Preparation of Highly Purified Stearidonic Acid from Echium Oil via an Enzymatic Method Combined with Preparative High Performance Liquid Chromatography

Ji Yeon Baik¹, Nam Ho Kim¹, Se-Wook Oh² and In-Hwan Kim^{1*}

¹ Department of Food and Nutrition, Korea University, Seoul, Republic of Korea

² Department of Food and Nutrition, Kookmin University, Seoul, 136-702, Republic of Korea

Abstract: Stearidonic acid (SDA), an *n*-3 polyunsaturated fatty acid (PUFA), can be obtained from plant origin oils and it can be a good source of PUFA for vegetarians. SDA can be easily converted to longer PUFA such as docosahexaenoic acid and eicosapentaenoic acid. Highly purified stearidonic acid (SDA) was prepared successfully from echium oil via an enzymatic method combined with preparative high performance liquid chromatography. In the 1st step, SDA enrichment was accomplished using *Candida rugosa* lipase and 39.5% of SDA was obtained in the fatty acid fraction. Subsequently, the 1st reaction mixture was used for the 2nd enzymatic esterification without any separation process. The 2nd esterification was conducted for further SDA enrichment in a packed-bed reactor using Lipozyme RM IM from *Rhizomucor miehei* and the SDA content increased in a very short residence time. Ethanol was selected as an appropriate alcohol to react as an acyl receptor, and the other conditions for SDA enrichment were optimized at 20°C of temperature, and 1:4 of molar ratio (i.e., fatty acid to ethanol). Under these conditions, 51.6% of SDA was obtained in the fatty acid fraction after a residence time of 15 min. Finally, highly purified SDA (purity, >99%) was obtained by prep-HPLC using the SDA-rich fraction obtained from the two-step lipase-catalyzed esterification.

Key words: echium oil, enzymatic method, packed-bed reactor, stearidonic acid

1 INTRODUCTION

Stearidonic acid (SDA), all-*cis*-6,9,12,15-octadecatetraenoic acid, is an *n*-3 polyunsaturated fatty acid (PUFA) and is considered to be a good source of health-promoting PUFA for vegetarians. SDA can be converted to longer chain highly unsaturated fatty acid, and it has similar biological properties to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)⁽¹⁾. There have been numerous studies of the concentration of *n*-3 PUFA such as EPA and DHA because of their physiological effects on cardioprotective mechanisms⁽²⁻⁵⁾. These *n*-3 PUFA are obtained mainly from fish oil and sea food products. However, these sources have limited applications in food, because of their poor stability, chemical safety and sustainability.

There is therefore an increasing demand for *n*-3 PUFA that can be obtained from natural plant oils, because EPA and DHA can be synthesized through a series of desaturation and elongation steps from other types of *n*-3 PUFA such as α -linolenic acid (ALA) and SDA present in vegetable

oils⁽⁶⁾. In the human body, SDA is the first metabolite in the conversion of ALA to longer chain highly unsaturated fatty acids such as EPA and DHA⁽⁷⁾. However, this conversion is inefficient because Δ 6-desaturase which is used to convert ALA to SDA is rate limiting in humans⁽⁸⁾. Because of the lack of Δ 6-desaturase, SDA can provide an efficient means of increasing EPA in human body.

SDA is found naturally in seafood and some species of seaweed, but it is a minor *n*-3 PUFA contributes to only 0.5-2% of the total fatty acids⁽⁹⁾. However, blackcurrant seed oil and oils derived from some members of the *Boraginaceae* family are rich in SDA⁽¹⁰⁾. Among these oils, echium oil can be considered to be an alternative plant source of *n*-3 PUFA because it has a unique ratio of *n*-3 PUFA to *n*-6 PUFA, and is also rich in SDA⁽⁷⁾.

Two-step lipase-catalyzed selective esterifications of fatty acids and alcohols have been used in attempts to concentrate PUFA from various oil sources. In these methods, fatty acid was separated from 1st reaction mixture and used

*Correspondence to: In-Hwan Kim, Department of Food & Nutrition Korea University, Jeongneung-Dong, Seongbuk-Gu Seoul, 136-703, Republic of Korea

E-mail: k610in@korea.ac.kr

Accepted March 9, 2015 (received for review November 5, 2014)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<http://www.jstage.jst.go.jp/browse/jos/> <http://mc.manuscriptcentral.com/jjocs>

as the substrate for the 2nd reaction. In our previous study, SDA from echium oil was enriched using a similar procedure. However, in this study, a novel strategy for SDA enrichment from echium oil by two-step lipase-catalyzed esterification was applied. In other words, the 1st reaction mixture was used as a substrate for the 2nd reaction without any separation process. The 2nd reaction for further SDA enrichment was carried out in a packed bed reactor (PBR) system. The 1st reaction mixture was prepared in a batch reactor under the optimum conditions which were determined in our previous study. The effects of alcohol, temperature and the molar ratio of fatty acid in the 1st reaction mixture to alcohol were investigated in a PBR system. Finally, highly purified SDA (purity, >99%) was prepared by prep-HPLC from a SDA-rich fatty acid obtained by two-step lipase-catalyzed esterification.

2 EXPERIMENTAL

2.1 Materials

Echium oil was purchased from De Wit Speciality Oils (Texel, Netherlands). Lipase OF from *Candida rugosa* (CRL) was purchased from Meito Sangyo Co., Ltd. (Nagoya, Japan). Lipozyme RM IM from *Rhizomucor miehei* was purchased from Novozymes, Ltd. (Seoul, Korea). Lauryl alcohol (purity, ≥ 99.0%) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Silica gel 60 for column chromatography was purchased from Merck KGaA (Darmstadt, Germany). All of other chemicals used in this study were purchased as the analytical grade unless otherwise noted.

2.2 Preparation of fatty acid from echium oil

Echium oil (150 g) was added to a solution of sodium hydroxide (60 g) in distilled water (150 mL) and ethanol (99%, 450 mL). The mixture was refluxed with stirring at 500 rpm for 1 h. Water (300 mL) was added to the saponified mixture and the aqueous layer containing the saponifiable matter was acidified by adding an aqueous solution of 6 N HCl and adjusted to pH 1 to release the free fatty acids. The upper layer containing the fatty acid was extracted into *n*-hexane (300 mL) and washed twice with distilled water (150 mL). The *n*-hexane layer containing the fatty acid was then dried over anhydrous sodium sulfate. Then *n*-hexane was removed from the fatty acid by evaporation in a rotary evaporator at 40°C. The residual *n*-hexane in the fatty acid was removed completely by nitrogen flushing in a water bath at 40°C.

2.3 1st esterification using *Candida rugosa* lipase in a batch reactor

The large-scale lipase-catalyzed esterification of the fatty acids from echium oil with lauryl alcohol was carried out in

a batch reactor using CRL according to the method reported by Baik *et al.*¹¹⁾. Briefly, equimolar amount of the fatty acid (299.18 g, 1.08 mol) and lauryl alcohol (200.82 g, 1.08 mol) were placed in a 1 L water-jacketed glass vessel and the vessel reactor was preheated to 30°C using water circulator. And 1.25 mL amount of initial water (0.25 wt% of total substrate) was added to the reaction mixture. The reaction was subsequently initiated by the addition of 10 g of enzyme (2 wt% of total substrate) and the stirring of the reaction mixture with a magnetic stirrer at 500 rpm. After the reaction time of 4 h, the reaction mixture was filtered through anhydrous sodium sulfate to remove any water and enzyme, and then stored at -40°C.

2.4 2nd esterification using Lipozyme RM IM in a PBR

Lipozyme RM IM-catalyzed selective esterification of 1st reaction mixture and ethanol was performed in a PBR system. The 1st reaction mixture consisted of lauryl ester, lauryl alcohol, and fatty acids. For the 2nd reaction, each alcohol was added based on the amount of fatty acid in the 1st reaction mixture.

A diagram of the apparatus is shown in Fig. 1. The small-scale PBR used for the 2nd reaction consisted of a stainless-steel column (length 5.1 cm and i.d. 0.48 cm) packed with 0.32 g of Lipozyme RM IM. The reaction mixture was continuously fed upward into the column using a syringe pump (Model 200, KD Scientific, New Hope, PA, USA). The column was kept in a water bath equipped with a water circulator and a digital controller (Model E 200, Lauda, Lauda-Königshofen, Germany) to maintain the reaction system at a constant temperature. Each experiment was initiated by flushing the reactor to equilibrate the system and remove bubbles in the column. The reaction mixture was fed at a rate corresponding to a reactor residence time of 1 min for 5 min (five void volumes of substrate mixture). The flow rate was then adjusted to produce the desired residence time. For each trial, samples were collected after the reactor had been conditioned with three void volumes of the substrate under the desired reaction conditions.

Fig. 1 A diagram of the packed-bed reactor (PBR) system for enzymatic reaction.

2.5 Production of highly purified SDA by prep-HPLC

Preparative HPLC (prep-HPLC) was used to produce highly purified SDA (purity, >99%) from SDA-rich fatty acid which was obtained by the two-step lipase-catalyzed esterification.

The SDA-rich fatty acid for prep-HPLC was separated from the 2nd reaction mixture according to our previous method¹¹⁾. The prep-HPLC (PU-2089; JASCO, Tokyo, Japan) system consisted of Athena C18 semi-prep column (250 × 10.0 mm i.d.; ANPEL Scientific Instrument Co., Ltd, Shanghai, China), and a Rheodyne injector with a 100 µL sample loop and photodiode array detector (MD-2018; JASCO, Tokyo, Japan). A mixture of acetonitrile, methanol, and hexane (900:80:15, v/v/v) with 0.2% of acetic acid was used as the mobile phase with a flow rate of 5.0 mL/min. The samples (200 mg) were dissolved in 1 mL of eluent and 50 µL of the solution was injected. The total running time was 15 min. Detection was performed by monitoring the absorbance signals at 208 nm.

2.6 Product analysis

30 mg of sample dissolved in a suitable amount of chloroform was loaded on a preparative TLC silica gel 60 F₂₅₄ plate (Merck KGaA, Darmstadt, Germany) and developed with petroleum ether/diethyl ether/acetic acid (100:20:1, v/v/v). The free fatty acids and fatty acid esters were detected by spraying the TLC plate with a 2,7-dichlorofluorescein

solution (0.2% (w/v) in 95% methanol). The bands corresponding to the free fatty acids and fatty acid esters were scraped off from the TLC plate and methylated with 14% BF₃ in methanol. The fatty acid methyl esters (FAMES) were analyzed using a Varian 3800 gas chromatograph (Varian Inc, Walnut Creek, CA, USA) equipped with a Supelcowax 10 fused-silica capillary column (30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA, USA) and a flame-ionization detector. The column was held at 180°C for 1 min and then heated to 210°C at a rate of 1.5°C/min. Helium was used as the carrier gas with a flow rate of 1 mL/min. The injector and detector temperatures were set at 240 and 250°C, respectively. The FAMES were identified by comparison with the retention times of standards. Heptadecanoic acid was used as an internal standard.

SDA content in the fatty acid fraction (mol%) and SDA yield in the fatty acid fraction (mol%) were calculated as follows:

$$\text{SDA content in fatty acid (mol\%)} = \frac{b}{a} \times 100$$

$$\text{SDA yield in the fatty acid (mol\%)} = \frac{b}{b+c} \times 100$$

where —

a: the mole of total fatty acid of the reaction product

b: the mole of SDA in the fatty acid of the reaction product

Table 1 Fatty acid composition (mol%) of echium oil, the fatty acid fractions obtained after *Candida rugosa* lipase-catalyzed esterification, Lipozyme RM IM-catalyzed esterification and final product obtained from prep-HPLC^a.

Fatty acids	Initial ^b	SDA-rich fraction 1 ^c	SDA-rich fraction 2 ^d	Final product ^e
C16:0	6.9 ± 0.3 ^f	4.4 ± 0.2	1.0 ± 0.1	0.0 ± 0.0
C18:0	3.3 ± 0.0	5.8 ± 0.1	0.8 ± 0.1	0.0 ± 0.0
C18:1n9	15.3 ± 0.4	4.6 ± 0.1	1.4 ± 0.1	0.0 ± 0.0
C18:1n7	0.3 ± 0.2	0.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
C18:2n6	15.0 ± 0.1	4.1 ± 0.0	1.3 ± 0.0	0.0 ± 0.0
C18:3n6	11.2 ± 0.2	32.4 ± 0.3	41.0 ± 0.5	0.1 ± 0.0
C18:3n3	33.6 ± 0.1	8.9 ± 0.1	2.9 ± 0.0	0.0 ± 0.0
C18:4n3	14.3 ± 0.3	39.5 ± 0.2	51.6 ± 0.3	99.9 ± 0.0

^a Tabular entries are the average of duplicate determinations of each sample from two experimental trials.

^b Echium oil used as a substrate.

^c Fatty acid fraction obtained after 4 h under the optimum conditions for *Candida rugosa* lipase-catalyzed esterification in a scaled-up batch reactor.

^d Fatty acid fraction obtained after 15 min under the optimum conditions for Lipozyme RM IM-catalyzed esterification in a packed-bed reactor.

^e SDA fraction obtained from SDA-rich fraction using prep-HPLC

^f Standard deviation

c: the mole of SDA in the fatty acid ester of the reaction product

3 RESULTS AND DISCUSSION

3.1 1st esterification using *Candida rugosa* lipase in a batch reactor

A large-scale version of the enzymatic esterification using CRL was conducted under the optimum conditions which were determined in our previous study¹¹⁾. The reaction mixture was consisted of fatty acid (15.7 wt%), lauryl alcohol (16.9 wt%), and lauryl ester (67.4 wt%). The fatty acid composition of fatty acid fraction from the 1st reaction is shown in Table 1. After the 1st esterification, SDA content increased from 14.3% to 39.5% in the fatty acid fraction. The 1st reaction mixture was used, without any separation process, to study the Lipozyme RM IM-catalyzed esterification in a PBR.

3.2 2nd esterification using Lipozyme RM IM in a PBR.

3.2.1 Effect of alcohol

Several studies have been performed to enrich PUFA by Lipozyme RM IM-catalyzed esterifications using various alcohols. γ -Linolenic acid (GLA) has been purified to 90% in the free fatty acid fraction after selective esterification of fatty acid from borage oil with *n*-butanol¹²⁾. Also, ethyl EPA was enriched by selective alcoholysis of ethyl esters with lauryl alcohol using Lipozyme RM IM¹³⁾.

In the present study, we performed experiments to select a suitable alcohol for the further SDA enrichment from the 1st reaction mixture via Lipozyme RM IM-catalyzed esterification in a PBR. As shown in Fig. 2, three alcohols, namely *n*-butanol, ethanol, and lauryl alcohol were used for the reactions. In these trials, temperature and molar ratio of fatty acid to alcohol were fixed at 20°C and 1:2, respectively. Each alcohol was added based on the amount of the fatty acid in the 1st reaction mixture. And the SDA content in the fatty acid fraction of 1st reaction mixture was 39.5%.

For the reaction using lauryl alcohol, the maximum SDA content of 48.0% was obtained at a residence time of 12 min, and then the SDA content slightly decreased when the residence time was further increased. For the reactions using ethanol and *n*-butanol, the SDA contents increased with increasing residence time up to 9 min, but decreased slightly after reaching the maximum levels. In the trials using ethanol and *n*-butanol, higher maximum SDA contents were observed compared with that in the experiment using lauryl alcohol. Meanwhile, there was no significant difference in the maximum SDA contents, and SDA yields between reactions using ethanol and *n*-butanol.

It is important to determine the appropriate alcohol for a lipase-catalyzed reaction¹⁴⁾. Ethanol is known to be one of

Fig. 2 Effects of alcohol on the SDA content (A) and the yield (B) in the fatty acid fraction after Lipozyme RM IM-catalyzed esterification with a molar ratio of 1:2 (fatty acid to alcohol) at 20°C as a function of residence time.

the most commonly used alcohols for enzymatic reactions, because it is safe for food applications.

Based on the results in this study, ethanol was selected as the suitable alcohol for the reaction and used as the substrate to further investigate the effect of temperature and molar ratio of fatty acid to alcohol.

3.2.2 Effect of temperature

Although high temperatures can lead to an increase in the initial reaction rate according to the Arrhenius equation, too high temperature can lead to reductions in the lipase stability and half-life¹⁵⁾. The reaction temperature has a significant impact on the reaction, and the effect differs depending on the reaction conditions, substrate, and enzyme^{3, 16)}. Therefore, selecting the optimum temperature is crucial for enzymatic reactions.

The effects of temperature as a function of residence time on SDA enrichment content were studied in the range from 15 to 40°C, and these results are shown in Fig. 3. Molar ratio of fatty acid in the 1st reaction mixture to ethanol was 1:2. The 1st reaction mixture contained lauryl

Fig. 3 Effects of temperature on the SDA content (A) and the yield (B) in the fatty acid fraction after Lipozyme RM IM-catalyzed esterification with a molar ratio of 1:2 (fatty acid to ethanol) as a function of residence time.

alcohol, and the melting point of lauryl alcohol is 24°C. The reactor was blocked by crystallization at temperatures less than 15°C as a result of the increased melting point of the total substrate mixture. For the experiments, temperatures less than 15°C were unsuitable for the reaction in the PBR system.

Although the initial reaction rate at 40°C was fastest, the SDA content decreased greatly after reaching the maximum SDA content at a residence time of 3 min. Furthermore, the yield of SDA at 40°C was significantly lower than those at other temperatures over the entire course. For the reactions conducted at 15°C, 50.1% of the maximum SDA content was obtained with 85.7% of yield at a residence time of 12 min. And 49.1% of SDA content was obtained with 85.8% of yield when reaction conducted at 20°C at a residence time of 9 min. Although the maximum SDA level at 15°C was slightly higher than that at 20°C, the yield of SDA at 20°C was higher than that at 15°C. Also, 15°C was not an appropriate reaction temperature, because more energy was required for cooling the re-

action system.

Even if the same enzyme is used in the esterification reactions, the optimum temperature can differ depending on other reaction conditions. In our previous study, the optimum temperature was selected at 30°C for the Lipozyme RM IM-catalyzed esterification in a batch reactor¹¹⁾. When a trial was carried out in a PBR system, temperature is related to the mass transfer limitations because higher temperature can change the viscosity of substrate mixture which can increase the substrate and product transfer to the enzyme particles¹⁵⁾.

It is very well known that temperature affect significantly on the mass transfer limitations because higher temperature can change the viscosity of substrate mixture which can increase the substrate and product transfer to the enzyme particles. Moreover, it has also been reported that there are differences between the mass transfer limitations of batch reaction and PBR systems¹⁷⁾. The optimum temperatures for batch reaction systems and PBR systems therefore differ because of different mass transfer limitations.

Consequently, 20°C was selected as the optimum temperature for SDA enrichment via Lipozyme RM IM-catalyzed esterification.

3.2.3 Effect of molar ratio.

There have been several studies of lipase-catalyzed esterifications using fatty acids and alcohols^{12, 13, 18)}. The appropriate molar ratio of fatty acid in the 1st reaction mixture to ethanol was examined in a PBR, and the results are shown in Fig. 4. For these trials, the temperature was fixed at 20°C and the range of molar ratio tested was between 1:1 and 1:6. The maximum SDA contents at molar ratios of 1:1 and 1:2 were significantly lower than those at other molar ratios and large decreases were observed after the residence time of 9 min. For the trials using a molar ratio of 1:3, the maximum SDA content was achieved at a residence time of 12 min and then decreased. The trial at molar ratio of 1:4 showed the maximum SDA content of 51.6% with the yield of 84.9% and SDA content slightly decreased after the reaction reached the maximum level at a residence time of 15 min.

Haraldsson *et al.*¹⁹⁾ reported that using an excess of alcohol can decrease the activities and performances of lipases, because not many lipases tolerate polar conditions. Although the maximum SDA content achieved at a molar ratio of 1:5 was almost the same as that with a molar ratio of 1:4, smaller amount of alcohol is preferred for enzymatic reactions for food application.

Consequently, a molar ratio of 1:4 was selected as the optimum condition for SDA enrichment via Lipozyme RM IM-catalyzed esterification in a PBR system considering the content and yield of SDA.

Fig. 4 Effects of molar ratio (fatty acid to ethanol) on the SDA content (A) and the yield (B) in the fatty acid fraction after Lipozyme RM IM-catalyzed esterification with ethanol at 20°C as a function of residence time.

3.3 Production of highly purified SDA by prep-HPLC

There are a number of papers on the preparation of highly purified PUFA. All of these reported methods include various pre-concentration steps, followed by isolation of valuable PUFA by liquid chromatography using analytical or prep-HPLC column. Urea complexation, fractional distillation, and low temperature crystallization are typical pre-concentration techniques^{20–22}. There are some papers on the production of the highly purified SDA from various oil sources using urea complexation as a pre-concentration step and prep-HPLC. However, urea complexation involves large amount of solvents, chemicals, and by-products and products can be obtained at low yields. Moreover, in these papers, SDA enrichments were less than 95%.

In this study, an enzymatic method was employed for pre-concentration step, and SDA was purified (purity, > 99%) by prep HPLC from the SDA-rich fraction obtained from the two-step lipase-catalyzed esterification. SDA-rich fraction was loaded onto a semi-preparative column, the resulting chromatogram is shown in **Fig. 5**. Two peaks namely F1, and F2 were identified and the retention times of these two peaks were 5.2 min. (F1), and 6.4 min. (F2). The fractions were identified as SDA (F1), and GLA (F2) by GC (**Fig. 6**). Consequently, the resulting purities of SDA, and GLA were > 99%, and 97%, respectively. We obtained ca. 0.15 g SDA (> 99% purity) and ca. 0.25 g GLA (ca. 97% purity) from 0.5 g of SDA-rich fraction 2 after 50 times of injection. This study is intended to be a first approach for the preparation of pure SDA (> 99%) by prep-HPLC.

Fig. 5 A prep-HPLC chromatogram of fatty acid fraction from the two-step lipase-catalyzed esterification. Semi-preparative column (250×10 cm, 5 μm, reverse phase C18). Mobile phase (acetonitrile : methanol : hexane = 900:80:15 (v/v/v) with 0.2% of acetic acid) with flow rate at 5.0 mL/min. Other detailed conditions see in Materials and Method.

Fig. 6 GC chromatograms of SDA (A) and GLA (B) fractions obtained by prep-HPLC.

4 CONCLUSIONS

SDA was successfully enriched via two-step enzymatic esterifications. In the 1st reaction, SDA enriched fatty acid fraction was prepared as the large-scale esterification in a batch reactor. In the 2nd reaction, Lipozyme RM IM-catalyzed esterification was performed in a PBR system. Without any separation process, 1st reaction mixture was used as substrate together with ethanol. The effects of alcohol, temperature and molar ratio were investigated, and their optimum conditions were selected considering both the content and yield of SDA. GLA was also enriched together with SDA in fatty acid fraction. After the two-step lipase-catalyzed esterification, SDA (purity, >99%) can be isolated from the reaction mixture by using prep-HPLC.

ACKNOWLEDGMENT

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science, and Technology (2013R1A1A2006050) and was a part of the project titled 'Development of rapid detection system for foodborne pathogens to strengthen the food safety and to promote the sea foods consumption' funded by the Ministry of Oceans and Fisheries (Project No. 20130281) Korea".

References

- 1) Coupland, K. Stearidonic acid: A plant produced omega-3 PUFA and a potential alternative for marine oil fatty acids. *Lipid Tech.* **20**, 152-154 (2008).
- 2) Jonzo, M. D.; Hiol, A.; Zagol, I.; Druet, D.; Comeau, L-C. Concentrates of DHA from fish oil by selective esterification of cholesterol by immobilized isoforms of lipase from *Candida rugosa*. *Enzyme Microb. Tech.* **27**, 443-450 (2000).
- 3) Li, Z-Y.; Ward, O. Lipase-catalyzed esterification of glycerol and *n*-3 polyunsaturated fatty acid concentrate in organic solvent. *J. Am. Oil Chem. Soc.* **70**, 745-748 (1993).
- 4) Lyberg, A. M.; Adlercreutz, P. Lipase-catalysed enrichment of DHA and EPA in acylglycerols resulting from squid oil ethanolsis. *Eur. J. Lipid Sci. Tech.* **110**, 317-324 (2008).
- 5) Hwang J.; Aki H.; Kawamot S.; Shigeta S.; Ono K.; Suzuki O. Enzymatic preparation of glycerides rich in docosahexaenoic acid from thraustochytrid single cell oil by *Candida rugosa* lipase. *J. Oleo Sci.* **51**, 447-455 (2002).
- 6) Wael, A.; Ahmad, M. Oleochemical industry future through biotechnology. *J. Oleo Sci.* **63**, 545-554 (2014).
- 7) Bilgiç, S.; Yeşilçubuk, N. Ş. Lipase-catalyzed acidolysis of olive oil with echium oil stearidonic acid: optimization by response surface methodology. *J. Am. Oil Chem. Soc.* **89**, 1971-1980 (2012).
- 8) Ifeduba, E. A.; Akoh, C. C. Chemoenzymatic method for producing stearidonic acid concentrates from stearidonic acid soybean oil. *J. Am. Oil Chem. Soc.* **90**, 1011-1022 (2013).
- 9) Whelan, J. Dietary stearidonic acid is a long chain (*n*-3) polyunsaturated fatty acid with potential health benefits. *J. Nutr.* **139**, 5-10 (2009).
- 10) Petrik, M. B. H.; McEntee, M. F.; Johnson, B. T.; Obukowicz, M. G.; Whelan, J. Highly unsaturated (*n*-3) fatty acids, but not α -linolenic, conjugated linoleic or γ -linolenic acids, reduce tumorigenesis in Apc^{Min/+} mice. *J. Nutr.* **130**, 2434-2443 (2000).
- 11) Baik, J. Y.; No, D. S.; Oh, S. W.; Kim, I. H. Enrichment of stearidonic acid from echium oil via a two-step lipase-catalyzed esterification. *Eur. J. Lipid Sci. Tech.* **116**, 618-626 (2014).
- 12) Jachmanian, I.; Schulte, E.; Mukherjee, K. Substrate selectivity in esterification of less common fatty acids catalysed by lipases from different sources. *Appl. Microbiol.* **44**, 563-567 (1996).
- 13) Shimada, Y.; Maruyama, K.; Sugihara, A.; Baba, T.; Komemushi, S.; Moriyama, S.; Tominaga, Y. Purification of ethyl docosahexaenoate by selective alcoholysis of fatty acid ethyl esters with immobilized *Rhizomucor miehei* lipase. *J. Am. Oil Chem. Soc.* **75**,

- 1565-1571 (1998).
- 14) De, B.; Bhattacharyya, D.; Bandhu, C. Enzymatic synthesis of fatty alcohol esters by alcoholysis. *J. Am. Oil Chem. Soc.* **76**, 451-453 (1999).
 - 15) Xu, X.; Fomuso, L. B.; Akoh, C. C. Synthesis of structured triacylglycerols by lipase-catalyzed acidolysis in a packed bed bioreactor. *J. Agr. Food Chem.* **48**, 3-10 (2000).
 - 16) Shen, Z.; Wijesundera, C. Evaluation of ethanolysis with immobilized *Candida antarctica* lipase for regiospecific analysis of triacylglycerols containing highly unsaturated fatty acids. *J. Am. Oil Chem. Soc.* **83**, 923-927 (2006).
 - 17) Xu, X.; Balchen, S.; Høy, C-E.; Adler-Nissen, J. Production of specific-structured lipids by enzymatic inter-esterification in a pilot continuous enzyme bed reactor. *J. Am. Oil Chem. Soc.* **75**, 1573-1579 (1998).
 - 18) Rahmatullah, M. S.; Shukla, V.; Mukherjee, K. γ -Linolenic acid concentrates from borage and evening primrose oil fatty acids via lipase-catalyzed esterification. *J. Am. Oil Chem. Soc.* **71**, 563-567 (1994).
 - 19) Haraldsson, G. G.; Kristinsson, B. Separation of eicosa-pentaenoic acid and docosahexaenoic acid in fish oil by kinetic resolution using lipase. *J. Am. Oil Chem. Soc.* **75**, 1551-1556 (1998).
 - 20) Grima, E. M.; Medina, A. R.; Giménez, A. G.; González, M. I. Gram-scale purification of eicosapentaenoic acid (EPA, 20:5 *n*-3) from wet *Phaeodactylum tricornutum* UTEX 640 biomass. *J. Appl. Phycol.* **8**, 359-367 (1996).
 - 21) Grima, E. M.; Pérez, J.; Camacho, F. G.; Medina, A. R.; Giménez, A. G.; Lopez Alonso, D. The production of polyunsaturated fatty acids by microalgae: from strain selection to product purification. *Process Biochem.* **30**, 711-719 (1995).
 - 22) Medina, A. R.; Grima, E. M.; Giménez, A. G.; Gonzalez, M. Downstream processing of algal polyunsaturated fatty acids. *Biotechnol. Adv.* **16**, 517-580 (1998).
-