elsevier_FOCH_18484

Enrichment of Oomega-3 Ffatty Aacids in Ccod Lliver Ooil via Aalternate Ssolvent Wwinterization and Eenzymatic Iinteresterification

Qiong Lei

Sai Ba

Hao Zhang

Yanyan Wei

Jasmine Yiqin Lee

Tianhu Li*

thli@ntu.edu.sg

Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, Singapore 637371, Singapore

*Corresponding author.

Abstract

Enrichment of omega-3 fatty acids in cod liver oil via alternate operation of solvent winterization and enzymatic interesterification was attempted. Variables including separation method, solvent, oil concentration, time and temperature were optimized for the winterization. Meanwhile, Novozyme 435, Lipozyme RM IM and Lipozyme TL IM were screened for interesterification efficiency under different system air condition, time and temperature. In optimized method, alternate winterization (0.1 g/mL oil/acetone, 24 h, −80 °C, precooled Büchner filtration) and interesterification (Lipozyme TL IM, N_2 flow, 2.5 h, 40 °C) successfully doubled the omega-3 fatty acid content to 43.20 mol%. ^{1}H NMR was used to determine omega-3 fatty acid content, and GC–MS to characterize oil product, which mainly contained DHA (15.81 mol%) and EPA (20.23 mol%). The proposed method offers considerable efficiency and reduce production cost drastically. Oil produced thereof is with high quality and of particular importance for the development of omega-3 based active pharmaceutical ingredients.

Keywords: Cod liver oil; Omega-3 fatty acids; Enrichment; Solvent winterization; Enzymatic interesterification

1 Introduction

Docosahexaenoic acid (DHA; 22:6 ω-3) and eicosapentaenoic acid (EPA; 20:5 ω-3) are the most common omega-3 polyunsaturated fatty acids (PUFA). They have been proved to be nutritionally important to the human body by reducing the risk of Alzheimer's disease, cardiovascular disease, cognitive decline and cancers, and by regulating inflammation, blood pressure and thickness, hormone production and the activities of the immune and central nervous systems (Augustsson et al., 2003; Calder, 2006; Caygill & Hill, 1995; De Deckere, 1999; Gleissman, Johnsen, & Kogner, 2010; Holub, 2009; Morris, Evans, Tangney, Bienias, & Wilson, 2005; Tengku-Rozaina & Birch, 2013). Generally, these fatty acids exist in form of triacylglycerol (TAG) in food and are more easily metabolized and absorbed by human body than their methyl or ethyl esters (El Boustani et al., 1987; Hamazaki et al., 1982; Lawson & Hughes, 1988; Shahidi & Wanasundara, 1998; Yang, Kuksis, & Myher, 1989). Due to the limited ability of human body to synthesize EPA and DHA *de novo*, direct intake of omega-3 fats is clinically recommended to maintain the balance of human body's metabolisms (Huber, Vasantha Rupasinghe, & Shahidi, 2009; Shin, Akoh, & Lee, 2010; Torres, Munir, Blanco, Otero, & Hill, 2002).

Source of EPA and DHA for human consumption nowadays comes mainly from oily fishes, such as sardine, anchovy, mackerel, menhaden and tuna (Jacobsen, 2016). For example, natural cod liver oil (CLO) in fact contains only ~21.2 wt% PUFA (~18.7 wt% omega-3 fatty acids) on average while the others are monounsaturated fatty acids (MUFA) (~53.0 wt%) and saturated fatty acid (SFA) (~23.2 wt%) (Kurata, Yamaguchi, & Nagai, 2005). Hence, enrichment of omega-3 PUFA content in oils is of great importance and has been studied extensively (Homayooni, Sahari, & Barzegar, 2014).

So far, two major methodologies have been developed to concentrate omega-3 PUFA from fish oils in natural TAG form. The first one mainly relies on the physical property differences between omega-3 fatty acid moieties and non-omega-3 fatty acid moieties, such as solvent winterization (Homayooni et al., 2014; Tengku-Rozaina & Birch, 2013). Despite the simplicity of procedure and low cost, the increments of omega-3 PUFA content are not so obvious. For the second methodology, it modifies the fatty acids on their glycerol backbone within the oil via enzymatic reactions, including transesterification, interesterification, hydrolysis and re-esterification (McNeill, Ackman, & Moore, 1996). Although remarkable

increase in omega-3 content has been observed (Lin, Chen, & Chang, 2006), the procedures actually generate various by-products (e.g., free fatty acids, monoacylglycerols and diacylglycerols), and thus require further purification steps (e.g., membrane filtration, molecular distillation and urea complexation), leading to possible higher production cost (Rubio-Rodríguez et al., 2010). In this light, combination of these methodologies, specifically solvent winterization and interesterification, is recommended to achieve better results, where advantages of both can be merged (Bockisch, 1998, chap. 6).

In the current study, we proposed an alternate operation of solvent winterization and enzymatic interesterification as efficient method to enrich the content of omega-3 fatty acids in form of TAG within CLO (Fig. 1). The process of winterization was optimized, while the enzymatic interesterification was investigated with different lipases (Novozyme 435, Lipozyme RM IM and Lipozyme TL IM) under different conditions. ^{1}H NMR analysis was employed to quantify the content of omega-3 PUFA, and GC–MS to profile the fatty acid compositions. Such combination of these two simple food techniques provide considerable efficiency and reduce production cost drastically. Moreover, oil produced in this method is not exposed to high temperatures and thus its quality would be significantly better than those obtained by fractional distillation, which is of particular importance for the development of omega-3 based active pharmaceutical ingredients.

Fig. 1 Illustration of the proposed method to enrich omega-3 PUFA in CLO in TAG form via alternate operation of solvent winterization and enzymatic interesterification. (P, palmitic; O, oleic; D, DHA. The designation POD, etc., does not imply the TAG POD alone, but a mixture of all positional isomers — POD, ODP and PDO, etc.).

2 Materials and methods

2.1 Materials

Arctic cod liver oil was purchased from Nordic Naturals® (Watsonville, CA, USA). Lipozyme RM IM (*Rhizomucor miehei* lipase, immobilized on anion exchange resin), Lipozyme TL IM (lipase from *Thermomyces lanuginosa*) and Novozyme 435 (Lipase acrylic resin from *Candida antarctica* B) were generous gifts from Novo Nordisk A/S (Bagsvaerd, Denmark). Sodium methoxide, sodium hydroxide, and boron trifluoride-methanol solution (14% in methanol) were obtained from Sigma-Aldrich® (Singapore). Acetone, hexane, ethanol and methanol were acquired from RCI Labscan Limited (Bangkok, Thailand). Deuterated chloroform ($CDCl_3$) containing tetramethylsilane (TMS, v/v 0.03%) was purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA).

2.2 Preliminary study

2.2.1 Solvent winterization

CLO (~1 g) was treated with different amounts of organic solvent (hexane, acetone or ethanol) in 15 mL centrifuge vial and winterized at different test temperatures (–80 and –20 °C) for a certain period of time. Samples were centrifuged at 6000 rpm for 2 min in a Hettich EBA 20 centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), followed by storage at the winterization temperature for another 3 min. Then the solid and liquid phases (SP and LP, respectively) were separated by decantation and subjected to rotary evaporation at 35 °C for solvent removal. Recoveries of the winterized oil were calculated with the following equation:

$$\text{Recovery}(\%) = \frac{\text{weight of dry fraction from LP}}{\text{weight of CLO before winterization}} \times 100$$

2.2.2 Enzymatic interesterification

A certain amount of the winterized CLO were added into a round bottom flask and sealed with N_2 or under vacuum at test temperature (30, 40, 50 and 60 °C) for 5 min. The interesterification reaction started immediately after the addition of lipase (5 wt%) under stirring at 300 rpm, and ended when the lipase was removed by filtration. Time course of the reaction was monitored in a series of batches under the optimal reaction conditions determined thereof.

2.3 Optimized method

In a further study, the separation of LP from SP after winterization was conducted by Büchner vacuum filtration where the funnel was precooled to the winterization temperature for at least one hour. The improved operation was merely applied to assays that provided best results in the preliminary study (0.1 g/mL oil/acetone, 24 h) while the winterization temperatures (–80 and –20 °C) were reevaluated. For the enzymatic interesterification, preliminarily optimized conditions were maintained (Lipozyme TL IM, 40 °C, 2.5 h).

2.4 Quantification of omega-3 PUFA content by ^{1}H NMR

Aliquots were collected from both SP and LP concentrates and dissolved in $CDCl_3$ at a ratio of 1:9 by volume and analyzed in 5 mm NMR tubes. Chemical shifts were referred indirectly to TMS signal (δ = 0.0 ppm) by calibrating the residual signal from CHD_2Cl_3 to 7.26 ppm. ^{1}H NMR spectra were recorded on Bruker Avance 400 spectrometer operating at 400 MHz with QNP probe (5 mm). Figures were processed with Bruker's TopSpin™ software.

Total content of omega-3 PUFA in the oil sample was determined by the following equation (Sacchi, Medina, Aubourg, Addeo, & Paolillo, 1993):

$$\text{Content(mol\%)} = \frac{m(\omega\text{-}3)}{m(\omega\text{-}3) + m(\text{non-}\omega\text{-}3)} \times 100 = \frac{A(0.96)}{A(0.96) + A(0.87)} \times 100$$

Where m(ω-3) and m(non-ω-3) indicate the molar amount of omega-3 fatty acid moieties and non-omega-3 fatty acid moieties, respectively; A(0.96) and A(0.87) indicate the area of the triplet at 0.96 ppm and that of the multiplet at 0.87 ppm, respectively. Each set of experiment was duplicated and the data shown are expressed as the mean values (within 3% relative standard deviation).

2.5 Fatty acid composition analysis by GC–MS

The fatty acid profiles of origin CLO and winterized oil product were analyzed after conversion to their fatty acid methyl esters (FAME) through boron trifluoride-methanol method (Vazquez & Akoh, 2012). About 70 mg of oil was weighed into a sample vial, and 2 mL of 0.5 M NaOH methanol solution was added. Blanketing with N_2, the vial was caped tightly and incubated at 100 °C for 5 min to saponify the lipid. After cooling, 2 mL of 14% boron trifluoride methanol solution were added and the sample was vortexed for 1 min after blanketing with N_2. Incubation at 100 °C for 5 min allowed the methylation. After the reaction, the FAME was extracted by adding 2 mL hexane and 2 mL saturated NaCl solution, followed by vortex for 2 min and centrifugation for 3 min at 2000 rpm. The upper organic phase was treated with anhydrous sodium sulphate and then prepared for GC–MS analysis.

The samples were analyzed on a Thermo TR-5MS column (30 m × 0.25 mm I.D. × 0.25 μm film), in a ThermoFinnigan PolarisQ MS system which is equipped with Thermo Trace GC. The injector and detector temperature were set at 250 °C, and the column temperature was programmed from 170 °C to 215 °C at 5 °C/min (holding for 1 min at 215 °C), from 215 °C to 220 °C at 1 °C/min, from 220 °C to 240 °C at 2 °C/min, and from 240 °C to 290 °C at 20 °C/min. The injection volume was 0.5 μL and sample concentration was 1 ppm. Each assay was analyzed by duplicating two independent experiments.

3 Results and discussion

3.1 Quantification of omega-3 PUFA content by ^{1}H NMR

Fig. 2 shows the ^{1}H NMR spectrum of origin CLO, in which the chemical shifts of the methyl protons of omega-3 fatty acids (H^a) are at ~0.96 ppm as a triplet while those of non-omega-3 fatty acids (H^b) are at ~0.87 ppm as a multiplet, and no overlap has been observed. This is due to the different chemical environments around the terminal protons between omega-3 fatty acid moieties and non-omega-3 fatty acid moieties. Specifically, the methyl protons of omega-3 fatty acids are positioned closer to omega-3 carbon–carbon double bonds while the ones in non-omega-3 fatty acids are either far away from omega-3 carbon–carbon double bonds or have no connection with carbon–carbon double bonds at all. Direct determination of total omega-3 PUFA content in oil samples can therefore be realized by the quantitative ^{1}H NMR method described in Section 2.4. It has already been well-studied by different research groups and good agreement have been achieved between ^{1}H NMR data and those obtained by GC (Igarashi et al., 2000; Nestor et al., 2010; Sacchi et al., 1993; Tyl, Brecker, & Wagner, 2008). Though GC is the routine method for fatty acid composition analysis, it requires prior conversion of TAG to methyl esters and standards for the identification of components. Whereas, with this ^{1}H NMR method, the lipid profile analysis can be performed using the oil directly and no internal standard was required, since the it is based on structure-specific spectroscopic measurement (Sacchi et al., 1993). In this light, we suggest ^{1}H NMR is an efficient and convenient method to quantify the content of omega-3 PUFA in oils.

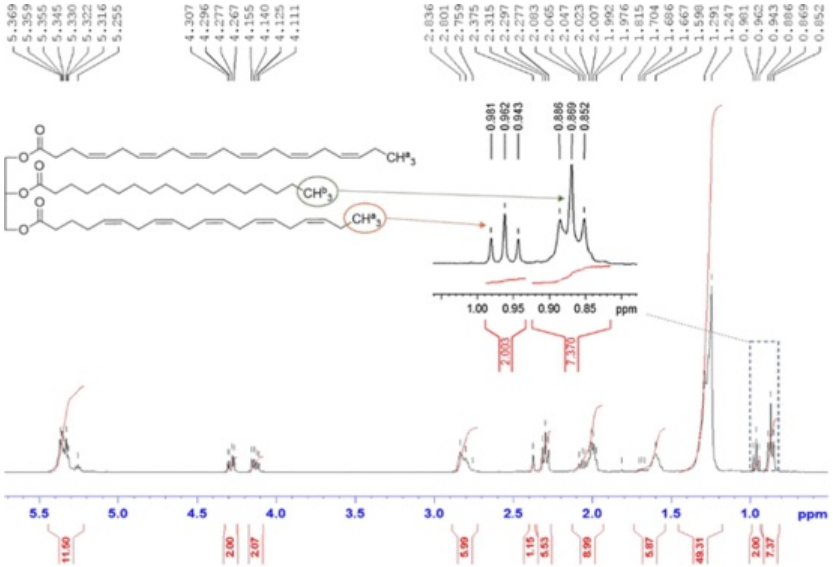

Fig. 2 ^{1}H NMR spectrum of the original CLO (400 MHz, 8 scans, $CDCl_3$) and the difference between the chemical shifts of terminal methyl hydrogen atoms in omega-3 fatty acid moieties and non-omega-3 fatty acid moieties in δ 0.80–1.00 regions. Assignments are as follows: δ(ppm) = 5.35 (m, 11.50H, —CH=CH— and —CH_2C**H**CH_2—), 4.28 (dd, J_1 = 12.0 Hz, J_2 = 4.4 Hz, 2.00H, —C**H**$_2$CHC**H**$_2$—), 4.15 (dd, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 2.07H, —C**H**$_2$CHC**H**$_2$—), 2.80 (m, 5.99H, =CHC**H**$_2$CH=), 2.38 (m, 1.15H, =CHC**H**$_2$C**H**$_2$COOR of DHA), 2.30 (m, 5.53H, —C**H**$_2$COOR of non-DHA fatty acids), 2.00 (m, 8.99H, —C**H**$_2$CH = CH—), 1.60 (m, 5.87H, —C**H**$_2$C**H**$_2$COOR of non-DHA fatty acids), 1.25 (m, 49.31H, —$(CH_2)_n$—), 0.96 (t, J = 7.6 Hz, 2.00H, —CH_3 of omega-3 PUFA), 0.87 (t, J = 6.8 Hz, 7.37H, —CH_3 of non-omega-3 fatty acids).

In order to improve precision, it is important to control the sample concentration and perform good shimming on the NMR instrument to avoid signal overlap (Barison et al., 2010). As shown in Table 1, results from our ^{1}H NMR analysis were equivalent with those from GC–MS. Opposite to the observations reported by other researchers, slightly higher values were obtained with ^{1}H NMR (Igarashi et al., 2000; Nestor et al., 2010). Oxidation of the oil sample during preparation of FAME before GC–MS is believed to contribute to these deviations.

Table 1 Composition of origin CLO and winterized oil product obtained from the optimized enrichment method analyzed by GC–MS and total omega-3 PUFA content analyzed by ^{1}H NMR.

Fatty acids	R_t (min)	Content of origin CLO		Content of winterized oil	
		(wt%)	(mol%)	(wt%)	(mol%)
C(14:0)	8.08 ± 0.02	4.03	4.91	0.85	1.06
C(16:1) ω-7	10.62 ± 0.02	12.57	13.81	16.04	18.15
C(16:0)	10.91 ± 0.02	16.58	18.07	0.83	0.93
C(18:4) ω-3	13.49 ± 0.02	3.49	3.54	5.25	5.48
C(18:2) ω-6	13.62 ± 0.02	0.96	0.96	1.67	1.72
C(18:1) ω-9	13.72 ± 0.02	20.65	20.54	23.73	24.30
C(18:1) ω-7	13.82 ± 0.02	5.19	5.16	5.18	5.31
C(18:0)	14.21 ± 0.04	1.83	1.80	nd	nd
C(20:5) ω-3	17.32 ± 0.02	10.53	9.81	21.08	20.23
C(20:1) ω-9 + 11	18.22 ± 0.02	13.69	12.44	6.89	6.45
C(22:6) ω-3	22.68 ± 0.02	7.63	6.57	17.83	15.81

C(22:1) ω-9 + 11 + 13	24.15 ± 0.04	2.85	2.39	0.65	0.56
∑SFA (GC–MS)	–	22.43	24.78	1.68	2.00
∑MUFA (GC–MS)	–	54.96	54.34	52.49	54.76
∑PUFA (GC–MS)	–	22.60	20.88	45.83	43.24
∑ω-3 PUFA (GC–MS)	–	21.65	19.92	44.16	41.52
∑ω-3 PUFA (NMR)	–	–	21.30	–	43.20

nd – not detected.

3.2 Preliminary study

3.2.1 Solvent winterization

Since acetone, hexane and ethanol are permitted organic solvent for food sector (Vazquez & Akoh, 2012), they were evaluated for the current winterization under different temperatures (–20 and –80 °C) when oil concentration and time were fixed. As shown in Fig. 3a, increments in the omega-3 PUFA content were not prominent in all assay, except for the one with acetone at –20 °C (4.33 mol%, recovery 68%). Such outperformance of acetone could be explained by its proper polarity which can deliver better selectivity on different TAG molecules while keeping separation process effective (Shahidi & Wanasundara, 1998). Hexane, with less polarity, remained such a high amount of oil in the LP that little was left in the SP, resulting in an ineffective separation (Vazquez & Akoh, 2012). In contrast, the polarity of ethanol decreased the solubility of oil molecules and thus diminished the selectivity, in consideration of the inconspicuous increment of omega-3 PUFA content with a particularly low recovery. Additionally, it was observed that all solvents winterized at –80 °C shared similarly low increments with high recoveries. Possible explanation refers to Fourier's law that heat transfer rate is proportional to the temperature difference within the same system. In this case, the big gap of temperature (from –80 °C to room temperature) accelerated the decalescence of winterized oil sample during the two-minute centrifugation, leading to fast thawing and futile winterization. Therefore acetone and –20 °C were chosen as the winterization solvent and temperature, respectively, for the rest of preliminary study.

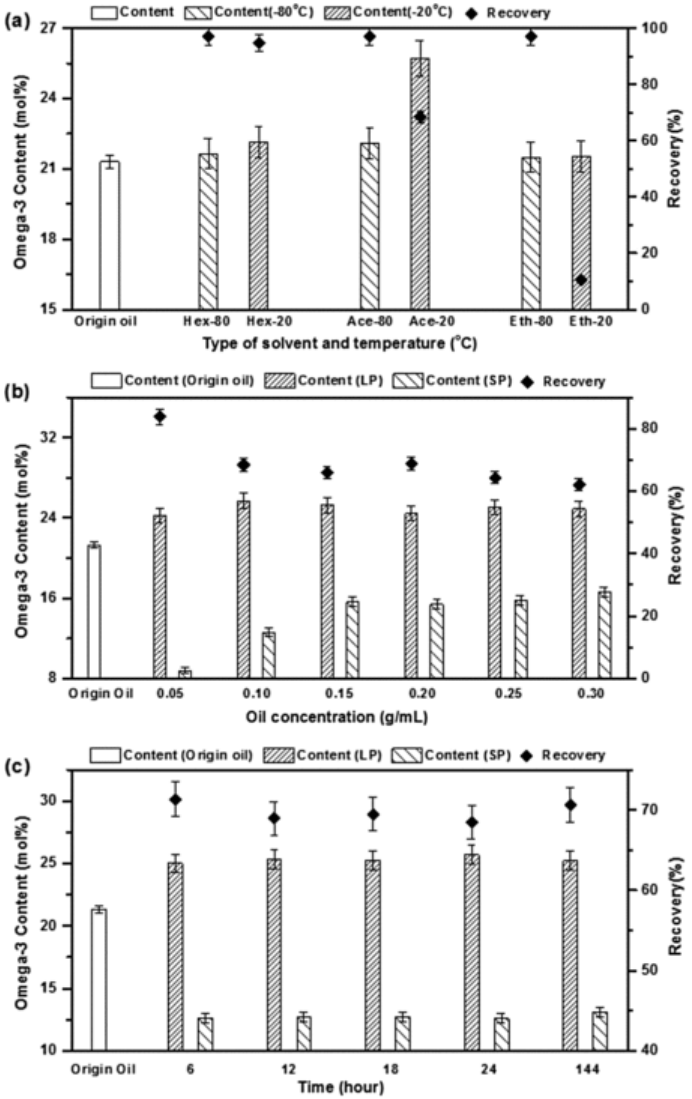

Fig. 3 Preliminary study on the effects of (a) type of solvent and temperature, (b) oil concentration in acetone solution, and (c) time length on the solvent winterization. Conditions: (Hex-20/80) hexane at –20/–80 °C; (Ace-20/80) acetone at –20/–80 °C; (Eth-20/80) ethanol at –20/–80 °C. SP, solid phase; LP, liquid phase.

Different oil concentrations for winterization in acetone were then examined as shown in Fig. 3b. The highest content increment of omega-3 PUFA (25.70 mol%) was attained in 0.1 g/mL assay, whereas further increase in oil concentrations elevated the value in SP rather than that in LP. This observation agrees well with that reported by López-Martínez et al., who suggested low oil concentration (10 wt%) would produce a meta-stable solution which promotes spontaneous crystal growth, while high oil content would generate supersaturated solution in which nucleation is favored against crystal growth and thus loses the selectivity upon different TAG molecules (López-Martínez, Campra-Madrid, & Guil-Guerrero, 2004). In Addition, samples with higher oil concentration were found to unfreeze faster during the centrifugation, causing a further decrease in the efficiency of separation. Although a concentration of 0.05 g/mL gave the lowest contents of omega-3 PUFA in SP, indicating an efficient removal of non-omega-3 fatty acids, the enrichment of omega-3 PUFA in LP was not efficient (24.26 mol%). Hence, 0.1 g/mL was determined as the optimal oil concentration.

The 0.1 g/mL oil/acetone solutions were then winterized at –20 °C for different time length. As shown in Fig. 3c, there is no significant difference in their results (ranging from 25.01 – to 25.70 mol%), suggesting that time is not a major variable in the

current solvent winterization. Similar conclusion was also drawn by Luis Vazquez and Casimir Akoh regarding the solvent winterization of modified soybean oil to enrich stearidonic acid content (Vazquez & Akoh, 2012). Since the 24 h assay provided the largest omega-3 PUFA content difference between LP and SP, suggesting a slightly better separation, it was selected as the optimal winterization time length.

3.2.2 Enzymatic interesterification

An stirring speed of 300 rpm was maintained in the current study as it is high enough for all the tested lipases to minimize mass transfer limitations as well as significant mechanical destruction on the lipase carrier (Criado, Hernández-Martín, López-Hernández, & Otero, 2007; Criado, Hernández-Martín, & Otero, 2007; Otero, Márquez, Criado, & Hernández-Martín, 2012). The lipase load was set at 5 wt% to maintain the catalytic efficiency and minimize the hindrance to mass transfer between substrates and lipase caused by high viscosity when high dosage of lipase was charged in oil reaction system (Arifin et al., 2012; Cao et al., 2013; Kristensen, Xu, & Mu, 2005). Interesterification efficiency was evaluated using the omega-3 PUFA content increment in next round of winterization.

Firstly, three immobilized lipases were screened for their catalytic efficiency on the current interesterification. Since omega-3 PUFA are highly vulnerable to oxidation and can generate genotoxic and cytotoxic compounds (Awada et al., 2012), the reaction was carried out under vacuum or in N_2 flow (Zhang et al., 2001). As shown in Fig. 4a, both Lipozyme TL IM and Lipozyme RM IM provided higher increment of omega-3 PUFA content in N_2 flow, while Novozyme 435 behaved contrarily. The reason might relate to the water content in lipase during reaction, seeing that the main difference between these two conditions is N_2 flow remains higher water content within the reaction system than vacuum. According to literatures, all the three lipases require a certain amount of water to maintain their enzyme activity, whereas a low water content is preferred in order to minimize the formation of by-products (Kowalska, Bekas, Kowalska, Lobacz, & Kowalski, 2007; Zhang et al., 2001). Specifically, Novozyme 435 is reported to perform better in drier condition (2 wt% water content), while Lipozyme TL IM and Lipozyme RM IM need slightly higher water content (3 and 4 wt%, respectively) during an interesterification reaction (Kowalska et al., 2007; Zhang et al., 2001), in accordance with our observations. Hence, vacuum was employed for Novozyme 435, and N_2 flow for the two Lipozymes in following studies.

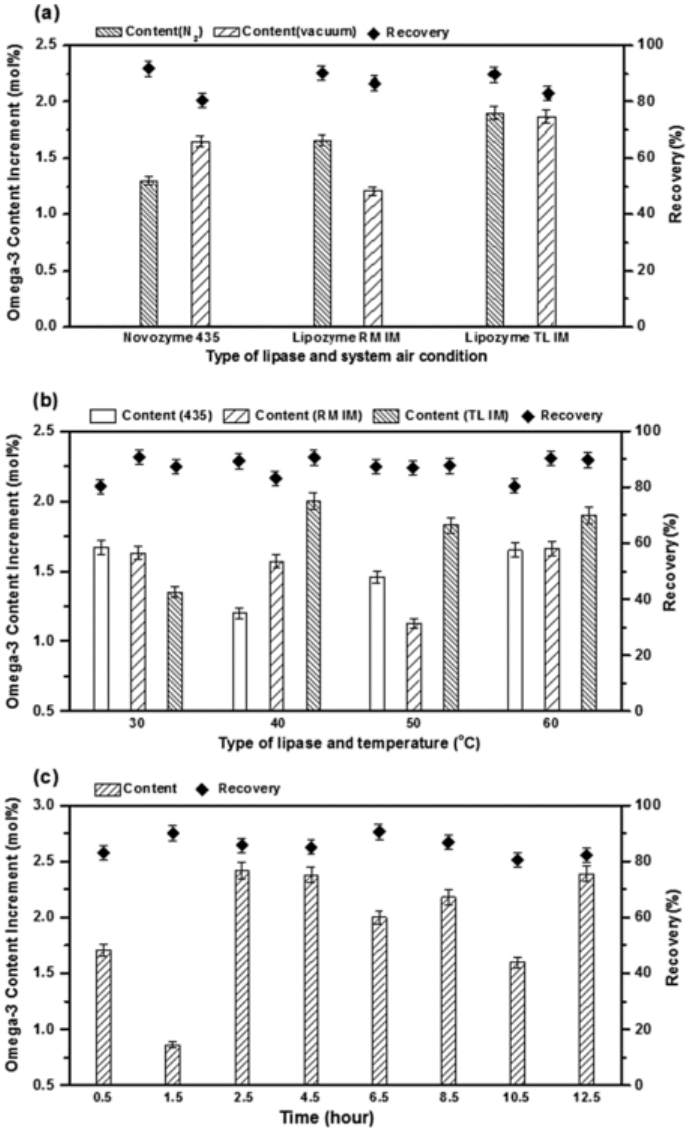

Fig. 4 Preliminary study on the effects of (a) type of lipase and reaction system air condition, (b) type of lipase and temperature, and (c) time length on the enzymatic interesterification. Reaction conditions: (a) 60 °C, 6 h; (b) 6 h, vacuum for Novozyme 435, and N_2 flow for Lipozyme TL IM and Lipozyme RM IM; (c) 40 °C, N_2 flow, Lipozyme TL IM. Lipase load of 5 wt% and agitation speed of 300 rpm were applied to all.

It is important to determine the proper temperature for each lipase to ensure its operational stability and catalytic activity (Xu, Fomuso, & Akoh, 2000), hence the three lipases are subjected to interesterification reactions at different temperatures. As shown in Fig. 4b, both of Novozyme 435 and Lipozyme RM IM provided moderate increments in omega-3 PUFA content, except for an exceptional low value at 40 and 50 °C, respectively. In the case of Lipozyme TL IM, it clearly preferred higher temperature (40–60 °C), equivalent to other's observation (Otero et al., 2012). Obviously, Lipozyme TL IM outperformed the other two lipases, and the highest increment of omega-3 PUFA content was 2.00 mol% (recovery 91%) achieved at 40 °C reaction. Based on these results, Lipozyme TL IM and 40 °C was selected for further studies.

Time course of the interesterification was recorded when Lipozyme TL IM was employed under its optimized reaction conditions (Fig. 4c). The maximum increment of omega-3 PUFA content (2.42 mol%) was obtained after 2.5 h reaction, suggesting a

higher amount of TAG molecules with higher melting point fatty acids was generated and subsequently removed by the second round of winterization. Although the rearrangement of fatty acid distribution during the interesterification catalyzed by Lipozyme TL IM is not as random as that by Novozyme 435 (Kowalska et al., 2007), our results revealed the possibility to make it favor the formation of TAG molecules with higher melting point fatty acids through time control. Therefore, 2.5 h was determined to be the optimum reaction time.

3.3 Optimized method

It was found that the overall increment of omega-3 PUFA content was not ideal, considering the omega-3 PUFA content only reached 30.43 mol% after five rounds of winterization as shown in Fig. 5. The less efficient separation after winterization was believed to be the main defect, since the thawing of TAG crystals during centrifugation could not be neglected. To address this problem, the separation process was improved by involving a precooled Büchner vacuum filtration. Benefits of such procedure include: (1) eliminating the temperature difference that the oil solution experienced and (2) shortening the time for separation, both of which contribute significantly to the prevention of crystal unfreezing.

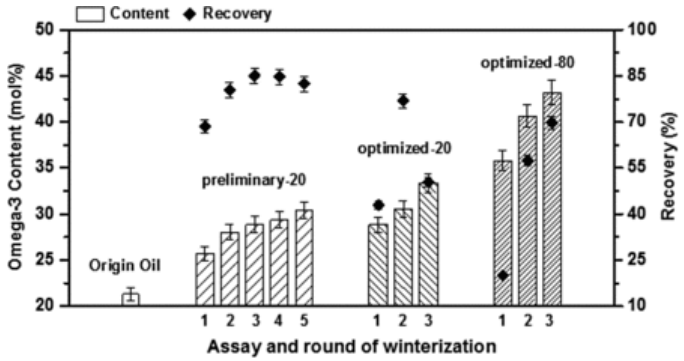

Fig. 5 Comparison between preliminarily studied method and optimized methods for their efficiency in omega-3 PUFA enrichment in CLO. Assay conditions: (preliminary-20) −20 °C, (optimized-20) −20 °C, (optimized-80) −80 °C. Oil/acetone concentration of 0.1 g/mL and 24 h applied to all winterization. Lipozyme TL IM (5 wt%), 40 °C, 2.5 h, N_2 flow and 300 rpm were applied to all interesterification.

Fig. 5 clearly shows better efficiency in concentrating omega-3 PUFA in CLO with the improved separation process. Winterization at −20 °C raised the omega-3 PUFA content to 28.82 mol% from origin CLO, and on this basis, to 33.34 mol% after another two rounds winterization. It should be noticed that there is moderate decrease in the recovery of each winterization, indicating more oil crystals were reserved during the separation and removed afterwards. This observation positively verified that precooled Büchner vacuum filtration was more efficient in preventing the thawing of oil crystal. In the case of −80 °C, the omega-3 PUFA content was boosted up to 35.78 mol% after the first winterization. Although the recovery was as low as 20%, it is still more efficient than the −20 °C assay, considering the net recovery of three times of winterization was only 17% with 33.34 mol% omega-3 PUFA in the oil product. Remarkably, a value of 43.20 mol% was achieved after another two rounds of winterization, which is the double of original level (21.30 mol%), suggesting −80 °C winterization is more efficient to enrich omega-3 PUFA in CLO.

Additionally, time was found to have an influence on the winterization efficiency, contrary to the previous conclusion in preliminary study. No oil crystal has been observed after refrigeration at −80 °C for 4 h during the third winterization, while a lot was obtained 20 h later. Further extension would hardly increase the crystal amount (data not shown), hence the optimal winterization time was still fixed as 24 h.

The experiment was scaled up to 50 g of oil, and no significant differences in the omega-3 PUFA content increment and recovery were detected. Hence the results gained at different reaction scales was combined in the study.

3.4 Fatty acid composition analysis of winterized CLO

The fatty acid profiles of origin CLO and the winterized oil product obtained from optimized enrichment method were analyzed by GC–MS. Based on the weight proportion (wt%) of each fatty acid obtained from GC and their molecular weight, calculations were made to convert the data into molar proportions (mol%). As shown in Table 1, the content of omega-3 PUFA was successfully enriched to 41.52 mol% (15.81 mol% DHA and 20.23 mol% EPA), equivalent with the result from ^{1}H NMR analysis (43.20 mol%). The majority of SFA was removed and only 2.00 mol% was left in the winterized oil product, while MUFA maintained the same content level as origin CLO (54.76 and 54.34 mol%, respectively), suggesting that the proposed method is efficient to concentrate PUFA, especially omega-3 PUFA in the case of CLO, by removing SFA.

4 Conclusions

The content of omega-3 PUFA in CLO has been successfully doubled to 43.20 mol% via the proposed alternate operation of solvent winterization and enzymatic interesterification. Non-omega-3 fatty acid fractions, especially SFA, were efficiently removed after winterization at −80 °C for 24 h in 0.1 g/mL oil/acetone solution. Meanwhile, Lipozyme TL IM catalyzed interesterification reaction at 40 °C for 2.5 h with N_2 flow, could favor the re-distribution of non-omega-3 fatty acid

fractions to form TAG molecules with higher melting point, facilitating their removal in the following winterization. DHA (15.81 mol%) and EPA (20.23 mol%) were the main omega-3 PUFA concentrated in the obtained oil product.

Our method realized the enrichment of omega-3 PUFA on the basis of two simple food techniques, and avoided costly separation and purification. Meanwhile, the method using ^{1}H NMR to determine omega-3 PUFA content simplified the characterization process greatly. Moreover, oil produced in this method is not exposed to high temperatures and thus possesses significantly better quality, which is of particular importance for the development of omega-3 based active pharmaceutical ingredients.

Acknowledgements

The authors wish to express their gratitude to Nanyang Technological University for financial supports through research grants, M4011062.110 (MOE Tier 1), M4011125.110 (MOE Tier 1), M4020244.110 (MOE Tier 2), M4081461.110 (iFood Grant), to Tianhu Li.

References

Arifin N., Koh S.-P., Long K., Tan C.-P., Yusoff M.S.A. and Lai O.-M., Modeling and optimization of Lipozyme RM IM-catalyzed esterification of medium- and long-chain triacyglycerols (MLCT) using response surface methodology, *Food and Bioprocess Technology* **5**, 2012, 216–225.

Augustsson K., Michaud D.S., Rimm E.B., Leitzmann M.F., Stampfer M.J., Willett W.C., et al., A prospective study of intake of fish and marine fatty acids and prostate cancer, *Cancer Epidemiology, Biomarkers & Prevention* **12**, 2003, 64–67.

Awada M., Soulage C.O., Meynier A., Debard C., Plaisancié P., Benoit B., et al., Dietary oxidized n-3 PUFA induce oxidative stress and inflammation: Role of intestinal absorption of 4-HHE and reactivity in intestinal cells, *Journal of Lipid Research* **53**, 2012, 2069–2080.

Barison A., Pereira da Silva C.W., Campos F.R., Simonelli F., Lenz C.A. and Ferreira A.G., A simple methodology for the determination of fatty acid composition in edible oils through ^{1}H NMR spectroscopy, *Magnetic Resonance in Chemistry* **48**, 2010, 642–650.

Bockisch M., Fats and oils handbook, 1998, AOCS Press; Champaign.

Calder P.C., N-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases, *The American Journal of Clinical Nutrition* **83**, 2006, 1505S–1519S.

Cao Y., Qi S., Zhang Y., Wang X., Yang B. and Wang Y., Synthesis of structured lipids by lipase-catalyzed interesterification of triacetin with camellia oil methyl esters and preliminary evaluation of their plasma lipid-lowering effect in mice, *Molecules* **18**, 2013, 3733–3744.

Caygill C.P.J. and Hill M.J., Fish, n-3 fatty acids and human colorectal and breast cancer mortality, *European Journal of Cancer Prevention* **4**, 1995, 329–332.

Criado M., Hernández-Martín E., López-Hernández A. and Otero C., Enzymatic interesterification of extra virgin olive oil with a fully hydrogenated fat: Characterization of the reaction and its products, *Journal of the American Oil Chemists' Society* **84**, 2007, 717–726.

Criado M., Hernández-Martín E. and Otero C., Optimized interesterification of virgin olive oil with a fully hydrogenated fat in a batch reactor: Effect of mass transfer limitations, *European Journal of Lipid Science and Technology* **109**, 2007, 474–485.

De Deckere E.A.M., Possible beneficial effect of fish and fish n-3 polyunsaturated fatty acids in breast and colorectal cancer, *European Journal of Cancer Prevention* **8**, 1999, 213–221.

El Boustani S., Colette C., Monnier L., Descomps B., Crastes De Paulet A. and Mendy F., Enteral absorption in man of eicosapentaenoic acid in different chemical forms, *Lipids* **22**, 1987, 711–714.

Gleissman H., Johnsen J.I. and Kogner P., Omega-3 fatty acids in cancer, the protectors of good and the killers of evil?, *Experimental Cell Research* **316**, 2010, 1365–1373.

Hamazaki T., Hirai A., Terano T., Sajiki J., Kondo S., Fujita T., et al., Effects of orally administered ethyl ester of eicosapentaenoic acid (EPA; C20:5, ω-3) on PGI_2-like substance production by rat aorta, *Prostaglandins* **23**, 1982, 557–567.

Holub B.J., Docosahexaenoic acid (DHA) and cardiovascular disease risk factors, *Prostaglandins, Leukotrienes & Essential Fatty Acids* **81**, 2009, 199–204.

Homayooni B., Sahari M.A. and Barzegar M., Concentrations of omega-3 fatty acids from rainbow sardine fish oil by various methods, *International Food Research Journal* **21**, 2014, 743–748.

Huber G.M., Vasantha Rupasinghe H.P. and Shahidi F., Inhibition of oxidation of omega-3 polyunsaturated fatty acids and fish oil by quercetin glycosides, *Food Chemistry* **117**, 2009, 290–295.

Igarashi T., Aursand M., Hirata Y., Gribbestad I.S., Wada S. and Nonaka M., Nondestructive quantitative determination of docosahexaenoic acid and n-3 fatty acids in fish oils by high-resolution ^{1}H nuclear magnetic resonance spectroscopy, *Journal of the American Oil Chemists' Society* **77**, 2000, 737–748.

Jacobsen C., Fish oils: Composition and health effects, In: Caballero B., Finglas P.M. and Toldrá F., (Eds.), *Encyclopedia of Food and Health,* 2016, Academic Press; Oxford, 686–692.

Kowalska M., Bekas W., Kowalska D., Lobacz M. and Kowalski B., Modification of beef tallow stearin by chemical and enzymatic interesterification with rapeseed oil, *American Journal of Food Technology* **2**, 2007, 521–528.

Kristensen J.B., Xu X. and Mu H., Process optimization using response surface design and pilot plant production of dietary diacylglycerols by lipase-catalyzed glycerolysis, *Journal of Agricultural and Food Chemistry* **53**, 2005, 7059–7066.

Kurata S., Yamaguchi K. and Nagai M., Rapid discrimination of fatty acid composition in fats and oils by electrospray ionization mass spectrometry, *Analytical Sciences* **21**, 2005, 1457–1465.

Lawson L.D. and Hughes B.G., Human absorption of fish oil fatty acids as triacylglycerols, free acids, or ethyl esters, *Biochemical and Biophysical Research Communications* **152**, 1988, 328–335.

Lin T.J., Chen S.W. and Chang A.C., Enrichment of n-3 PUFA contents on triglycerides of fish oil by lipase-catalyzed trans-esterification under supercritical conditions, *Biochemical Engineering Journal* **29**, 2006, 27–34.

López-Martínez J.C., Campra-Madrid P. and Guil-Guerrero J.L., Γ-Linolenic acid enrichment from Borago officinalis and Echium fastuosum seed oils and fatty acids by low temperature crystallization, *Journal of Bioscience and Bioengineering* **97**, 2004, 294–298.

McNeill G.P., Ackman R.G. and Moore S.R., Lipase-catalyzed enrichment of long-chain polyunsaturated fatty acids, *Journal of the American Oil Chemists' Society* **73**, 1996, 1403–1407.

Morris M.C., Evans D.A., Tangney C.C., Bienias J.L. and Wilson R.S., Fish consumption and cognitive decline with age in a large community study, *Archives of Neurology* **62**, 2005, 1849–1853.

Nestor G., Bankefors J., Schlechtriem C., Brännäs E., Pickova J. and Sandström C., High-resolution ^{1}H magic angle spinning NMR spectroscopy of intact Arctic Char (*Salvelinus Alpinus*) muscle. Quantitative analysis of *n* – 3 fatty acids, EPA and DHA, *Journal of Agricultural and Food Chemistry* **58**, 2010, 10799–10803.

Otero C., Márquez P., Criado M. and Hernández-Martín E., Enzymatic interesterification between pine seed oil and a hydrogenated fat to prepare semi-solid fats rich in pinolenic acid and other polyunsaturated fatty acids, *Journal of the American Oil Chemists' Society* **90**, 2012, 81–90.

Rubio-Rodríguez N., Beltrán S., Jaime I., de Diego S.M., Sanz M.T. and Carballido J.R., Production of omega-3 polyunsaturated fatty acid concentrates: A review, *Innovative Food Science and Emerging Technologies* **11**, 2010, 1–12.

Sacchi R., Medina I., Aubourg S., Addeo F. and Paolillo L., Proton nuclear magnetic resonance rapid and structure-specific determination of ω-3 polyunsaturated fatty acids in fish lipids, *Journal of the American Oil Chemists' Society* **70**, 1993, 225–228.

Shahidi F. and Wanasundara U.N., Omega-3 fatty acid concentrates: Nutritional aspects and production technologies, *Trends in Food Science & Technology* **9**, 1998, 230–240.

Shin J.-A., Akoh C.C. and Lee K.-T., Enzymatic interesterification of anhydrous butterfat with flaxseed oil and palm stearin to produce low-trans spreadable fat, *Food Chemistry* **120**, 2010, 1–9.

Tengku-Rozaina T.M. and Birch E.J., Enrichment of omega-3 fatty acids of refined hoki oil, *Journal of the American Oil Chemists' Society* **90**, 2013, 1111–1119.

Torres C.F., Munir F., Blanco R.M., Otero C. and Hill C.G., Jr., Catalytic transesterification of corn oil and tristearin using immobilized lipases from *Thermomyces lanuginosa*, *Journal of the American Oil Chemists' Society* **79**, 2002, 775–781.

Tyl C.E., Brecker L. and Wagner K.H., ^{1}H NMR spectroscopy as tool to follow changes in the fatty acids of fish oils, *European Journal of Lipid Science and Technology* **110**, 2008, 141–148.

Vazquez L. and Akoh C.C., Enrichment of stearidonic acid in modified soybean oil by low temperature crystallisation, *Food Chemistry* **130**, 2012, 147–155.

Xu X., Fomuso L.B. and Akoh C.C., Synthesis of structured triacylglycerols by lipase-catalyzed acidolysis in a packed bed bioreactor, *Journal of Agricultural and Food Chemistry* **48**, 2000, 3–10.

Yang L.Y., Kuksis A. and Myher J.J., Lumenal hydrolysis of menhaden and rapeseed oils and their fatty acid methyl and ethyl esters in the rat, *Biochemistry and Cell Biology* **67**, 1989, 192–204.

Zhang H., Xu X., Nilsson J., Mu H., Adler-Nissen J. and Høy C.E., Production of margarine fats by enzymatic interesterification with silica-granulated *Thermomyces lanuginosa* lipase in a large-scale study, *Journal of the American Oil Chemists' Society* **78**, 2001, 57–64.

Highlights

- Alternate winterization and interesterification enriched omega-3 PUFA efficiently.
- The omega-3 PUFA content in cod liver oil was successfully doubled to 43.20 mol%.
- Oil was winterized in acetone for 24 h at –80 °C before precooled vacuum filtration.
- Optimum interesterification was with Lipozyme TL IM at 40 °C for 2.5 h in N_2.
- ^{1}H NMR analysis was efficient and authentic for omega-3 PUFA content determination.

Queries and Answers

Query: Your article is registered as a regular item and is being processed for inclusion in a regular issue of the journal. If this is NOT correct and your article belongs to a Special Issue/Collection please contact n.selvaraj@elsevier.com immediately prior to returning your corrections.
Answer: This is correct.

Query: The author names have been tagged as given names and surnames (surnames are highlighted in teal color). Please confirm if they have been identified correctly.
Answer: They have been identified correctly.

Query: The country name has been inserted for the affiliation. Please check, and correct if necessary.
Answer: Yes

Query: One or more sponsor names may have been edited to a standard format that enables better searching and identification of your article. Please check and correct if necessary.
Answer: These are correct.

Query: The country names of the Grant Sponsors are provided below. Please check and correct if necessary. ‘Nanyang Technological University’ - ‘Singapore’.
Answer: Correct.