glycerides without the usage of organic solvents (Chen et al., 2023).

This study aims to develop and optimize an enzymatic method to produce DHA-enriched oil using low concentration of lipase (<5 % of oil weight). The optimized benchtop reaction conditions were up-scaled up to a 5 L scale production using stirred tank reactor. The obtained DHA-enriched oil was purified using molecular distillation and evaluated in terms of structure (using NMR spectroscopy, gas chromatography and liquid chromatography), *in-vitro* bioavailability (Caco-2 cell monolayer model) and pro-apoptotic behavior.

2. Materials and methods

2.1. Materials

Lipase AY "Amano" 400SD (from *Candida rugosa*) was purchased from Amano Enzyme China Ltd. Chemicals (including potassium hydroxide and sodium bisulfate anhydrous) and organic solvents (including methanol, *n* - hexane, ethanol, isopropanol, formic acid) were purchased from Shanghai Macklin Biochemical Co., Ltd. Tuna oil was obtained from oil Qingdao Seawit Life Science Co., Ltd. DMEM (Dulbecco's Modified Eagle Medium), Fetal Bovine Serum (FBS), Penicillin - Streptomycin, Non - Essential Amino Acids 9 (NEAA) were purchased from Gibco Life Technologies. Phosphate - Buffered Saline (PBS) and Hank's Balanced Salt Solution (HBSS) was purchased from HyClone Biochemical Product (Beijing) Co., Ltd. Human colon cancer cell line Caco-2 was purchase from Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

2.2. Lipase-catalyzed preparation of DHA-enriched tuna oil

2.2.1. Single-factorial experiment

Candida rugosa lipase has been reported to selectively enrich DHA in the glycerol backbone (Chen et al., 2019) and thus was used in this study. The lipase catalyzed reaction was optimized in terms of lipase concentration (1 %, 2 %, 3 %, 4 %, 5 % relative to the weight of tuna oil), mass ratio of water to oil (1:3, 1:4, 1:5,1:6 and 1:7), reaction time (1 h, 2 h, 3 h, 4 h), and reaction temperature (40 °C, 45 °C, 50 °C, 55 °C, 60 °C) using a single factorial experiment.

Briefly, tuna oil was mixed with distilled water in a jacketed reaction vessel. The jacketed reaction vessel was heated to the required reaction temperature using circulation water bath. Lipase was then added to catalyze the selective hydrolysis under a stirring speed of 300 rpm. At the end of the reaction time, the reaction mixture was terminated by heating the reaction mixture at 95 °C for 10 min. The water and oil mixture were separated by centrifugation (1698×*g*, 10 min) and the oil phase was saponified to remove the hydrolyzed free fatty acids to obtain the glycerides fraction. Saponification was conducted according to previously reported method (Chen et al., 2019). Briefly, the hydrolyzed oil (200 μL) was dissolved in ethanol, mixed with distilled water (both 2 mL), and saponified using ethanolic KOH (1 mL, 0.5 mol/L). The glyceride fraction was extracted twice by hexane (4 mL).

Acid value and fatty acid compositions the product of hydrolysis or glyceride fraction were analyzed. The acid values were estimated and calculated using AOCS Ca - 5a - 40 method with slight modifications (**AOCS, 1997**). Briefly, hydrolyzed oil sample was titrated with NaOH standard solution using phenolphthalein as indicator. Acid value is calculated as follows:

$$\text{Acid value}(\text{mg}/\text{g}) = \frac{\text{volume of alkali} * \text{strenghth of alkali} * 40(\textit{relative molecular mass})}{\textit{mass, g of test portion}}$$

Fatty acid composition was determined according to previously published method (Yu et al., 2023). Oil samples (10–20 μL) were dissolved in 2 mL of *n*-hexane. Potassium hydroxide - methanol solution (0.5 mL, 2 mol/L) was added to the dissolved lipid, incubated for 2 min, and mixed with a vortex mixer. Sodium bisulfate anhydrous was added to the mixtures and allowed to stand for 2 min. The upper layer of the mixtures was passed through a 0.22 μm organic filter membrane and analyzed using gas chromatography (Nexis GC - 2030) equipped with flame ionization detector, and a CP – Si188 fused capillary column (100 m × 0.25 mm × 0.2 m, Agilent, Santa Clara, CA, U.S.A.). The temperature program was set as follows: The initial oven temperature was set at 45 °C for 4 min, then increased to 175 °C at 13 °C/min and held for 27 min, and finally increased to 215 °C at 4 °C/min and held for 35 min. The detector and injector temperatures were set at 250 °C. The gas flow rates were 24 mL/min for nitrogen, 32 mL/min for hydrogen and 200 mL/min for air, respectively. The injection volume was 1 μL, and the split ratio was 1:29 (Wu et al., 2020). Standard solution containing a mixture of 37 fatty acids methyl esters (Supelco® 37 Component FAME Mix) was used for peak identification. The fatty acid content was calculated using the area normalization method.

2.2.2. Orthogonal experimental design for process optimization

The lipase catalyzed partial hydrolysis process was optimized to obtain high DHA content in tuna oil using an orthogonal experimental design which was designed based on the results from the single factorial study. The reaction conditions include enzyme concentration (3 %, 4 % and 5 % of oil weight), reaction time (2 h, 3 h and 4 h), temperature (45 °C, 50 °C and 55 °C) and water to oil ratio (1:3, 1:4 and 1:5). Fatty acid composition was determined according to method described in 2.2.1.

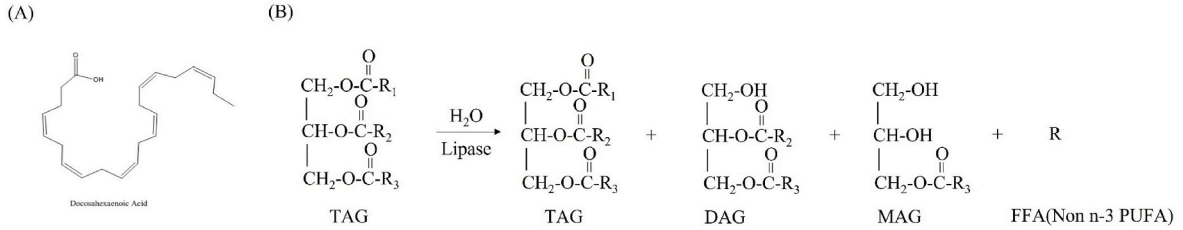

Fig. 1. (A) Molecular structure of DHA and (B) reaction scheme of the lipase catalyzed selective enrichment of ω-3 PUFAs.

2.2.3. Up-scaling and purification process of the lipase-catalyzed preparation of DHA-enriched tuna oil

Three liters of the optimized reaction substrate (tuna oil and water) was added to a 5 L jacketed batch reactor, heated to optimized reaction temperature and stirred at 300 rpm. The reaction was initiated by addition of lipase solution. At the end of the reaction, the reaction mixture was stood to allow for phase separation. The oil and aqueous phase were collected separately. The aqueous phase containing enzyme solution was reused. The obtained oil phase was subjected to purification using a three-step distillation process using a short path distillation (SPD) system (VKL70 - 5S, VTA GMBH & CO.KG, Germany).

In the first step, the distillation temperature was kept at 110 °C, the wiped - film speed was set 300 rpm and the material flow rate was 10 mL/min. In the second step, the distillation temperature was increased to 160 °C, the wiped-film speed was increased to 350 rpm and the material flow rate was reduced to 5 mL/min. In the third step, the distillation temperature was kept at 180 °C, the wiped - film speed was 400 rpm and the material flow rate was 2 mL/min. The system was kept at low atmospheric pressure between 2.5 and 9.1 Pa (Solaesa et al., 2016).

The residue fractions obtained after each distillation steps (kept under nitrogen atmosphere and stored at −80 °C) were analyzed in terms of fatty acids and glycerides compositions. Fatty acids composition was evaluated according to method described in 2.2.1. Glycerides compositions were determined using HPLC-RID (Waters 1525 Series HPLC) equipped with a silica gel column (4.6 mm 250 mm 5 m; Phenomenex, USA). The oil sample (25 μL) was diluted in *n* - hexane (1000 μL) and passed through a 0.45 μm organic filter membrane. The mobile phase (*n* - hexane/isopropanol/formic acid (15:1:0.003, v/v/v) was set at a constant flow rate of 1.0 mL/min. Injection volumes was set at 20 μL (Huang et al., 2023). Glyceride composition, including triglycerides, diglycerides, monoglycerides and free fatty acids, were quantified using area normalization.

The peroxide values were determined according to China National Standards (GB5009.227–2016) (National Standards of China, 2016). Briefly, oil sample was titrated with $Na_2S_2O_3$ standard solution using starch as indicator. Peroxide values is calculated as follows:

$$\text{Peroxide value}(\%) = \frac{\text{standard solution volume} * \text{standard concentration} * 0.1269}{mass, g\ of\ test\ portion} * 100$$

2.3. Positional distribution of the fatty acids in purified DHA-enriched tuna oil

Positional distribution of the fatty acids in DHA-enriched tuna oil was determined using NMR (Bruker, Rheinstetten, Germany) according to previously published method (Tengku-Rozaina et al., 2014). The oil samples (100 mg) were dissolved in 1000 μL of $CDCl_3$, centrifuged (10000 r/min, 5min) and the upper layer was pipetted into a 5 mm NMR tube. The experiment was conducted at the following acquisition parameters: 1024 scans, 10.76 pulse width, 1.3631 s acquisition time, 238 ppm scan width, 2 s relaxation time, 64 K time domain, 30 pulse sequence zgpg, 30 °C excitation pulse.

Peak assignment for the ^{13}C NMR spectrum was done according to previously published method (Ahmmed et al., 2021; Tengku-Rozaina & Birch, 2014). Fig. S1 shows the peak assignment for ^{13}C NMR spectrum. The percentage area of fatty acids in the carbonyl region was obtained using Mestrenova 14.0 software.

2.4. Bioavailability of the DHA-enriched tuna fish oil

2.4.1. Cell culture and cytotoxicity

Caco-2 cells were cultured in DMEM containing 10 % FBS and 1 % penicillin-streptomycin and 1 % non - essential amino acids at 37 °C and 5 % CO_2. The medium was changed every 2 days and the cells used for the experiments were 20–30 generations.

For proliferation experiments, we seeded 100 μl of cell suspension into 96-well plates with a density of 10,000 cells per well for 24 h. The culture solution was totally replaced with fish oil samples of different concentrations (2–400 μg/mL). Each concentration was set up with five replicates. After 24 h of incubation, the Cell Counting Kit - 8 (CCK8, Beyotime Institute of Biotechnology, China) was used to measure cell proliferation. 10 μL of CCK-8 solution was added to each well. The absorbance at 450 nm was measured after 2 h incubation avoid light. The calculation of cell viability is shown below:

$$\text{Cell viability} = \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}}$$

A_{sample} was the absorbance of sample; $A_{control}$ was the absorbance of control (no samples, just cells) and A_{blank} was the absorbance of blank (neither cells nor samples) respectively.

2.4.2. Cell uptake and transport of DHA-enriched tuna oil

Caco-2 cells suspension (0.5 mL) were seeded at a density of 1.0×10^5 cells/mL on the apical side of 12 - well transwell plate and 1.5 mL of cell culture solution was added on the basolateral side. The medium was replaced every day after the Trans Epithelial Electrical Resistance (TEER) values were tested using Millicell® ERS - 2 (Electrical Resistance system) (Millipore, Billerica, U.S.A.). Caco-2 cells monolayers with TEER values higher than 2000 Ω/cm^2 at day 21 were used for uptake and transport analysis.

Firstly, the monolayers were washed twice with HBSS (pH 7.4, 37 °C). DHA-enriched tuna oil (0.5 mL, 100 μg/mL) were added to the apical chamber, and 1.5 mL of HBSS was added (pH 7.4) to the basolateral chamber. After 2 h of incubation, cells in apical chamber and the medium in basolateral chamber were collected. Lipid were extracted from both chambers according to previous method (Yu et al., 2023) and analyzed for their fatty acid composition using the method of 2.2.1. Cell uptake refers lipids extracted from cells in apical chamber and lipid transport refers to lipids in the basolateral medium.

2.5. Functional properties of DHA-enriched tuna oil

Caco-2 cells were cultured in 6 - well plates and treated with tuna oil and DHA-enriched tuna oil (100 μg/mL) for 24 h. The cells were then harvested using EDTA - trypsinization according to the protocol specified in the Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, Nanjing, China). The Caco-2 cells were resuspended in binding buffer (100 μL) after washing with PBS buffer. The cell suspension was incubated in the dark for 10 min after addition of Annexin V-FITC and PI buffer (5 μL respectively). The cells apoptosis was analyzed by flow cytometry (Thermo-life Attune NxT, Thermo Fisher Scientific, USA). Data analysis was performed with FlowJo (FlowJo LLC, Ashland, OR) software version 10.8.1.

2.5.1. Intracellular reactive oxygen species (ROS) generation

Intracellular ROS generation was analyzed using reactive oxygen species assay kit (CA1410, Solarbio). Caco-2 cells were cultured in 96 - well plates and treated with tuna oil and DHA-enriched tuna oil (100 μg/mL) for 24 h. The media was removed, and 100 μL of diluted fluorescent probe (DCFH-DA) was added to each well and incubated at 37 °C in the dark for 20 min. The cells were washed with PBS for three times (0.01

M). Finally, 100 μl of fresh PBS was added to each well and fluorescence was detected through a confocal laser scanning microscopy (Leica DM3000, Germany).

2.5.2. Mitochondrial membrane potential analysis

Mitochondrial membrane potential was measured by mitochondrial membrane potential assay kit with JC-1(C2003S, Beyotime). Caco-2 cells were cultured in 6 - well plates and treated with tuna oil and DHA-enriched tuna oil (100 μg/mL) for 24 h. The cells were then harvested through EDTA - trypsinization. Cells were washed with PBS and stained with 500 μL of JC - 1 solution and incubated for 20 min at 37 °C. The cells mitochondrial membrane potential was analyzed by flow cytometry (Thermo-life Attune NxT, Thermo Fisher Scientific, USA). Data analysis was performed with FlowJo (FlowJo LLC, Ashland, OR) software version 10.8.1.

2.6. Statistical analysis

SPSS software (version 26.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. One - way analysis of variance (ANOVA), Duncan's multiple range test (group >2) and independent samples t -test were performed to compare significant difference between the estimated means of two groups. The p value less than 0.05 indicates significant differences between the samples. Graphpad Prism 9.4.1 (San Diego, CA, USA) was used generate all the graphs. Graphical abstract was created using BioRender software (http://biorender.com).

3. Results and discussion

3.1. Lipase-catalyzed preparation of DHA-enriched tuna oil

3.1.1. Effects of lipase concentration

Fig. 2(A) shows the effect of lipase concentration on acid value and DHA concentration of the tuna oil. Both acid value and DHA concentration of the tuna oil increased with lipase concentration. As lipase concentration increased from 1 % to 4 %, acid value and DHA concentration of the tuna oil increased from 54.64 mg/g to 74.72 mg/g and 38.55 %–43.67 %, respectively. However, both acid value and DHA concentration significantly decreased ($p < 0.05$) at high lipase concentration of 5 %. Therefore, 4 % of lipase concentration was considered as optimum lipase concentration to obtain high yield of glycerides enriched in DHA. Our result is in consistent with previously reported study which found a significant decrease in the DHA concentration in the glyceride fraction at high enzyme concentration (Ma et al., 2014). Lipase promotes hydrolysis reaction. However, excessive lipase concentration will not increase reaction rates due to formation of lipase aggregates which reduces lipases accessibility to the reaction substrates (Jiang et al., 2023).

3.1.2. Effects of reaction temperature

Reaction temperature affects lipase activity and stability. The relationship between reaction temperature and lipase activity can be described as "inverted U" shape, where the enzymatic activity is highest at optimum reaction temperature, and a too high or too low reaction temperature is detrimental to enzymatic activity (Chen et al., 2023). As reaction temperature increased from 45 °C to 50 °C, acid value and DHA concentration of tuna oil increased from 62.45 mg/g to 76.78 mg/g and 41.90 %–44.28 %, respectively. However, both acid value and DHA concentration decreased significantly ($p < 0.05$) when the temperature exceeded 55 °C. Therefore, 50 °C was considered as optimum reaction temperature to obtain high yield of glycerides enriched in DHA. This is in consistent with previously reported study in which 50 °C was regarded as the optimum temperature where *Thermomyces lanuginosus* lipase-catalyzed hydrolysis of tuna oil (Sun et al., 2023).

3.1.3. Effects of reaction time

Fig. 2(C) shows the effects of reaction time on acid value and DHA concentration of the tuna oil. Acid value increased from 0.11 mg/g to 49.85 mg/g after 1 h of reaction time, and gradually increased up to 62.45 mg/g after 4 h of reaction time. Meanwhile, the DHA

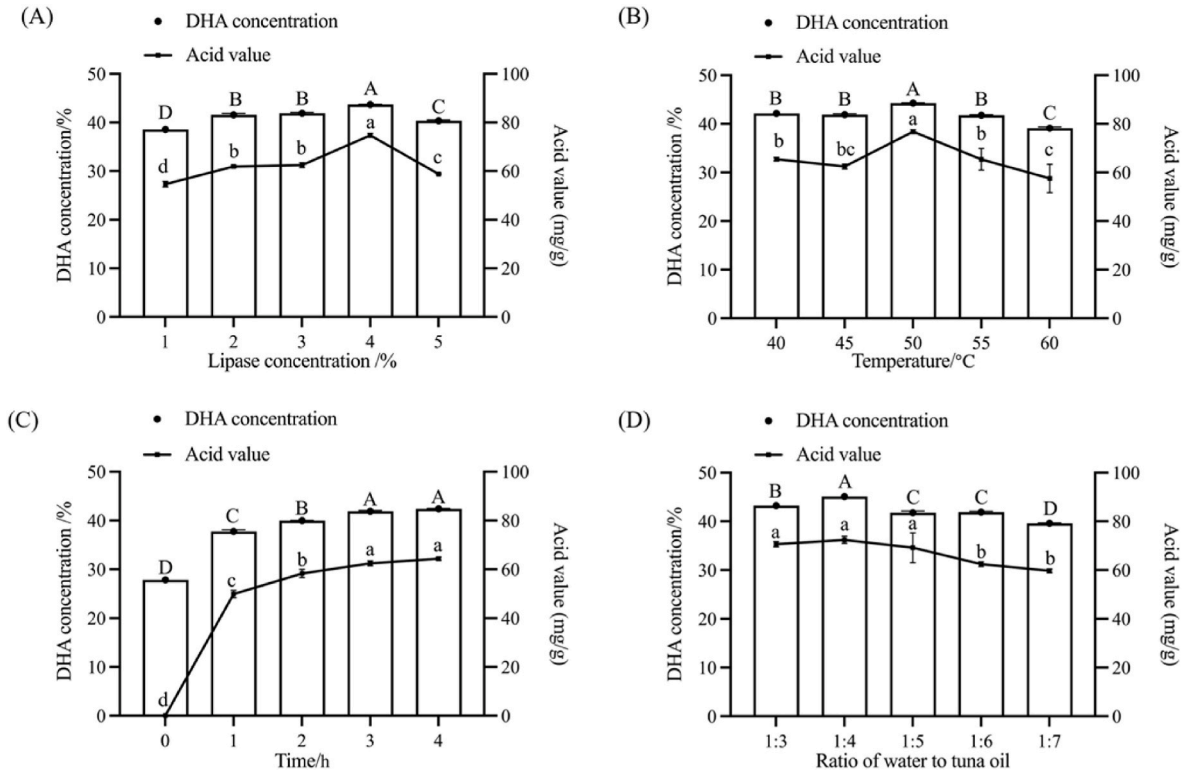

Fig. 2. Effects of lipase concentration (A), reaction temperature (B), reaction time (C), ratio of water to tuna oil(D) on acid value and DHA concentration of tuna oil.

concentration in tuna oil increased sharply from 27.83 % to 39.98 % during the first 2 h of the reaction time. Following that, the DHA concentration increased gradually from 41.90 % to 42.41 % at 4 h ($p < 0.05$). Therefore, 4 h of reaction time was selected as optimum reaction time to obtain high yield of glycerides enriched in DHA. Our result is in consistent with previously reported study which found the optimum reaction time was 4 h (Yang et al., 2020).

3.1.4. Effects of the ratio of water to oil

Lipase can catalyze hydrolysis of triglyceride with increased activity in the presence of an oil/water interface. As shown in Fig. 2(D), DHA concentration in the glyceride fraction increased significantly ($p < 0.05$) as the mass ratio of water to tuna oil increased from 1:7 to 1:5 with a maximum DHA concentration of 45.11 % at water to tuna oil ratio of 1:4. Thus, water to tuna oil ratio of 1:4 was selected as optimum water to tuna oil ratio to obtain high yield of glycerides enriched in DHA. Water promoted the reaction by providing more substituents (hydroxyl groups) for hydrolysis. However, a too high proportion of water can also affect the polarity of the system, thus inhibiting lipase activity through competitive inhibition with substrate for binding to active site of the lipase and subsequently reducing the enrichment effect (Hosseini et al., 2018; Jiang et al., 2023). This is in agreement with a previously reported findings which low water reduces the catalytic activity of the lipase used and high water leads to saturating effects and inhibition of the substrate by the lipase (Sun et al., 2023).

3.1.5. Orthogonal experimental design for process optimization

Based on the findings of the single factorial study, orthogonal experiment was designed to optimize lipase concentration, water to oil mass ratio, reaction time and temperature to obtain DHA-enriched tuna oil. Unprocessed tuna oil has 27.83 % DHA. At the optimal reaction conditions (lipase concentration of 4 %, ratio of water to tuna oil of 1:3, reaction temperature of 55 °C, reaction time of 4 h), this work enriched the DHA concentration in the glyceride fraction to 46.12 % (Table 1). Range analysis (R_n value) was conducted to investigate the impact of each factor on DHA enrichment. The R_n value of the reaction conditions were: lipase concentration = 2.18 > reaction time = 2.16 > the ratio of water to tuna oil = 1.52 > reaction temperature = 1.51. This result indicated that lipase concentration had the highest impact on DHA enrichment in tuna oil.

3.1.6. Up-scaling and purification process of the lipase-catalyzed preparation of DHA-enriched tuna oil

Tuna oil (2.76 kg) and distilled water (0.92 kg) were added to a 5 L stirred tank glass reactor and stirred at 200 rpm. As the temperature of the reaction mixture reached 55 °C, 110.4 g of lipase was added to activate the partial hydrolysis reaction for 4 h. Acid value and DHA concentration in the hydrolyzed tuna oil were 62.75 mg/g and 45.3 %, respectively.

Molecular distillation is commonly used to purify thermally unstable compounds under vacuum conditions to minimize thermal degradation (Zhang et al., 2018). The acid value, peroxide value and DHA concentration of unprocessed tuna oil were 0.11 mg/g, 0.45 % and 25.24 %, respectively. Following enzymatic and distillation process, the purified DHA-enriched tuna oil has an acid value, peroxide value and DHA concentration of 0.85 mg/g, 1.2 % and 46.28 %, respectively which meet the requirement of first grade fish oil as stipulated by Chinese aquatic industry standard for fish oil(SC/T 3502-2016, 2016). The yield of the DHA-enriched oils following molecular distillation was 32.6 % (wt % of the tuna oil used as substrate).

In this study, a three-step distillation process is used to remove the free fatty acids (FFA) and monoglycerides (MAG) in the glyceride fractions. Fig. 3(A) shows the glycerides compositions in unprocessed tuna oil which contain mostly TAG (98.03 %). The DAG, MAG, and FFA concentration of unprocessed tuna oil were 0.34 %, 0.50 % and 1.13 %, respectively. Following first distillation step, the residue contained 41.84 % of FFA, 3.53 % of MAG, 23.06 % of DAG, 31.57 % of TAG. After the second distillation step, the residue contained 15.07 % of FFA, 3.73 % of MAG, 33.44 % of DAG, 47.75 % of TAG. Following this final step, the DHA-enriched tuna oil contained mainly by TAG (57.91 %) and DAG, (40.58 %) with low concentration of MAG (0.49 %) and FFA (1.02 %). This result is consistent with previously reported findings which reported high concentration of DAG and TAG in DHA-enriched purified oil (Sun et al., 2023). High concentration of DAG in the purified oil may offer potential health benefits as DAG has been reported to reduce visceral fat and inhibit weight gain as compared to TAG (Jiang et al., 2022).

Table 1
Orthogonal experimental design for optimization of the lipase-catalyzed selective enrichment of DHA.

No.	Time/ h	Lipase concentration (%)	Ratio of water to tuna oil	Temperature (°C)	DHA concentration (%)
1	2	3	1:3	45	41.31 ± 0.88
2	2	4	1:4	50	43.42 ± 0.09
3	2	5	1:5	55	40.33 ± 0.20
4	3	3	1:4	55	40.61 ± 0.37
5	3	4	1:5	45	42.85 ± 0.25
6	3	5	1:3	50	44.25 ± 0.08
7	4	3	1:5	50	42.93 ± 0.30
8	4	4	1:3	55	46.12 ± 0.08
9	4	5	1:4	45	43.50 ± 0.51
K_1	125.06	124.85	130.68	127.66	–
K_2	127.70	131.39	127.52	130.60	–
K_3	131.55	128.07	126.11	126.06	–
k_1	41.69	41.62	43.56	42.55	–
k_2	42.57	43.80	42.51	43.53	–
k_3	43.85	42.69	42.04	42.02	–
R	2.16	2.18	1.52	1.51	–

K_n represents the sum of DHA concentration for a factor at level n; $\mathbf{k_n}$ represents the average of DHA concentration for a factor at level n; $\mathbf{R_n}$ represents the range of the average value of the DHA concentration for a factor at level n.

Feed flow rate during the SPD process is an important variable affecting efficiency of the purification process. High feed flow rate shortens the operation time but leads to lower purification (Yeoh, 2014). Thus, we have reduced the feed flow rate from 10 mL/min in the first step to 5 mL/min in the second and to 2 mL/min in the third step. Increased distillation temperature can gradually remove FFA, however a high temperature of more 200 °C might causing PUFA degradation (Messina et al., 2022). Thus, we have not increased the distillation temperature beyond 180 °C in the three distillation steps.

Fig. 3(B) shows the fatty acid compositions in the unprocessed tuna oil and residue fractions of the purified tuna oil. Unprocessed tuna oil contains 31.39 % of SFA, 30.22 % of MUFA and 38.39 % of PUFA. During the first two steps of the distillation process, SFA decreased significantly from 13.43 % to 9.88 % ($p < 0.05$), with no significant changes in MUFA and PUFA. Following the final distillation step, a PUFA (61.49 %) enriched tuna oil was obtained with 28.25 % of MUFA and 10.26 % of SFA.

As shown in Fig. 3(B), DHA and palmitic acids are the two most abundant fatty acids in the unprocessed tuna oil which accounted for 25.24 % and 20.54 %, respectively. This is in agreement with a previous finding which reported found 28–30 % palmitic acids and 24–28 % of EPA and DHA in fish oil(Solaesa et al., 2016). Following the first two step of molecular distillations, palmitic acid decreased from 20.54 % to 6.60 % ($p < 0.05$). Meanwhile, DHA in glyceride fractioin increased to 46.28 %. This is consistent with the finding of Yang and colleagues that the DHA content in the glyceride fraction of tuna oil increased significantly from 24.21 % to 45.38 % using *Candida rugosa* lipase catalyzed selective hydrolysis (Yang et al., 2020). FAs with low molecular weights (such as C14:0, C16:0) were distilled completely (Tenllado et al., 2011).

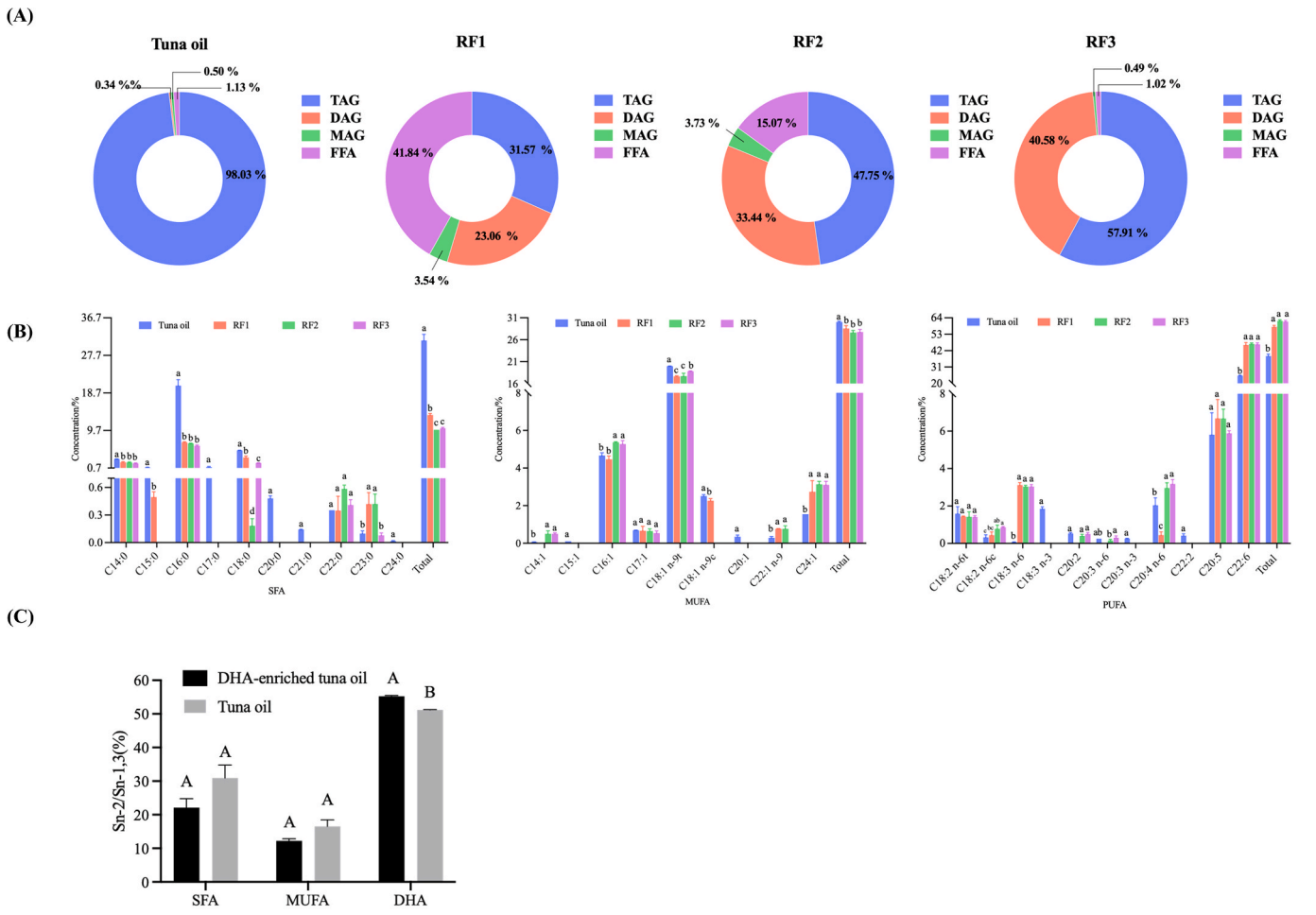

Fig. 3. (A) Glycerides and (B) fatty acid compositions in the distillate fractions. (C) regio - distribution of SFA, MUFA and DHA in tuna oil and DHA-enriched tuna oil. RF1: Residue fractions after first step of distillation; RF2: Residue fractions 2 after second step of distillation; RF3: Residue fractions 3 after third step of distillation; MAG: monoacylglycerol; DAG: Diacylglycerol; TAG: Triacylglycerol; FFA: Free fatty acid: SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid. Different letters show significant differences between treatments ($p < 0.05$).

3.1.7. Positional distribution of the fatty acids in purified DHA-enriched tuna oil

Bioavailability of EPA and DHA located at *sn*-2 position has been found to be more efficiently absorbed than those distributed at the *sn*-1 or *sn*-3 position(Jin et al., 2020). Positional distribution of the fatty acids in purified DHA-enriched tuna oil was determined using ^{13}C NMR spectroscopy. Fig. 3(C) shows unprocessed tuna oil contains 51.17 % of DHA concentration in the *sn-2* position. Purified DHA-enriched tuna oil contained significantly ($p < 0.05$) higher DHA concentration in the *sn*-2 position (55.24 %) Thus, the DHA-enrichment in tuna oil is mainly due to the fatty acid selectivity of *Candida rugosa* lipase rather than positional selectivity. *Candida rugosa* lipase discriminated against DHA due to additional double bond in DHA resulting in a larger spatial site barrier and inability to access the lipase active site (Hosseini et al., 2018).

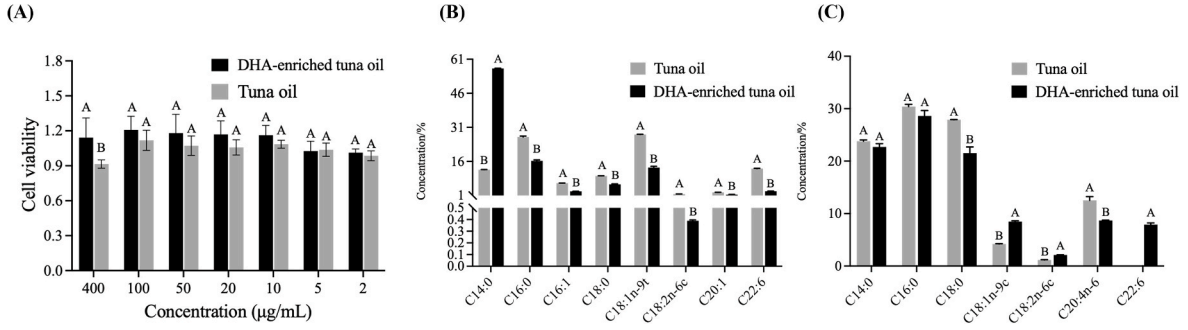

Fig. 4. (A) Cytotoxicity of the unprocessed tuna oil and DHA-enriched tuna oil. Cellular transport (B) and (C) cell uptake of the unprocessed tuna oil and DHA-enriched tuna oil.
Different letters show significant differences between treatments ($p < 0.05$).

3.2. Bioavailability of the DHA-enriched tuna fish oil

Viability assay is conducted to investigate the effects on DHA-enriched tuna fish oil on cell cytotoxicity. The general criterion for a sample to be nontoxic to cells at a particular concentration is having a cell viability of more than 0.9 (Zhou et al., 2018). Caco-2 cells viability following treatment of 2–400 μg/mL of tuna oil and DHA-enriched tuna oil ranged from 0.98 to 1.20 indicating both samples were non-toxic to the Caco-2 cells. An oil concentration of 100 μg/mL was chosen for the following experiments based on highest cell viability following treatment.

Fig. 4(B) shows the cellular transport of DHA-enriched tuna oil. Eight FAs were detected in the basolateral medium of DHA-enriched tuna oil as compared to unprocessed tuna oil. Among these fatty acids, significant concentration of C14:0 ($p < 0.05$) was transported and significant lower concentration of DHA ($p < 0.05$) was transported. In terms of cellular uptake, C16:0 in both DHA-enriched tuna oil and unprocessed tuna oil was the most uptake fatty acids (Fig. 4(C). There was 7.89 % of the DHA uptake in the DHA-enriched oil, DHA-enriched whereas no DHA uptake was in the unprocessed tuna oil. This result is consistent with the study of Song and colleagues which shows significant DHA and EPA cellular uptake in DHA-enriched oil as compared to unprocessed oil (Song et al., 2023). Otherwise, there is no observed DHA uptake from tuna oil possibly due to not enough time of uptake and was consist of that the release rate of long-chain PUFA was lower *in-vitro* digestion (Ye et al., 2019).

3.3. Functional properties of DHA-enriched tuna oil

Pro-apoptotic behavior of the tuna oil and DHA-enriched tuna oil was investigated using Annexin V-FITC/PI. Both tuna oil and DHA-enriched tuna oil induced early and late apoptosis (Fig. 5(A)). However, DHA-enriched tuna oil (16.03 %) shows significantly ($p < 0.05$) higher apoptosis rate as compared to tuna oil (14.06 %). This result demonstrated that n-3 unsaturated fatty acids promoted apoptosis.

Overproduction of ROS is a negative signal in cell which lead to failure to inhibit anti-apoptotic factors, cause damage to cell membrane and eventually trigger apoptosis (Ruiz et al., 2022; Wang et al., 2023). We found that DHA-enriched tuna oil caused overproduction of ROS which is evident from enhanced fluorescence intensity of DHA-enriched tuna oil group as compared to tuna oil treated group and untreated group (blank). It has been reported that unoxidized lipids of n-3 PUFA are more susceptible to uptake than lipids in a more oxidized state (Dasilva et al., 2018). It's worth indicating that the increase of oxidative

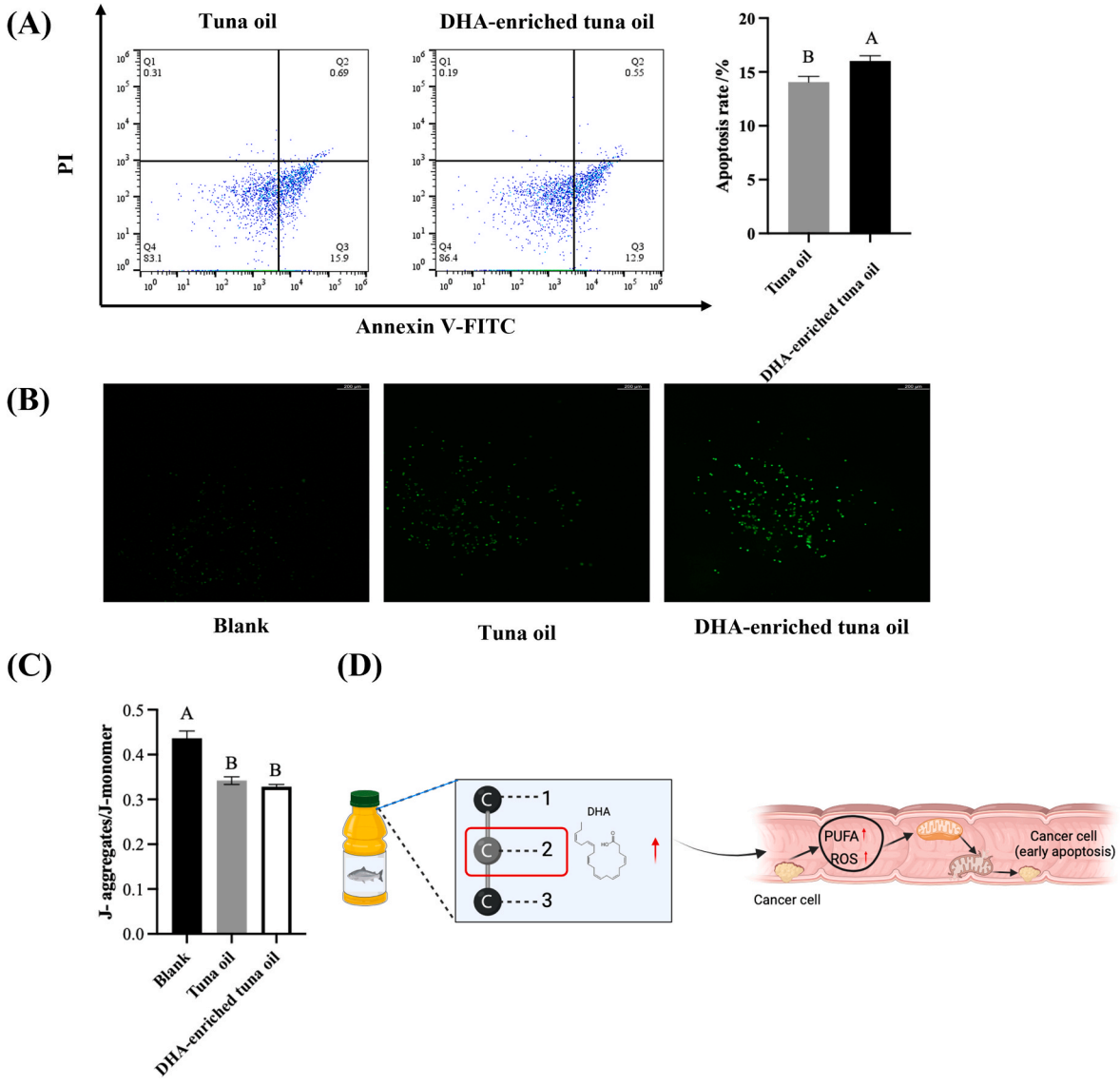

Fig. 5. (A) Caco2 cell apoptosis rate and (B) ROS level and (C) membrane depolarization and (D) the pathway of early apoptosis in Caco2 cells.

stress markers was due to intestinal absorption rather than auto-oxidation of tuna oil. In addition, Fig. 5(C) shows that the tuna oil treated group showed the ratio of j-aggregates to j-monomers reduced significantly as compared to untreated group (blank). Therefore, the tuna oil with n-3 unsaturated fatty acids (DHA) are more conducive to promoting the early apoptosis of colorectal cancer cells.

Therefore, we speculate the higher pro-apoptotic behavior in DHA-enriched tuna oil to be related to its better uptake by Caco-2 cells as more n-3 polyunsaturated fatty acids (DHA) are positioned at *sn*-2 position. Better uptake of the DHA-enriched tuna oil by Caco-2 cells subsequently generated large amount of ROS, increased mitochondrial depolarization and thus triggering early apoptosis in the Caco-2 cells (Fig. 5(D)). Similar to the findings of Zhang and colleagues, n-3 fatty acids (EPA, DHA) induced ROS generation, promoted Ca^{2+} accumulation, activated caspases, and lowered mitochondrial membrane potential thereby causing apoptosis in colon cancer cells(Zhang et al., 2015).

4. Conclusions

Candida rugosa lipase was used to catalyze the selective hydrolysis of tuna oil for high yield preparation of DHA-enriched tuna oil. DHA-enriched tuna oil produced at 5 L scale production (lipase concentration of 4 %, ratio of water to tuna oil of 1:3, reaction temperature of 55 °C, reaction contained 46.12 % of DHA, in which 55.24 % of DHA was located in *sn*-2 position of the glyceride fraction. *In - vitro* bioavailability study shows the DHA-enriched oil had better DHA uptake than the unprocessed oil. In addition, the DHA-enriched oil demonstrated increased intracellular oxidation and mitochondrial damage leading to pro-apoptosis of the human colon cancer cells.

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CRediT authorship contribution statement

Jingwen Yu: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Cai Shen:** Writing – review & editing, Visualization, Resources, Methodology, Data curation. **Haohui Chen:** Validation, Software, Methodology. **Min Luo:** Software, Methodology. **Linshang Zhang:** Validation, Software, Methodology. **Yanan Liu:** Validation, Methodology, Investigation. **Feng Xu:** Supervision, Resources, Methodology, Data curation. **Chin-Ping Tan:** Visualization, Resources. **Ling-Zhi Cheong:** Writing – review & editing, Writing – original draft, Software, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116341.

References

Ahmmed, M. K., Ahmmed, F., Stewart, I., Carne, A., Tian, H., & Bekhit, A. E.-D. A. (2021). Omega-3 phospholipids in Pacific blue mackerel (*Scomber australasicus*) processing by-products. *Food Chemistry, 353*, Article 129451. https://doi.org/10.1016/j.foodchem.2021.129451

AOCS. (1997). *Method Ca 5a-40, Free fatty acids, official methods and recommended practices of the AOCS*. Champaign: AOCS Press.

Chen, Y., Cheong, L.-Z., Zhao, J., Panpipat, W., Wang, Z., Li, Y., Lu, C., Zhou, J., & Su, X. (2019). Lipase-catalyzed selective enrichment of omega-3 polyunsaturated fatty acids in acylglycerols of cod liver and linseed oils: Modeling the binding affinity of lipases and fatty acids. *International Journal of Biological Macromolecules, 123*, 261–268. https://doi.org/10.1016/j.ijbiomac.2018.11.049

Chen, Y., Liu, K., Yang, Z., Chang, M., Wang, X., & Wang, X. (2023). Lipase-catalyzed two-step hydrolysis for concentration of acylglycerols rich in ω-3 polyunsaturated fatty acids. *Food Chemistry, 400*, Article 134115. https://doi.org/10.1016/j.foodchem.2022.134115

Dasilva, G., Boller, M., Medina, I., & Storch, J. (2018). Relative levels of dietary EPA and DHA impact gastric oxidation and essential fatty acid uptake. *The Journal of Nutritional Biochemistry, 55*, 68–75. https://doi.org/10.1016/j.jnutbio.2017.11.007

Efsa, E. F. S. A. (2010). *Scientific opinion on dietary reference values for dat, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol*.

Hosseini, H., Ghorbani, M., Jafari, S. M., & Mahoonak, A. S. (2018). Investigating the effect of lipase from Candida rugosa on the production of EPA and DHA concentrates from *Kilka* fish (*Clupeonella cultiventris caspia*). *LWT - Food Science and Technology, 93*, 534–541. https://doi.org/10.1016/j.lwt.2018.03.066

Huang, Y., Li, H., Wang, Z., Fu, Y., Chen, Y., & Wang, X. (2023). Enzymatic synthesis of branched chain fatty acid-enriched structured triacylglycerols via esterification with glycerol. *Food Chemistry, 429*, Article 136943. https://doi.org/10.1016/j.foodchem.2023.136943

Jiang, C., Chen, Y., Wang, Y., Li, C., Wang, X., Meng, Z., & Wang, X. (2022). Concentration of n-3 polyunsaturated fatty acid glycerides by *Candida antarctica* lipase A-catalyzed selective methanolysis. *Food Bioscience, 46*, Article 101562. https://doi.org/10.1016/j.fbio.2022.101562

Jiang, C., Wang, Z., Huang, Y., Wang, X., Chang, M., & Wang, X. (2023). Enzymatic enrichment of acylglycerols rich in n-3 polyunsaturated fatty acids by selective methanolysis: Optimization and kinetic studies. *Journal of Food Science, 88*(7), 2858–2869. https://doi.org/10.1111/1750-3841.16618

Jin, J., Jin, Q., Wang, X., & Akoh, C. C. (2020). High *Sn*-2 docosahexaenoic acid lipids for Brain benefits, and their enzymatic Syntheses: A review. *Engineering, 6*(4), 424–431. https://doi.org/10.1016/j.eng.2020.02.009

Jin, J., Zhang, S., & Akoh, C. C. (2022). Preparation and characterization of *sn*-2 polyunsaturated fatty acids-rich monoacylglycerols from menhaden oil and DHA-single cell oil. *LWT - Food Science and Technology, 156*, Article 113012. https://doi.org/10.1016/j.lwt.2021.113012

Karrar, E., Albakry, Z., Mohamed Ahmed, I. A., Zhang, L., Chen, C., Wu, D., & Li, J. (2023). Docosahexaenoic acid and eicosapentaenoic acid from microalgae: Extraction, purification, separation, and analytical methods. *Algal Research, 103365*. https://doi.org/10.1016/j.algal.2023.103365

Ma, N., Hong, S. I., Zhao, T., No, D. S., Kim, C.-T., Kim, Y., & Kim, I.-H. (2014). Enrichment of docosahexaenoic acid from tuna oil via lipase-mediated esterification under pressurized carbon dioxide. *The Journal of Supercritical Fluids, 87*, 28–33. https://doi.org/10.1016/j.supflu.2013.12.024

Messina, C. M., Arena, R., Manuguerra, S., La Barbera, L., Curcuraci, E., Renda, G., & Santulli, A. (2022). Valorization of side Stream products from Sea cage Fattened Bluefin tuna (*Thunnus thynnus*): Production and in *vitro* Bioactivity evaluation of enriched ω-3 polyunsaturated fatty acids. *Marine Drugs*.

National Standards of China. (2016). *National food safety standard - determination of peroxide value in food, GB5009* pp. 227–2016).

Newell, M., Brun, M., & Field, C. J. (2019). Treatment with DHA Modifies the response of MDA-MB-231 breast cancer cells and tumors from nu/nu Mice to Doxorubicin through apoptosis and cell Cycle Arrest. *The Journal of Nutrition, 149*(1), 46–56. https://doi.org/10.1093/jn/nxy224

Patel, A., Karageorgou, D., Katapodis, P., Sharma, A., Rova, U., Christakopoulos, P., & Matsakas, L. (2021). Bioprospecting of thraustochytrids for omega-3 fatty acids: A sustainable approach to reduce dependency on animal sources. *Trends in Food Science & Technology, 115*, 433–444. https://doi.org/10.1016/j.tifs.2021.06.044

Pettersen, K., Monsen, V. T., Hakvåg Pettersen, C. H., Overland, H. B., Pettersen, G., Samdal, H., Tesfahun, A. N., Lundemo, A. G., Bjørkøy, G., & Schønberg, S. A. (2016). DHA-induced stress response in human colon cancer cells – Focus on oxidative stress and autophagy. *Free Radical Biology and Medicine, 90*, 158–172. https://doi.org/10.1016/j.freeradbiomed.2015.11.018

Ruiz, M. C., Perelmulter, K., Levín, P., Romo, A. I. B., Lemus, L., Fogolín, M. B., León, I. E., & Di Virgilio, A. L. (2022). Antiproliferative activity of two copper (II) complexes on colorectal cancer cell models: Impact on ROS production, apoptosis induction and NF-κB inhibition. *European Journal of Pharmaceutical Sciences, 169*, Article 106092. https://doi.org/10.1016/j.ejps.2021.106092

SC/T 3502-2016. (2016). *Fish oil: Aquatic industry standard (2016)*.

Solaesa, Á. G., Sanz, M. T., Falkeborg, M., Beltrán, S., & Guo, Z. (2016). Production and concentration of monoacylglycerols rich in omega-3 polyunsaturated fatty acids by enzymatic glycerolysis and molecular distillation. *Food Chemistry, 190*, 960–967. https://doi.org/10.1016/j.foodchem.2015.06.061

Song, R., Li, W., Deng, S., Zhao, Y., & Tao, N. (2023). Assessment of lipid composition and eicosapentaenoic acid/docosahexaenoic acid bioavailability in fish oil obtained

through different enrichment methods. *Frontiers in Nutrition, 10*, Article 1136490. https://doi.org/10.3389/fnut.2023.1136490

Sun, C., Chen, Y., Zhang, H., Zhu, L., Li, X., & Liang, S. (2023). Preparation of glycerides enriched with n-3 long-chain polyunsaturated fatty acids by *Thermomyces lanuginosus* lipase-catalyzed hydrolysis of tuna oil. *LWT - Food Science and Technology, 182*, Article 114875. https://doi.org/10.1016/j.lwt.2023.114875

Tengku-Rozaina, T. M., & Birch, E. J. (2014). Positional distribution of fatty acids on hoki and tuna oil triglycerides by pancreatic lipase and ^{13}C NMR analysis. *European Journal of Lipid Science and Technology, 116*(3), 272–281. https://doi.org/10.1002/ejlt.201300357

Tenllado, D., Reglero, G., & Torres, C. F. (2011). A combined procedure of supercritical fluid extraction and molecular distillation for the purification of alkylglycerols from shark liver oil. *Separation and Purification Technology, 83*, 74–81. https://doi.org/10.1016/j.seppur.2011.09.013

Wang, G., Qiu, J., Li, A., Ji, Y., & Zhang, J. (2023). Apoptosis and oxidative stress of mouse breast carcinoma 4T1 and human intestinal epithelial Caco-2 cell lines caused by the phycotoxin gymnodimine-A. *Chemico-Biological Interactions, 384*, Article 110727. https://doi.org/10.1016/j.cbi.2023.110727

Wen, M., Zhao, Y., Shi, H., Wang, C., Zhang, T., Wang, Y., & Xue, C. Short-term supplementation of DHA as phospholipids rather than triglycerides improve cognitive deficits induced by maternal omega-3 PUFA deficiency during the late postnatal stage. Food & Function, 564-572. https://doi.org/10.1039/D0FO02552F.

Wu, Y., Zhang, N., Deng, Z. Y., Zhang, H., & Li, J. (2020). Effects of the Major structured triacylglycerols in human milk on lipid metabolism of Hepatocyte cells *in vitro*. *Journal of Agricultural and Food Chemistry*, (69), 9147–9156. https://doi.org/10.1021/acs.jafc.0c06976

Xia, Q., Akanbi, T. O., Wang, B., Li, R., Liu, S., & Barrow, C. J. (2020). Investigation of enhanced oxidation stability of microencapsulated enzymatically produced tuna oil concentrates using complex coacervation. *Food & Function, 11*, 10748–10757. https://doi.org/10.1039/d0fo02350g

Yang, Z., Jin, W., Cheng, X., Dong, Z., Chang, M., & Wang, X. (2020). Enzymatic enrichment of n-3 polyunsaturated fatty acid glycerides by selective hydrolysis. *Food Chemistry*. , Article 128743. https://doi.org/10.1016/j.foodchem.2020.128743

Ye, Z., Li, R., Cao, C., Xu, Y.-J., Cao, P., Li, Q., & Liu, Y. (2019). Fatty acid profiles of typical dietary lipids after gastrointestinal digestion and absorbtion: A combination study between *in-vitro* and *in-vivo*. *Food Chemistry, 280*, 34–44. https://doi.org/10.1016/j.foodchem.2018.12.032

Yeoh, C. M., TongTang, E., KimSiew, T., LinAbdullah, W., ChuahChoong, L., & Yaw, T. S. (2014). Molecular distillation and characterization of diacylglycerol-enriched palm olein. *European Journal of Lipid Science and Technology, 116*(12), 1654–1663. https://doi.org/10.1002/ejlt.201300502

Yu, J., Wu, Y., Yao, D., Song, S., Zhang, H., Xu, X., & Cheong, L.-Z. (2023). The effects of maternal and perinatal factors on human milk lipids composition. *Journal of Food Composition and Analysis, 123*, Article 105596. https://doi.org/10.1016/j.jfca.2023.105596

Zhang, H., Secundo, F., Sun, J., & Mao, X. (2022). Advances in enzyme biocatalysis for the preparation of functional lipids. *Biotechnology Advances, 61*, Article 108036. https://doi.org/10.1016/j.biotechadv.2022.108036

Zhang, Y., Wang, X., Xie, D., Li, Y., & Wang, X. (2017). One-step concentration of highly unsaturated fatty acids from tuna oil by low-temperature Crystallization. *Journal of the American Oil Chemists' Society, 94*(3), 475–483. https://doi.org/10.1007/s11746-017-2949-3

Zhang, Y., Wang, X., Xie, D., Zou, S., Jin, Q., & Wang, X. (2018). Synthesis and concentration of 2-monoacylglycerols rich in polyunsaturated fatty acids. *Food Chemistry, 250*, 60–66. https://doi.org/10.1016/j.foodchem.2018.01.027

Zhang, C., Yu, H., Shen, Y., Ni, X., Shen, S., & Das, U. N. (2015). Polyunsaturated fatty acids trigger apoptosis of colon cancer cells through a mitochondrial pathway. *Archives of Medical Science, 11*(5), 1081–1094. https://doi.org/10.5114/aoms.2015.54865

Zhou, F., Huang, W., Li, M., Zhong, Y., Wang, M., & Lu, B. (2018). Bioaccessibility and absorption Mechanism of Phenylethanoid Glycosides using Simulated digestion/Caco-2 intestinal cell models. *Journal of Agricultural and Food Chemistry*, 4630–4637. https://doi.org/10.1021/acs.jafc.8b01307