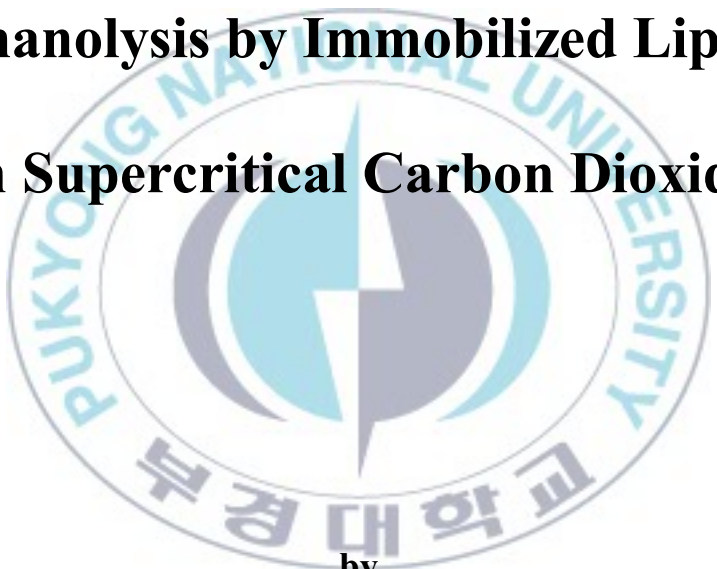
Thesis for the Degree of Master of Engineering

Characterization of Reaction Products from Krill (*Euphausia superba*) Oil Ethanolysis by Immobilized Lipase in Supercritical Carbon Dioxide



by

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Department of Food Science & Technology

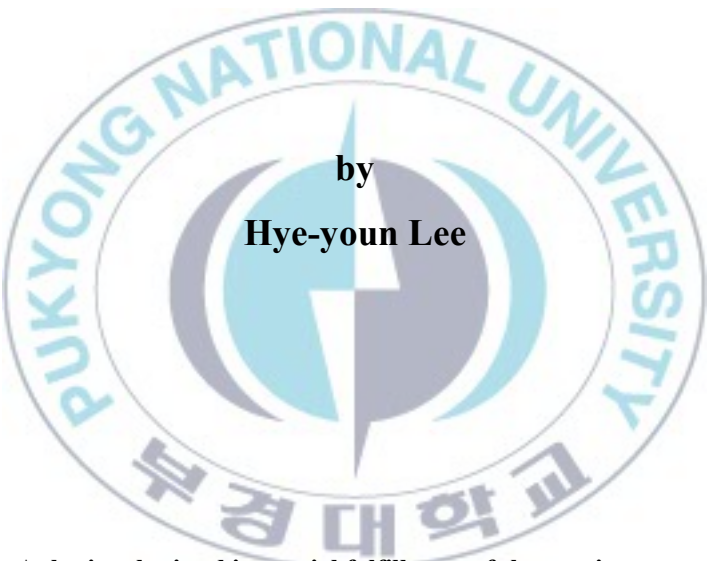
The Graduate School

Pukyong National University

February 2013

초임계 이산화탄소에서 고정화 리파아제에 의해 생성된 크릴 오일 반응물의 특성

Advisor: Prof. Byung-Soo Chun



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Contents

초임계 이산화탄소에서 고정화 리파아제에 의해 생성된 크릴 오일 반응물의 특성

이혜연

부 경 대 학 교 대 학 원 식 품 공 학 과

요 약

남극 크릴새우 (*Euphausia superba*)는 난바다곤쟁이목 난바다곤쟁이과에 속하는 종으로 남극해 생태계의 중심역할을 한다. 다른 수산자원들에 비하여 높은 수획량(60-155만톤)을 가지며 수산업에 새로운 가능성이 대두되고 있는 자원이다. 그러나 영양적인 가치에도 불구하고 현재 남극 크릴 새우는 양식 및 어업에 이용되고 있을 뿐 식생활에 이용도가 높지 않다. 수산가공산업에 있어서 남극 크릴 새우에 함유되어 있는 유용한 물질들의 이용은 경제적으로나 환경적 측면으로 볼 때, 많은 이익을 창출하게 해줄 수 있을 것으로 전망된다.

이산화탄소는 낮은 임계점과 무독성, 그리고 용매의 사용량이 적고 부식성이 없이 친환경적이기 때문에 가장 널리 이용되는 초임계 유체이며 기존의 추출법이 가지는 어려움을 해결할 수 있는 새로운 혁신기술로서 주목받고 있다.

본 논문에서는 초임계 이산화탄소를 이용하여 남극 크릴 새우 오일을 추출하였고, 추출된 크릴 오일에서 효소적 에탄올화 반응을 실시하였고 그 기능성에 대하여 연구하였다.

고도불포화 지방산 함유 diglyceride, monoglyceride 및 fatty acid methyl ester를 생성하기 위해 고정화 효소인 lipozyme TL-IM을 사용하여 크릴 오일의 효소적 에탄올화 반응을 수행하였다. 반응은 에탄올, 온도, 효소량, 반응시간에 따른 크릴 오일의 전환율을 실험해 보았고, 초임계 이산화탄소를 이용하여 추출한 오일과 일반 유

기용매를 이용하여 추출한 오일의 전환율을 비교하였다.

초임계 유체 추출은 20 MPa, 50℃에서 2시간 동안 추출한 오일을 이용하였다. 오일의 지방산 및 크릴 오일의 분자량을 계산하기 위하여 gas chromatography를 이용하여 분석하였으며 TLC나 HPLC를 이용하여 지질의 정성분석을 하였다.

상압에서는 반응된 크릴 오일의 전환율을 비교해보았을 때 같은 조건에서 에탄올의 몰함량 1배수, 반응 온도 50℃, 효소 사용량 2 w% 일 때, 최대 반응 전환율을 보였으며 초임계 이산화탄소 상태에서 반응해 보았을 때는 10 MPa, 50℃에서 최대 반응 전환율을 보였다.

반응 후 생성된 반응물의 안정성을 확인하기 위하여 산가, 과산화물가, 유리지방산 함량, 산화안정성(DPPH 라디칼 소거능, rancimat test)을 통해 항산화능을 알아보았다. 그 결과 유리지방산 함량에서는 유기용매 추출 오일보다 초임계 이산화탄소를 이용하여 추출한 크릴 오일이 안정성이 높았으며, 40℃에서 반응 조건에서 생성된 소재가 가장 안정성이 높았으며 이는 반응 온도가 높을수록 소재의 산화가 빨리 진행됨을 추측할 수 있다.

1. INTRODUCTION

1.1 Background

Antarctic krill (*Euphausia superba*), with an estimated biomass of approximately 500 million tones and with its ecological role as the most important trophic link between primary production and vertebrate predators, is incontestably the keystone species in the Antarctic marine ecosystem. Antarctic krill is a rich source of polyunsaturated fatty acids (PUFAs), mainly the long-chain omega-3 fatty acids eicosapentaenoic acid (EPA, C 20:5 n-3) and docosahexaenoic acid (DHA, C 22:5), which have been attracted much attention for health benefits. In addition, ω-3 fatty acids are essential for normal growth and development of the brain and the nervous system and may also play an important role in the prevention and treatment of coronary artery disease, hyperten-sion, arthritis, others inflammatory and autoimmune disorders, and cancer. Since PUFAs are easily oxidized, they should be preserved in the forms of triglycerides or esters. Accordingly, krill oil needs to be treated with transesterification. Alcoholysis can convert free fatty acid (FFA) into ester.

Transesterification or alcoholysis is the displacement of alcohol from an ester by another. It can enhance the value and the applicability of oils. Low-value oils

and fats can be converted to bio- diesel, trans-free fats, medium chain-length triglycerides and ω-3 polyunsaturated fatty acids (PUFAs) rich krill oils by several methods including: methanolysis, interesterification and acidolysis. Transesterification is a reversible reaction and is accelerated in the presence of a catalyst. In conventional chemical processing, the synthesis of esters by transesterification is achieved from either acid or alkaline esterification. These catalytic reactions have low selectivity and undesirable side reactions. Moreover, the process is not ecologically friendly. As an alternative, lipases have been used as biocatalysts for alcoholysis. Enzymatic catalysis by lipase allows modifying the properties of lipids by altering the locations of fatty acids in glycerides or replacing one or more of the fatty acids with new one. These exchange reactions usually work with high regio- and/or enantio-selectivity, making lipase an important group of catalysts.

Conventional methods based on solvent extraction from natural matrices are time-consuming as they involve a multiple extraction steps and require large amounts of organic solvents, which are often expensive and potentially hazardous (AOAC, 1992;SOP, 1992). The problems associated with traditional solvent extraction techniques have aroused growing interest in developing simpler, faster, more efficient methods for the extraction of carotenoids from foods and natural products (Illés et al., 1999; Careri et al., 2001). Decomposition or degradation of thermolabile compounds cannot be avoided in a conventional extraction method,

since relatively high temperatures are required for these processes. Organic solvents are also harmful to human health as well as the environment.

In recent years, supercritical fluid extraction technology (SFE) which is used as an alternative for lipid extraction to organic solvent extraction; has received much attention, because it allows a reduction in extraction time, requires little sample manipulation, and involves a much lower solvent consumption, leading to extracts of increased purity. Supercritical fluids have a relatively high liquid-like density as well as a relatively low viscosity and high diffusivity. These properties provide a unique solvent that is both effective at dissolving materials as well as penetrating solid matrices. These characteristics can provide appropriate circumstance for transesterification in terms of the coefficient of mass transfer. Lipase is well suited for applications in supercritical carbon dioxide (SC-CO_2) because their catalytic feature involves a lipid–water interface.

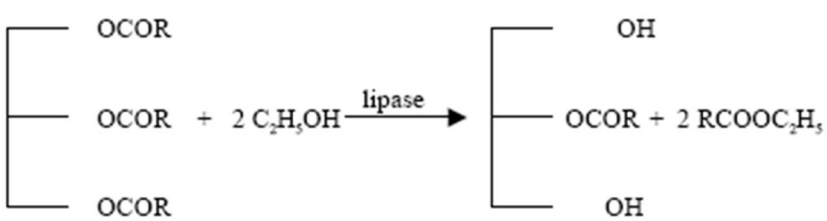


Fig. 1. Enzymatic ethanolysis of triglyceride by immobilized lipase.

1.2 Scope

The objective of this study is to analyze the reaction products from krill (*Euphausia superba*) oil ethanolysis by immobilized lipase in supercritical carbon dioxide. For this purpose, the compositions of fatty acids from extracted krill oil were evaluated by GC analysis. TLC and HPLC were used for analyzing the mixture containing tri-, di-, mono-glycerides and fatty acid esters obtained by the enzymatic ethanolysis of krill oil. In addition, the antioxidant activity of enzymatic ethanolysis reactant was compared with that of krill oil.

2. MATERIALS AND METHODS

2.1 Materials

The krill (*Euphausia superba*) were collected from Dongwon F & B Co., S. Korea. The krill blocks were stored at -80°C for no longer than 1 year before being used experimentally. Carbon dioxide (99.99% pure) was supplied by KOSEM, Korea. Immobilized commercial lipases of specifical lipozyme TL-IM (thermomuces lanuginosa immobilized on silica gel) was purchased by Novozymes (Bagsvaerd, Danmark). Lipid standard (Mono, Di, Triglyceride Mix., Supelco, USA) was used for identification. HPLC grade methanol, isopropanol, hexane and absolute ethanol (99.9%) were purchased from Burdick & Jackson.

2.2 Sample preparation

The krill samples were dried in a freeze-drier for 72 h. The dried samples were crushed and sieved (700 μm) by a mesh. The dried samples of freeze-dried raw krill were then stored at -80°C until using for SC-CO_2 and organic solvent extraction.

2.2.1 Supercritical carbon dioxide extraction

The extraction scale of SFE process used in this work in shown in Fig. 2. The krill oil was extracted by supercritical carbon dioxide for a laboratory scale. This apparatus can be operated at pressure up to 30 MPa. Thirty five grams of freeze dried krill sample were applied in 200 mL stainless steel extraction vessel containing a thin layer of cotton at the bottom. Before plugging with cap another layer of cotton was used at the top of the sample.

CO_2 was pumped to the extraction vessel by a high-pressure pump (pu-2-88, Jasco) up to the desired pressure which was regulated by a back-pressure regulator.

CO_2 was pumped into the vessel by high pressure pump up to the desired pressure, which was regulated by a back pressure regulator. The vessel temperature was maintained by heater. Flow rates and accumulated gas volume passing through the apparatus were measured using a gas flow meter. The oil was extracted by supercritical carbon dioxide (SC-CO_2) at temperature of 50°C and pressure of 20 MPa. The total extraction time was 2.5 hours. The flow rate of CO_2 was kept constant at 22 g/min for all extraction conditions and CO_2 volume passing through the apparatus were measured using a dry gas meter. The extracted oil was collected on the glass separation vessels. After that, the extracted oil was stored at -30°C until further analysis.

1 : CO_2 tank
2 : Chiller
3 : Pump
4 : Extractor
5 : Heat exchanger
6 : Thermometer
7 : Seperator
v : Safety valve
g : Pressure gauge
F : Flow meter

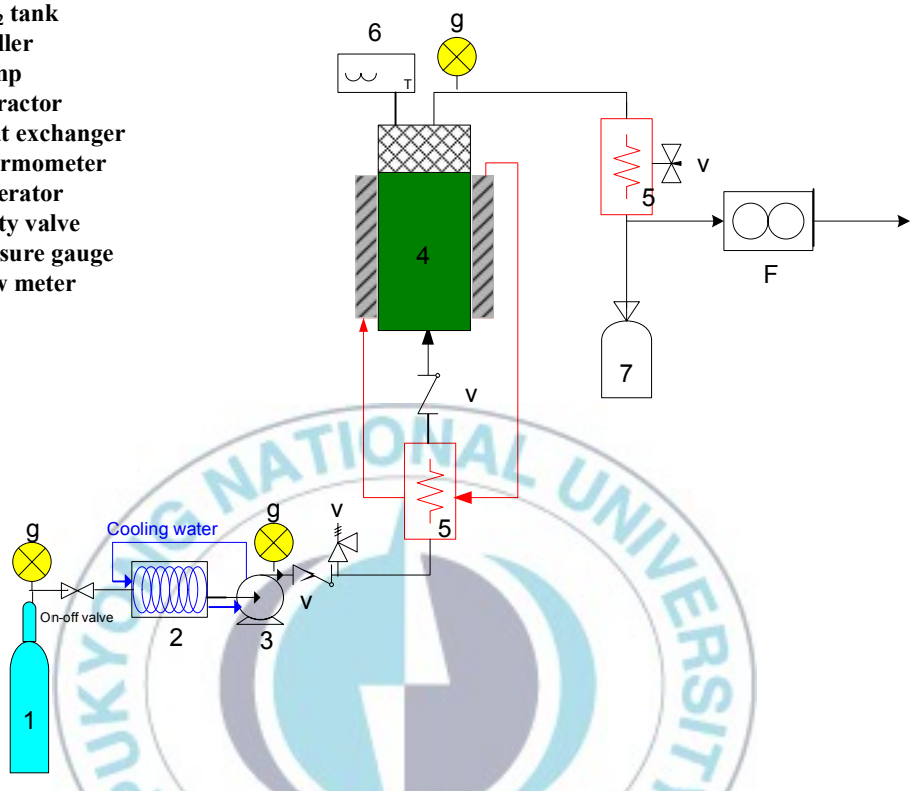


Fig. 2. The diagram of supercritical carbon dioxide extraction system.

2.2.2 Soxhlet extraction by hexane

In order to compare bioactive functionalities of krill by SC-CO_2 extraction and conventional organic solvent extraction, soxhlet extraction was selected. The extraction was carried out in a soxhlet apparatus using hexane as solvent. Three gram of the freeze dried raw krill sample was placed in a thimble holder that is gradually filled with condensed extraction solvent from a distillation flask. When the liquid of hexane layer reaches the over flow level, a siphon aspirates the solute from the thimble-holder and unloads it back into the distillation flask. Oil was extracted with 150 mL of hexane for 24 h. Soxhlet extractions were conducted in triplicate. After extraction, hexane was evaporated by a rotary vacuum evaporator (EYELA, Japan) and the obtained oil was collected in a vial. Then, krill oil was stored at -30°C until further analysis.

2.2.3 Ethanolysis of krill oil in non-pressurized system

In this experiment, krill oil and ethanol was tested for ethanolysis reaction using immobilized enzyme (Lipozyme TL-IM) in non-pressurized systems. Experiments which may affect the reaction by varying the conditions (krill oil and ethanol ratio; 1.0 to 4.0 mole ration, temperature; 40℃ to 60℃, enzyme load; 1-5 w% of krill

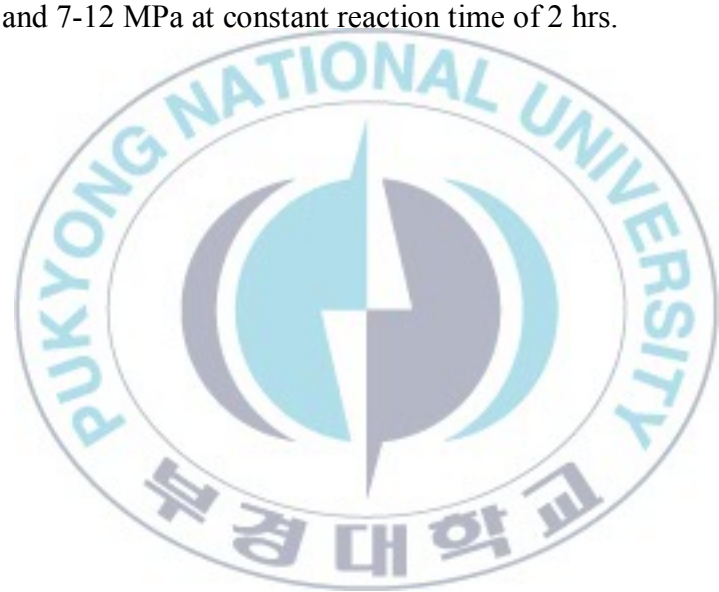
oil, reaction time; 1 to 20hr) were carried out ethanolysis. The molecular weight of krill based on the triglyceride molecular weight was 828.6g/mole. And ethanol molecular weight was 46g/mole and density of krill oil was 0.922g/ml and ethanol was 0.733g/ml. The reactions were carried out in 25ml Erlenmeyer flasks containing mixtures of 5g krill oil and ethanol (99.9%) ranging from 1.0 to 4.0 mole ratio based on krill oil using Lipozyme TL-IM.

The krill oil was mixed with ethanol for emulsification of the reaction mixture, after which immobilized lipases was added. The reaction mixture was incubated at 40 to 60^{o}C, 150 rpm in shaking incubator (LSI-3016R: Lab Tech Co., LTD, Korea). The reaction products were obtained by removing the debris and the enzyme and dissolved in the mixture of methanol, were analyzed by HPLC-UV. The reaction products were analyzed during storage in the freezer at -15^{o}C.

2.2.4 Ethanolysis of krill oil in pressurized system

The set up of a laboratory scale of SC-CO_2 system is shown in Fig. 4. The stainless steel reaction vessel which was 55 mL in internal volume (112 mm x 25 mm i.d) consists of a stainless (SS-316), sight glass, spin bar stirrer and water jacket. Samples (extracted by SC-CO_2) were loaded in to the stainless steel reaction vessel with ethanol and enzyme. After injection, reactor was closed with

reactor's head pressure was increased. The reaction temperature was maintained by connecting the reaction vessel with water bath and temperature was measured using FLUKE 52(FLUKE, USA) continuously. Samples of the effluent were taken in to sealed vials at periodic intervals (at 10, 20, 30, 60, 120 min, respectively). Finally, the reactor was depressurized to atmospheric and hexane added to the residue for washing. The effect of temperature and pressure studied at 40-60℃ and 7-12 MPa at constant reaction time of 2 hrs.



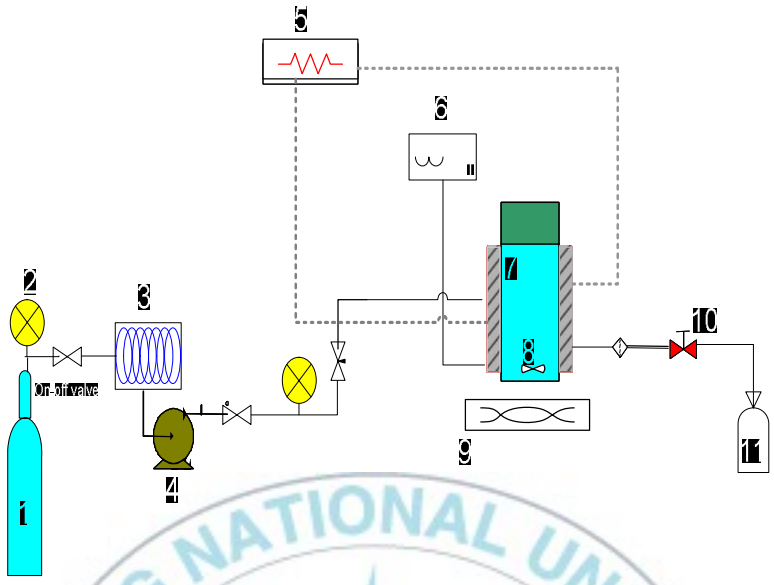


Fig. 3. Schematic diagram of the apparatus for enzymatic ethanolysis in supercritical carbon dioxide extraction system : 1. CO_2 cylinder, 2. Pressure gauge, 3. Cooling bath, 4. High pressure pump, 5. Heat exchanger, 6. Thermometer, 7. Reactor, 8. Magentic bar, 9. Stirrer, 10. Mitering valve, 11. Separator.

2.2.5 Recovery and purification of 2-monoacylglycerols

Recovery of 2-monoacylglycerols (MAGs) from the ethanolysis reaction was performed by solvent extraction following the methodology specified by Muñío et al. which is summarized in Fig. 4. First, residual ethanol were eliminated from the reaction mixture and the resulting 2-MAG–ethyl ester mixture was dissolved in an ethanol/water mixture (90:10, v/v) in the proportion 1:9 (v/v); for extracting ethyl esters this mixture was extracted three times with an equal volume of hexane. Finally the hydroethanolic solution rich in 2-MAGs was stored at -30ºC until analysis by GC.

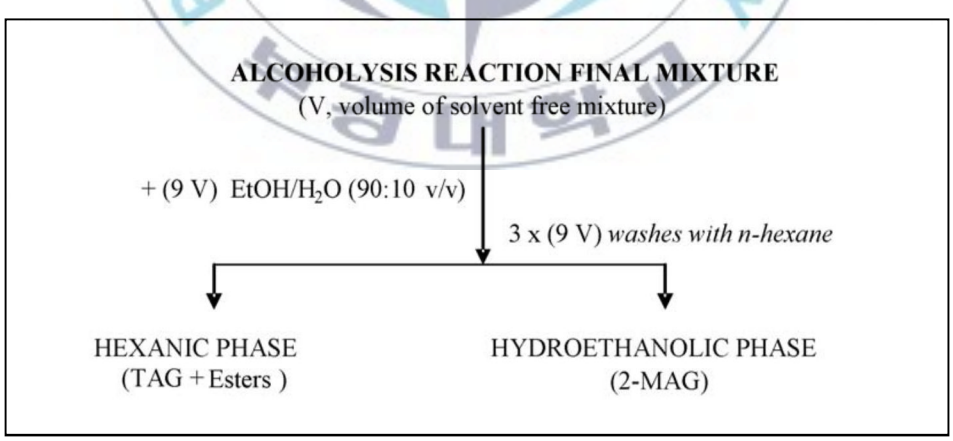


Fig. 4. Experimental procedure for the recovery of 2-MAGs from the final alcoholysis mixture by solvent extraction.

2.3 Analytical methods

2.3.1 Fatty acid analysis by gas chromatography (GC) analysis

The fatty acids profiles of both krill oil obtained by SC-CO_2 and organic solvent, hexane extraction were analyzed by GC-flame ionization detector (FID) using a Agilent Technologies 6890N gas chromatograph (Agilent Technologies, USA). The fatty acid methyl esters (FAMEs) were prepared firstly and then separated using an SPTM-2560, Fused Silica capillary column (100 m length x 0.25 mm internal diameter, 0.20 μm of film). Helium at a flow rate 0.9 ml/min was used as a carrier gas of fatty acid methyl esters. The split ratio was fixed at 10:1. The oven temperature was programmed starting at a constant temperature of 140°C for 5 min, and then increased to 240°C at a rate of 3.5°C/min and hold at 240°C for 15 min. Injector and detector temperatures were 250°C. FAMEs were identified by comparison of retention time with standard 37 Component FAMEs mixture (SuplecoTM, USA).

2.3.2 Thin layer chromatography (TLC)

TLC for the separation of lipid mixtures was performed on 20 x 20 cm aluminum foil-backed plates pre-coated with 0.2 mm layer of a silica gel 60

(ALUGRAM®SIL G/UV254; Macherey-Nagel, Germany). The lipid solutions were mixed with isopropanol/hexane (5:4, v/v; 4000 ppm). The mixtures (5 mL) were spotted onto a chromatographic plate. The lipid compounds were separated using cyclohexane/ethyl acetate (3:2, v/v) in the development tank. The development tank was saturated with solvent for 20min. The chromatogram was developed at the room temperature. After the solvent had risen to near the top of the plate (about 1cm from the top), the plate was removed. The plate was kept in the hood until the majority of the eluting solvent had evaporated from the plate about 10 min. Iodine vapor was used for the detection of the spot. Spots were visualized by iodine vapor.

2.3.3 High-performance liquid chromatography (HPLC)

HPLC analysis was carried out on a Waters HPLC equipped with a model 600 E system controller, a model 484 UV/VIS detector and a Atlantis®dC18 column (5μ, 4.6 × 150 mm, Waters, USA). The different species of lipids as tri-, di-, mono-glycerides and ethyl esters were analyzed by the method of Holcapek et al. (Table 1). The mobile phase consisted of (A) methanol and (B) isopropanol-hexane (5:4, v/v). A linear solvent gradient program was employed: at 0 min, 100/0; at 15min, 50/50; at 25min, 50/50; at 30min, 0/100 (%A/%B, respectively).

Samples (30μl) were dissolved in isopropanol-hexane (5:4, v/v) (1ml). The injection volume was 20μl and the flow-rate was 1ml/min. Mono-, di, triglycerides were detected at wavelength of 205nm. The amounts of MG, DG and TG in the extract were measured based on peak area of standard mixture of mono-, di-, triglycerides (Supleco, USA).

Table 1. HPLC conditions for the detection of mono-, di-, triglyceride.

Instrument	:	Waters 600E
Column	:	Atlantis® dC18 5 μ(4.6×150 mm, Waters, USA)
Column temp.	:	Room temp
Detector	:	Waters 484 UV detector
Wavelength	:	205 nm
Injection volume	:	20 μL
Flow rate	:	1.0 ml/min
Mobile phase	:	Methanol Hexane : Isopropanol (4:5)(v/v)

2.4 Measurement of oil stability

2.4.1 Free fatty acid content of ethanolysis reactants

Free fatty acids (FFAs) of extracted oil from ethanolysis reactants were analyzed as describe by Bernardez et al. Accurately, 50 mg of oil was placed into pyrex tubes with the addition of 3 mL of cyclohexane and then 1 mL of cupric acetate-pyridine reagent was added. Tubes were vortexed for 30 sec. After centrifugation at 2000 g for 10 min, the upper layer was read at 710 nm. The FFA content of oil was measured on a calibration curve constructed from oleic acid standard (Fig. 5). Copper reagent was prepared according to Lowry and Tinsley. Briefly, 5% (w/v) aqueous solution of cupric acetate was prepared and filtered. Then, the pH of cupric acetate solution was adjusted to pH 6.1 using pyridine.

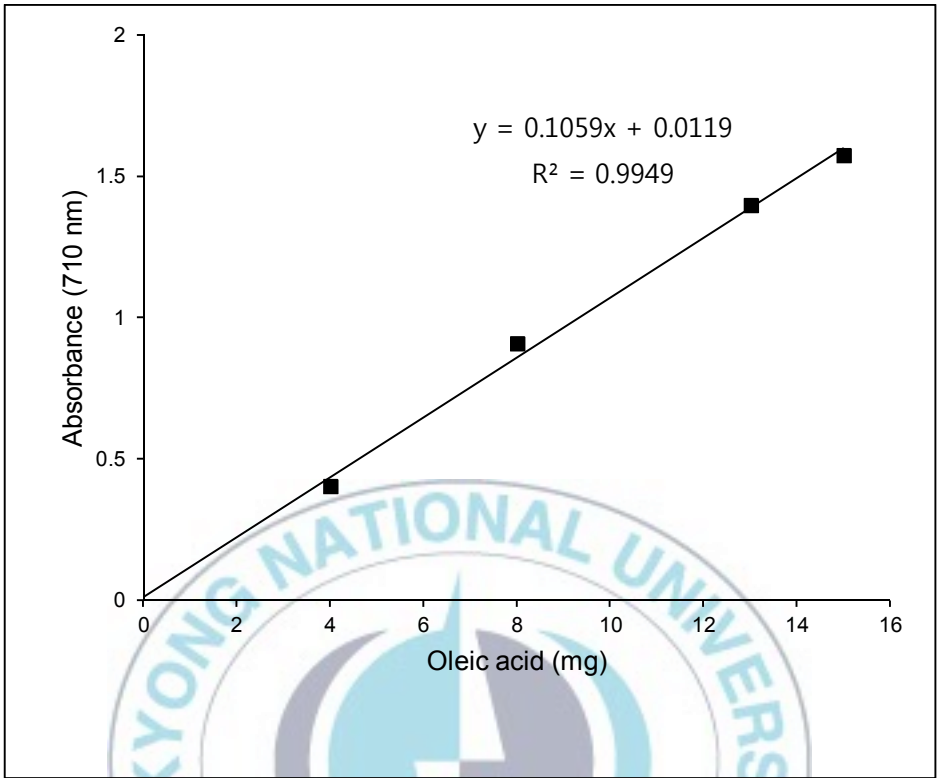


Fig. 5. Calibration curve of oleic acid for estimation of free fatty acids in ethanolysis reactants.

2.4.2 Acid value

The acid value was assessed according to the method described previously by P. Sun et al. One gram of sample was dissolved in 100 mL of ether : ethanol (1:1) and shaking. Then, phenolphthalein as an indicator was added by drop wise addition. Acid value of oil was analyzed by titration with 0.1N KOH–ethanol solution until the pink colour persists for at least 30 s. The acid value was calculated using the following equation.

$$\text{Acid value (AV)} = 5.611 * A * F/S$$

A : Volume of the KOH-ethanol solution of the titration (mL)
F : Concentration of the KOH-ethanol factor
S : Mass of oil (g)
5.611 : Molecular weight of KOH in mg

2.4.3 Peroxide value

Peroxide value was determined according to the AOCS method Cd 8-53 (AOCS, 1998) with modified amount of the sample taken. One gram of krill oil was dissolved in 6 mL of acetic acid-chloroform (3:2) solution. Then, 0.1 mL of saturated potassium iodine (KI) solution was added to the mixtures and allowed to stand with occasional shaking for 1 min. Distilled water (6 mL) was immediately added to the solution to allow the mixture to stand. The solution was titrated with

0.1N of sodium thiosulfate until the yellow iodine colour had almost disappeared. Then 0.4 mL of starch indicator solution was added with shaking to extract iodine from chloroform layer, and again titrated until the blue colour was disappeared. A blank determination was performed with the same procedure. Peroxide value was expressed as milliequivalents peroxide/1000 g sample.

$$\text{Peroxide value} = \frac{(A - B) \times M \times 1000}{W}$$

A = Volume of titrant, mL of sample

B = Volume of titrant, mL of blank

M = Molarity of sodium thiosulfate solution

W = Weight of sample, g

2.4.5 DPPH radical-scavenging activity

The scavenging effects of crude methanolic extracts were determined by the method of Ten and Chen, with slight modification. 3.9 mL of methanolic DPPH was firstly mixed with 0.1 mL of undiluted crude extract or methanol (as control) and they were stored in dark environment at room temperature for 30 min. Subsequently, the absorbance of crude extract and control were measured against methanol (as blank) at 517 nm using spectrophotometer (UVIKON 933, Kontron

Instrument). The percentage of DPPH radical-scavenging capacity was calculated using this equation:

$$\text{Scavenging effect (\%)} = [1-(A_{517\ nm,\ sample}/\ A_{517\ nm,\ blank})] \text{ X } 100$$

Trolox was used for calibration of standard curve and the results were expressed as μmol trolox equivalent antioxidant capacity per 100 g dry weight sample (μmol TEAC/100 g DW). The calibration equation for trolox was y=37.284x (R^2=0.9997).

3. RESULTS AND DISCUSSION

3.1 Fatty acid composition

Fatty acids are merely carboxylic acids with long hydrocarbon chains which are either saturated or unsaturated. It can be an indicator of its stability, physical properties, and nutritional value. The fatty acid compositions of the oil obtained by SC-CO_2 and hexane extraction are shown in Table 2. As shown in Table 2 krill extracted oil distributed 7 different fatty acids. The components are myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic aicd (C18:2n6), cis-5, 8, 11, 14, 17-eicosapentaenoic acid (C20:5n3) and cis-4, 7, 10, 13, 16, 19 docosahexaenoic acid (C22:6n3). The most prominent fatty acid is myristic acid and it was higher amounts in supercritical fluids extraction than that of hexane extraction. The average molecular weight of krill oil was calculated 828.6g/mol from fatty acid composition.

Table 2. Fatty acid composition and molecular weight of krill oils extracted with hexane and supercritical fluids.

Fatty acid	Krill oil				Fatty acid methyl ester (Mw)
	Organic solvent (hexane)		SC-CO_2 extraction (200 bar, 50℃)		
	Area	Amount(%)	Area	Amount(%)	
C14:0	1458.18	12.68	2538.52	20.39	228.4
C16:0	2364.28	20.19	2931.81	23.55	256.0
C18:0	250.84	2.14	321.52	2.58	284.5
C18:1	1624.12	13.87	2162.45	17.37	282.5
C18:2n6	61.23	0.52	13.05	0.10	280.5
C20:5n3 (EPA)	1752.96	14.97	545.55	4.38	302.5
C22:6n3 (DHA)	374.26	3.20	205.69	1.65	328.5

3.2 Ethanolysis of krill oil in non-pressurized system

3.2.1 Analysis of ethanolysis products by TLC and HPLC

For fatty acid analysis, the individual mono-, di-, triglycerides and esters were separated by TLC (Fig.6). TLC is a relatively simple experiment which is able to find the composition of the lipids rapidly. Therefore, it was performed before HPLC analysis.

Fig. 6 shows the ethanolysis products of krill oil by lipozyme TL-IM. Lipozyme TL-IM generated 1,2-diglycerides which was higher than 1,3-diglycerides. 1,3-Lipase is apt to react with fatty acids in the sn-1 and sn-3 positions

Before and after the reaction, chromatograms of krill oils are shown in the Fig. 7 and 8, respectively. After reaction, triglyceride contents were significantly decreased and mono- , diglyceride and ester contents were increased. The results in all the reaction were measured by conversion to total%.

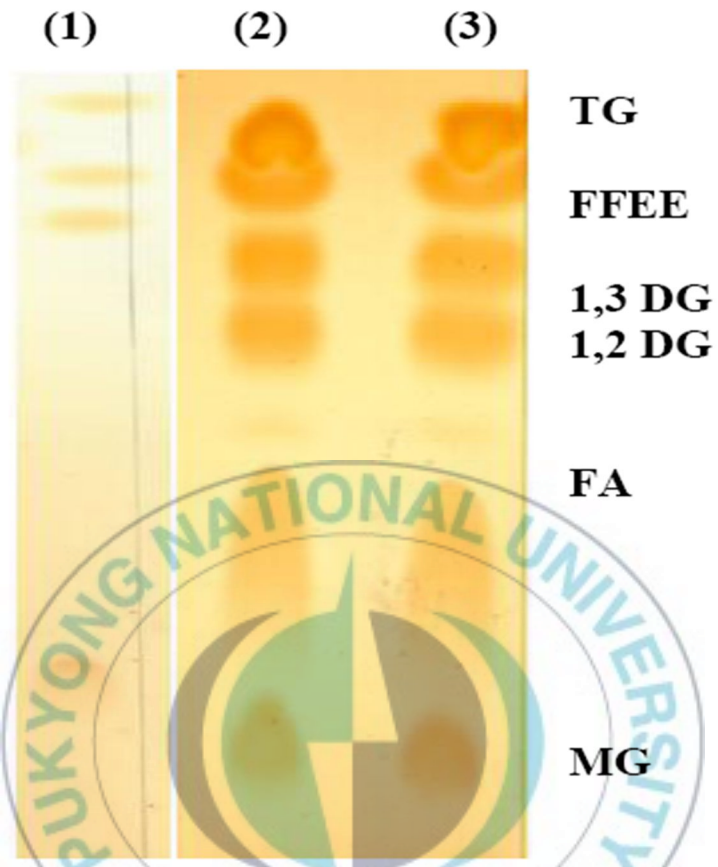


Fig. 6. TLC-analysis of lipid compounds converted from krill oil (1) lipid standard (mono-, di-, tri-olein), (2,3) enzymatic ethanolysis of krill oil in SC-CO_2 system.

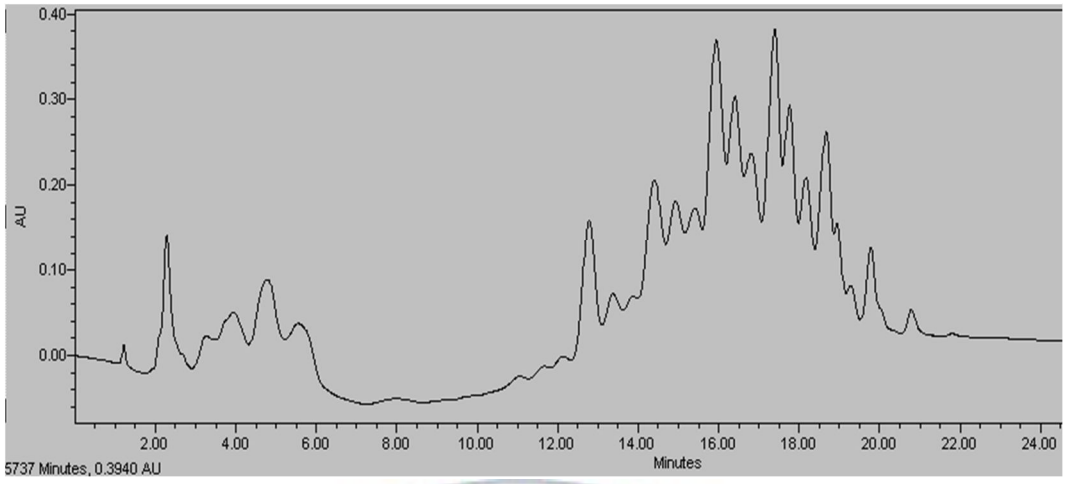


Fig. 7. HPLC-analysis of lipid compounds of krill oil.

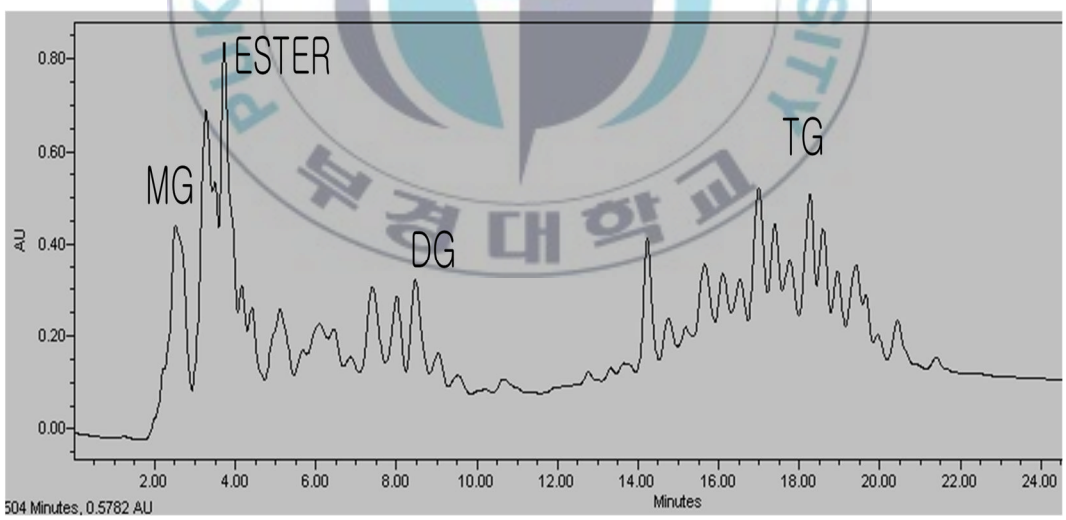


Fig. 8. HPLC-analysis of lipid compounds of krill oil after ethanolysis.

3.2.2 Effect of substrate ratio on enzymatic ethanolysis

Generally, the high substrate ratio favors formation of the products. However, in enzymatic ethanolysis, the highest ethanolysis of krill oil by immobilized lipases occurred at near a mole ratio of 1 to 5 ethanol to krill oil (Re =1 or 1.5). From this Fig. 9 the optimized ethanol ratio for obtaining highest conversion is 1:1 ratio using lipozyme TL-IM. When the mole ratio increased to 2, the enzyme activity significantly decreased. The reason is thought that ethanol inhibit enzyme activity (Simada et al., 2002).

3.2.3 Effect of temperature on enzymatic ethanolysis

The influence of temperature on the enzymatic ethanolysis was studied in a fixed condition at mole ratio (Re) of 1.0, 5w% lipase, 150 rpm shaking speed and 8hrs of reaction time. The krill oil used supercritical fluid extraction at 20 MPa, 40℃.

Experiment temperatures were the range of 40 to 60°C. The effect of temperatures on enzymatic ethanolysis of krill oil is shown in Fig. 10. The optimized temperature for obtaining the highest conversion is 60°C.

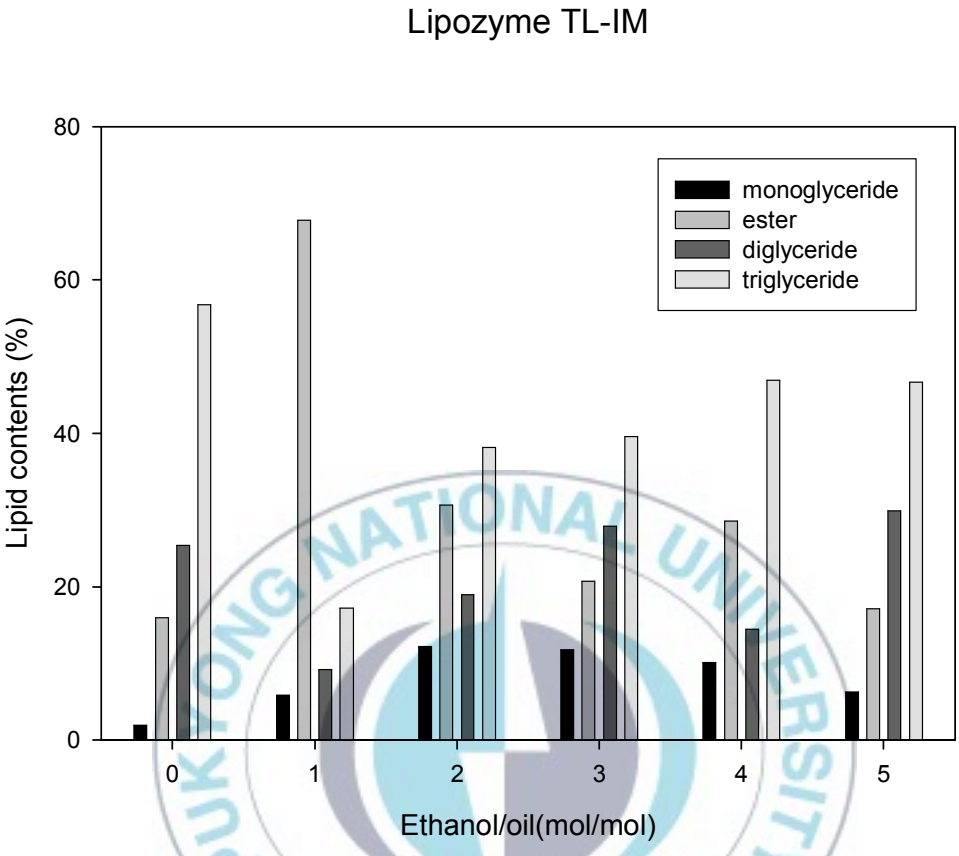


Fig. 9. Effect of ethanol substrate ratio on krill oil at 60°C, 3% lipozyme TL-IM, 150 rpm, 8hr.

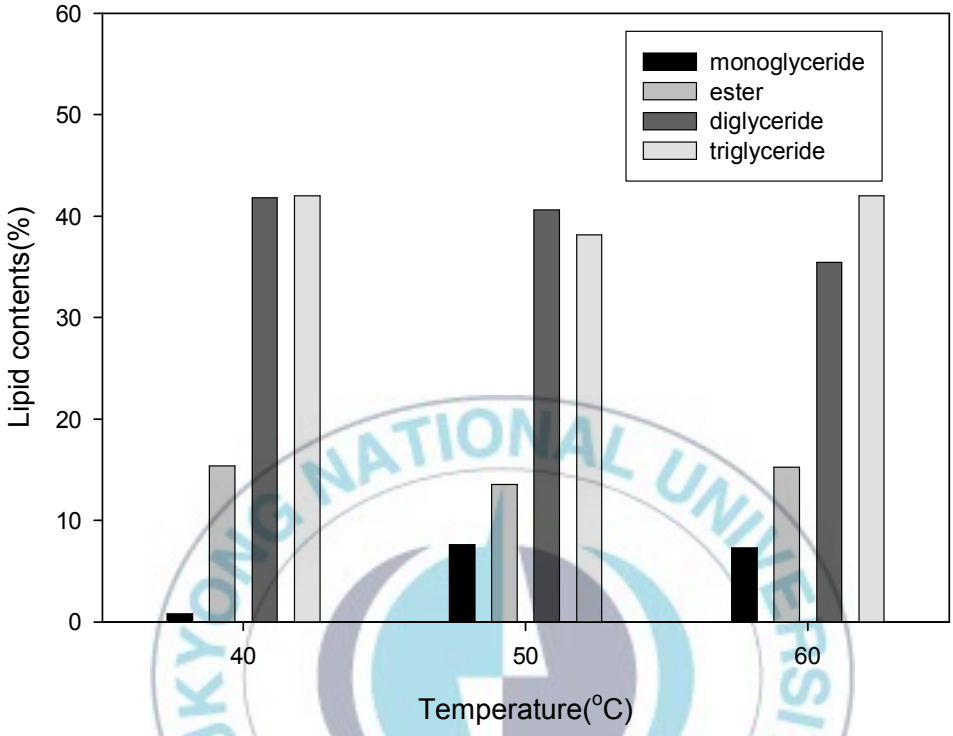


Fig. 10. Effect of temperature on krill oil at 1.0 ethanol mole ratio, 5% lipozyme TL-IM, 150 rpm, 8hrs.

3.2.4 Effect of enzyme load on enzymatic ethanolysis

The influence of enzyme load on the enzymatic ethanolysis was studied in a fixed condition at mole ratio (Re) of 1.0, 60℃, 150 rpm shaking speed and 8hrs of reaction time. The krill oil were used for supercritical fluid extraction at 20 MPa, 50°C.

Enzyme concentration was the range of 2 to 5 wt%. The effect of enzyme concentration on enzymatic ethanolysis of krill oil is shown in Fig. 11. From this Fig. 11, at the optimized temperature, the highest conversion was 2 wt%. It means that even adding more than 2 wt% of the enzyme usage does not affect the yield of MG and DG.

3.2.5 Comparison of conversion in organic extraction and SC-CO_2 extraction

In order to compare with organic solvent extraction and SC-CO_2 extraction was performed enzymatic ethanolysis at mole ratio of 1.0, 60℃, 4 wt% lipase and 150 rpm shaking speed (Fig. 12).

From this Fig. 12, conversion of organic solvent extraction was higher than SC-CO_2 extraction.

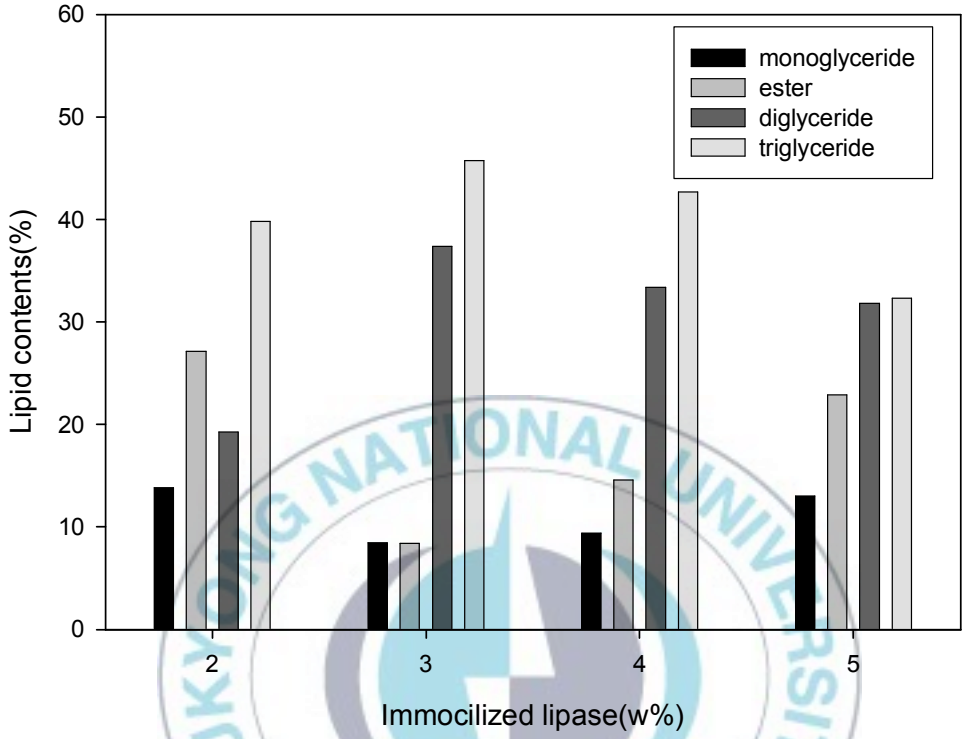


Fig. 11. Effect of enzyme concentration on krill oil at 1.0 ethanol mole ratio, 60°C, 150 rpm, 8hr.

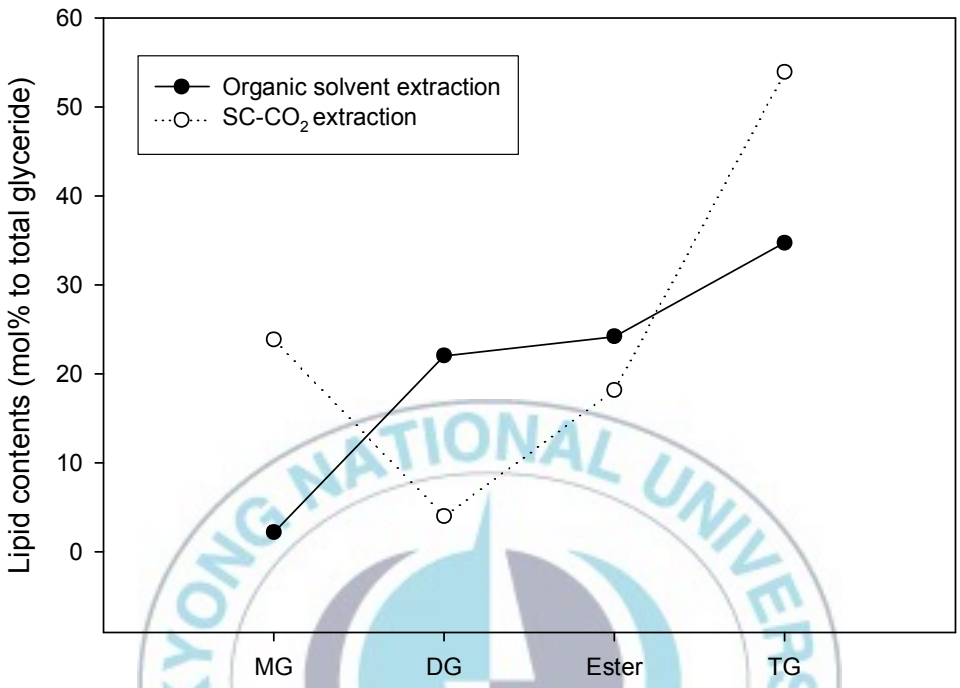


Fig. 12. Comparison of lipid contents between organic solvent extraction and SC-CO_2 extraction.

3.3 Ethanolysis of krill oil in pressurized system

3.3.1 Effect of temperature in SC-CO_2 system

The influence of temperature in SC-CO_2 system was studied in a fixed condition at mole ratio (Re) of 1.0, 4w% lipase and 2hrs of reaction time. The krill oil used supercritical fluid extraction at 20 MPa, 50°C.

Experiment temperatures ranging from 40 to 60°C were set up, at constant pressure of 8 Mpa. The effect of temperatures on enzymatic ethanolysis of krill oil is shown Fig. 13. The optimized temperature for obtaining the highest conversion is 50°C.

As increasing temperature increases has a positive effect on the kinetic constant, as defined by the transition state theory. However, high temperatures may promote side reactions, such as dehydration and denaturation if their activation energy is higher than that of esterification, reducing the ester yield (Hassan et al., 2006).

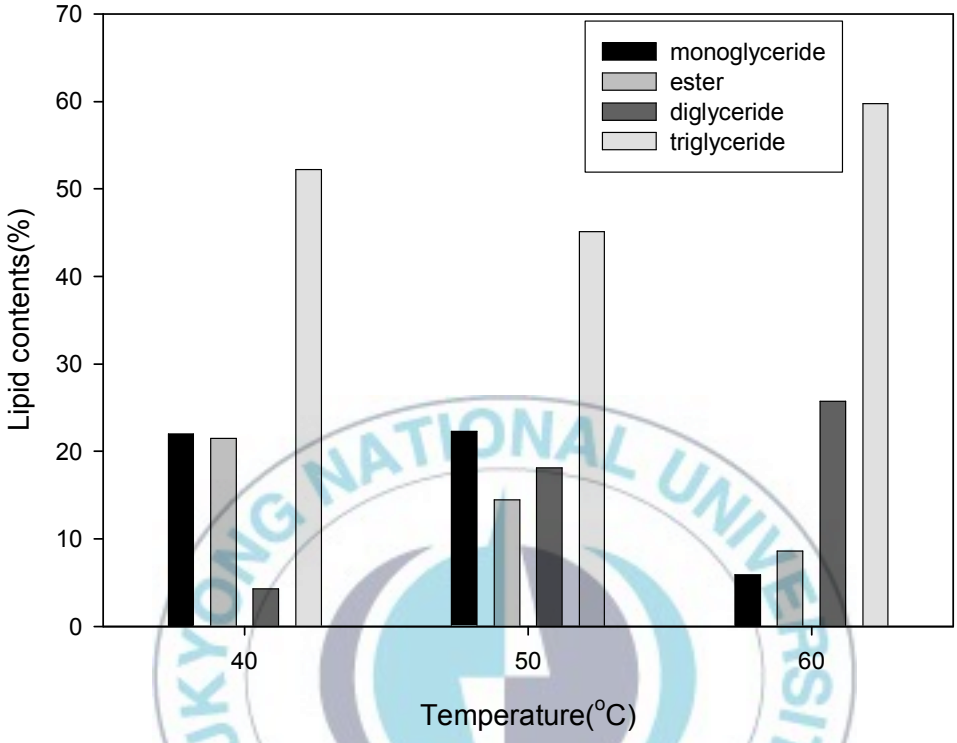


Fig. 13. Effect of temperature on krill oil at 1.0 ethanol mole ratio, 4% lipozyme, 8 MPa, 2 hrs.

3.3.2 Effect of pressure in SC-CO_2 system

Generally, pressure affects reaction rate in supercritical fluids can vary some physical parameters, such as the partition coefficient, dielectric constant, and fluid Hildebrand solubility parameter (Hassan et al., 2006).

The influence on enzymatic ethanolysis in SC-CO_2 system was performed in a fixed condition at mole ratio (Re) of 1.0, 4w% lipase and 2hrs of reaction time.

The krill oil was used for supercritical fluid extraction at 20 MPa, 50°C. Experiments pressures ranging from 6 to 10 MPa were set up. Extraction temperature was fixed at 50°C because it is related with enzyme activity and stability.

As it is shown in Fig. 14, the conversion yield was increased as the pressure was increased. However, Kwon et al. (2009) reported that the effect of increasing pressure diminished above 12.5 MPa. Drescher et al. (2002) also reported that the concentration of ethanol in liquid phase was slowly increased when pressure was increased from 7 to 10 MPa at constant temperature of 50ºC.

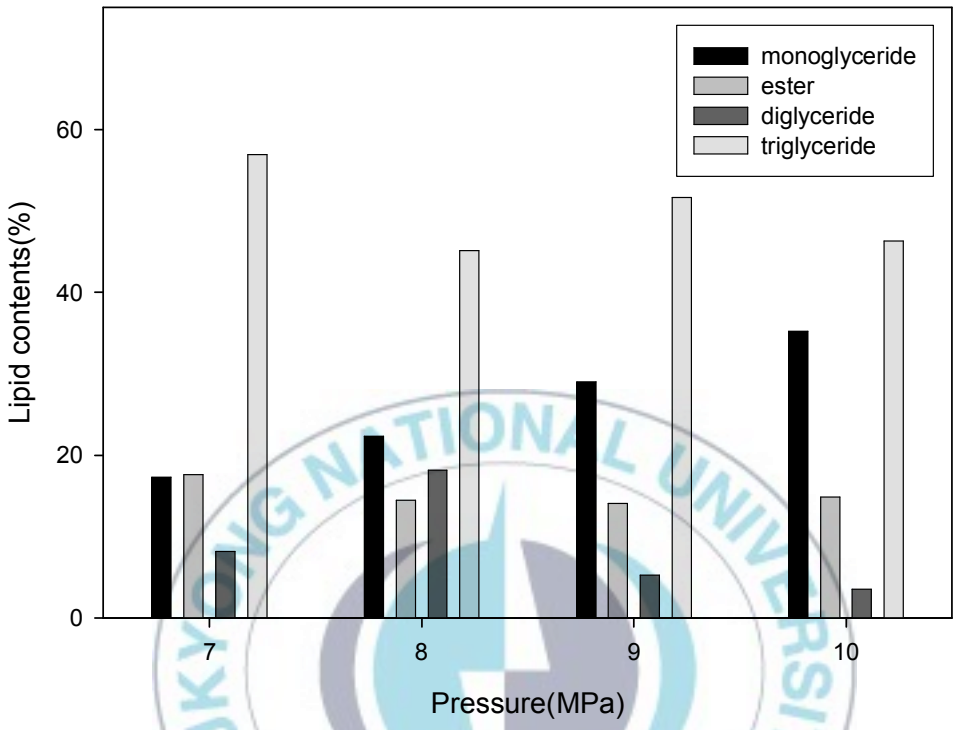


Fig. 14. Effect of pressure on krill oil at 1.0 ethanol mole ratio, 4% lipozyme, 50ºC, 2 hrs.

3.4 Regiospecific analysis of triacylglycerols

The fatty acids composition at position 2 of the TAGs obtained was analyzed by ethanolysis with immobilized lipase, lipozyme TL-IM following the method proposed by Shimada et al. Table 3 shows the fatty acids composition krill oil ethanolysis in different processes (non-pressurized and pressurized system).

After purification 2-MAGs from enzymatic reaction of krill oil, EPA, DHA is more higher than only enzymatic reaction oil.

Table 3. The krill oil ethanolysis in different processes (non-pressurized and pressurized system) and SCO_2 extracted krill oil for comparison of the fatty acids composition.

Fatty acid (%)	2-MAG fraction after enzymatic reaction of krill oil		SCF extraction of oil from krill
	In non-pressurized system (60ºC, 150 rpm)	In pressurized system (60ºC, 8 MPa)	
C14:0	16.6	18.3	19.6
C16:0	17.8	19.3	22.6
C16:1	11.6	13.1	14.6
C18:0	2.1	2.2	2.5
C18:1	7.4	7.2	7.5
C18:2	2.2	2.0	1.8
C20:0	2.0	1.5	1.0
C20:1	0.5	0.2	0.8
C20:2	6.2	5.3	3.7
C20:5(EPA)	10.2	8.6	4.2
C22:6(DHA)	4.5	4.4	1.6
Others	18.9	17.9	20.1

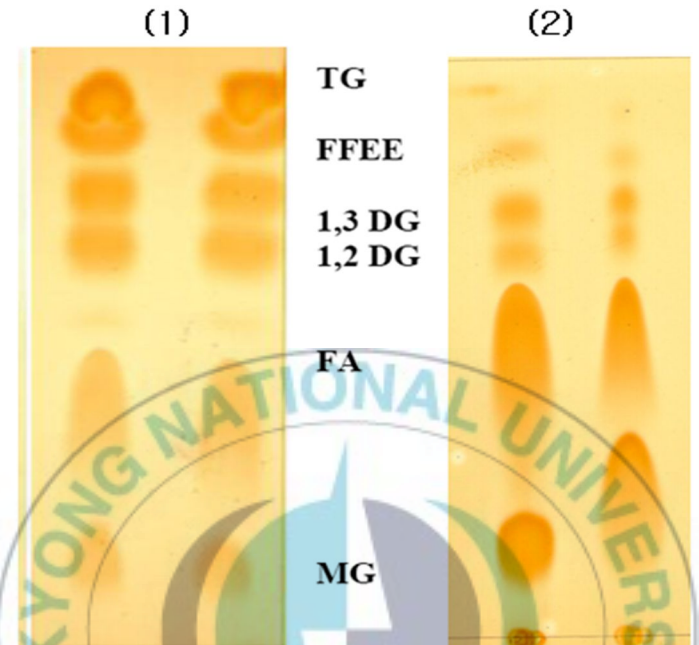


Fig. 15. TLC-analysis of lipids abtained from (1) enzymatic ethanolysis of krill oil in SC-CO_2 system and (2) krill oil after the recovery of 2-MAG.

3.5 Characterization of ethanolysis

3.5.1 Comparison of free fatty acid of enzymatic ethanolysis reactants and krill oil

The degree of oxidation depends strongly on the level of unsaturation, the presence of antioxidants and prior storage condition. High level of PUFAs is found in marine fish oil. Peroxide value and FFA analyses give an idea of how good or bad oil is at a particular time. FFAs are responsible for the acidity of oil. In this study, FFAs of enzymatic ethanolysis reactants in different processes (non-pressurized and pressurized system) were compared and are given in Table 4. It was found that the oil obtained by SC-CO_2 extraction showed more stability compared to the oil extracted by hexane. On the other hand, ethanolysis reactants in pressurized system were higher than non-pressurized system.

Table 4. Free fatty acids of krill oil by ethanolysis reactants.

	Krill			
	Organic solvent extraction(Hexane)	SC-CO_2 extraciton (50°C, 20 MPa)	Ethanolysis in non-pressurized system (50ºC, 150 rpm)	Ethanolysis in pressurized system (50ºC, 8 MPa)
Free fatty acids (g/100g oil)	11.97 ± 0.06	8.31 ± 0.10	7.22 ± 0.09	11.67 ± 0.06

3.5.2 Comparison of acid value and peroxide of enzymatic ethanolysis reactants and krill oil

Acid value (AV) and peroxide value (POV) was carried out to measure the quality of the oil and oxidation state of lipid. It is used to measure the rancidity which occurs by auto oxidation state of lipid.

It was found that as the temperature increase, the amount of AV and POV increase (Fig. 16 and Fig. 17). This result associated with FFAs contents. It also found that higher temperature caused a significant increment of the AV and POV.

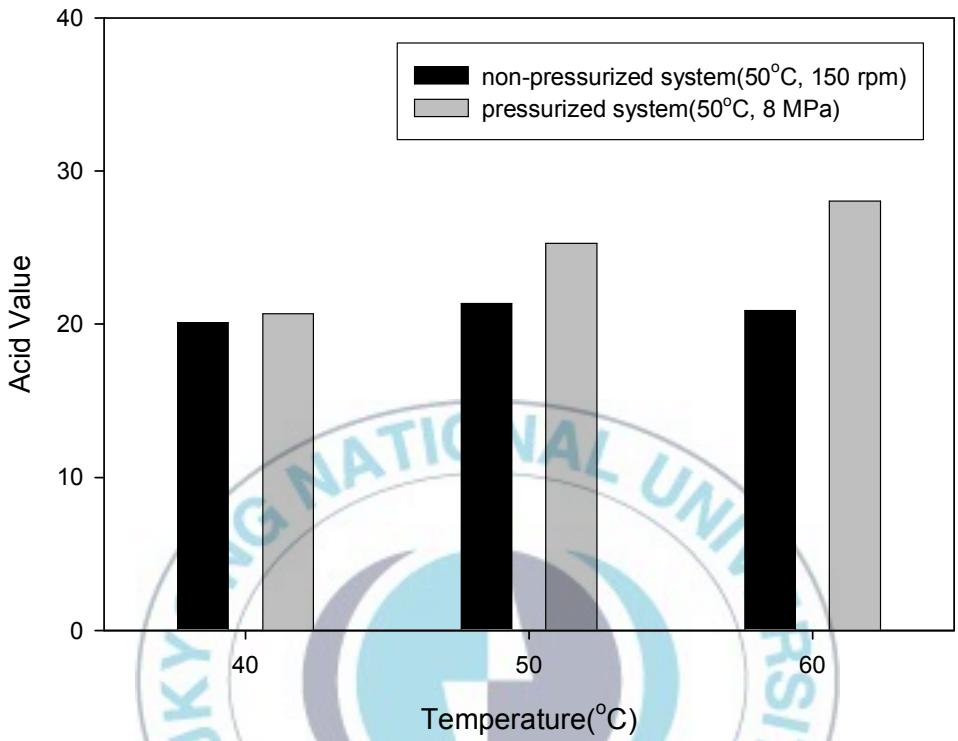


Fig.16. Comparison of the acid value of enzymatic ethanolysis of reactants at different systems.

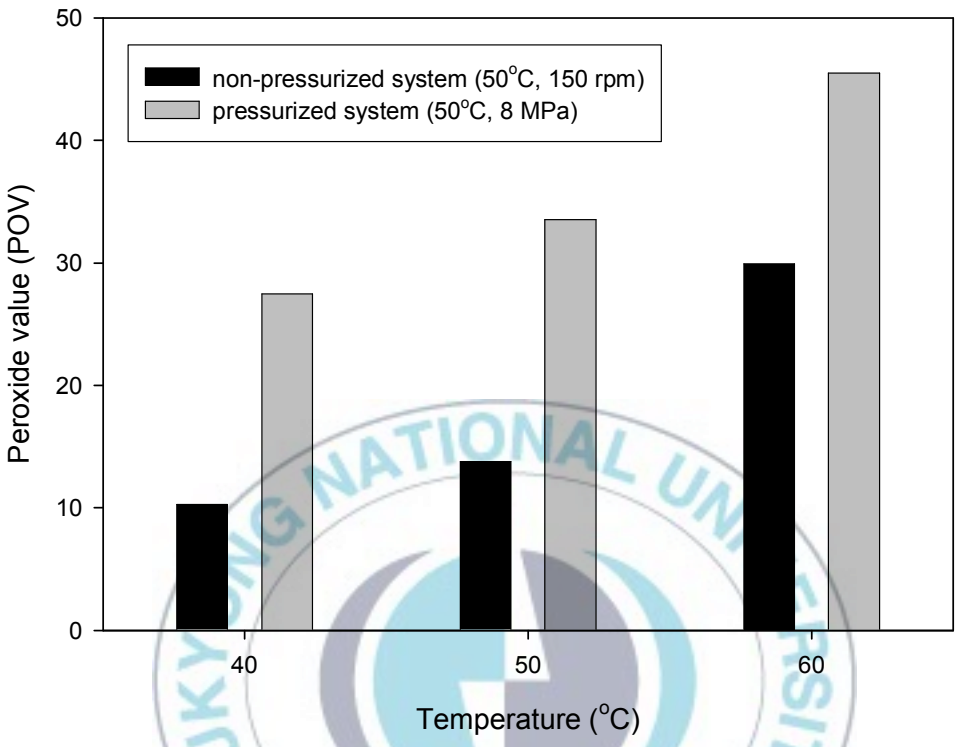


Fig.17. Comparison of the peroxide value of enzymatic ethanolysis of reactants at different systems.

3.5.3. DPPH radical scavenging effect

The DPPH radical scavenging effect of enzymatic reaction of krill oil is shown in Fig. 18. According to Fig. 18, it has been observed that the DPPH radical scavenging activities were significantly high in SC-CO_2 extracted oil than hexane extracted. It was also found that the DPPH radical scavenging activity of ethanolysis reactant in non-pressurized system was higher than ethanolysis in pressurized system.

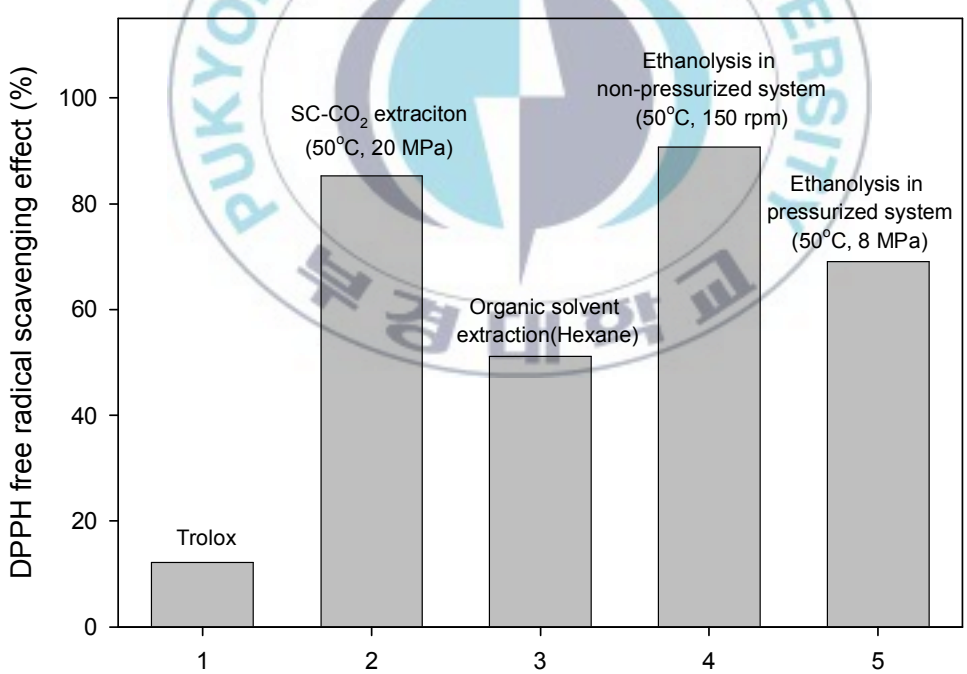


Fig. 18. DPPH free radical scavenging effect of krill oil by ethanolysis reactants.

4. CONCLUSION

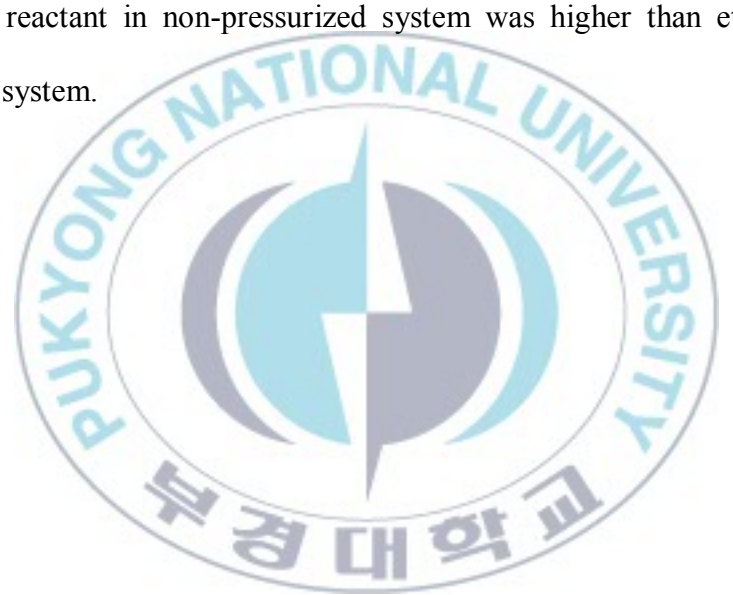
Production of rich monoglyceride, diglyceride, and ethyl ester from krill oil and ethanol with immobilized 1, 3-regiospecific lipases was performed. Changing main experimental parameters affecting reaction, the temperature, enzyme amount, reaction time and enzyme, the optimal reaction conditions were established. And oxidation stability compared enzymatic ethanolysis reactant with krill oil.

The optimized ethanol ratio for obtaining highest conversion is mole ratio 1 using lipozyme TL-IM. For non-pressurized system, the optimal temperature and enzyme load are 60^{o}C, 2% lipase. However, in pressurized system, the optimal temperature was found 50^{o}C. The increasing temperature has a positive effect. However, high temperatures may promote dehydration and denaturation (Hassan et al., 2006). When pressure ranging from 6 to 10 MPa was set up, the conversion yield was increased as the pressure increased.

Recovery of 2-MAGs from the ethanolysis reaction was performed by solvent extraction following the methodology specified by Muñío et al. The result is able to confirm that EPA and DHA being concentrated.

Krill oils obtained by enzymatic ethanolysis was characterized by measuring the FFA content, acid value, peroxide value, DPPH scavenging effect. Comparing the FFA, it was found that the oil obtained by SC-CO_2 extraction showed more

stability compared to the oil extracted by hexane. On the other hand, ethanolysis reactants in pressurized system were higher than non-pressurized system. The condition of 40^{o}C shows the highest oil stability among the various conditions. Comparing the DPPH radical scavenging, it has been observed that the DPPH radical scavenging activities were significantly high in SC-CO_2 extracted oil than hexane extracted. It was also found that the DPPH radical scavenging activity of ethanolysis reactant in non-pressurized system was higher than ethanolysis in pressurized system.



5. ACKNOWLEDGEMENT

석사 논문이 완성되기까지 도움을 주신 많은 분들께 감사의 인사를 올립니다. 먼저 석사과정 동안 학문의 길로 인도해주시고 부족한 저에게 아낌없는 격려와 지도를 해주신 전병수 교수님께 깊은 감사의 인사를 드립니다. 그리고 바쁘신 와중에도 저희 논문 심사를 맡아주신 김선봉 교수님과 이양봉 교수님께도 깊은 감사의 인사를 드립니다. 그리고 많은 가르침을 주신 조영제 교수님, 양지영 교수님, 안동현 교수님, 김영목 교수님께도 진심으로 감사의 말씀을 올립니다. 또한 실험실 생활 하는데 많은 도움을 주신 해림이 언니(언니 완전 사랑합니다), 신상규 선배님, Ali Nehari Abdelkader 박사님, Asaduzzaman 박사님, Tanbirul 박사님께(Dear. Asadu, Tanbirul Thanks for my birthday gift ! I won't forget about that.) 감사 드리며 함께 고생한 정은이, 승미, 정남이, 준호, 주희, 령희, Evi, Meillisa 에게도 감사합니다. 학부생으로서 제 실험을 묵묵히 도와준 준구, 인정이, 선준이, 창주, 동학이(그래서 치킨은 대체 언제 사줄꺼야?), 태근이, 효정이, 미경이, 은주에게 너무 고맙다는 말을 전하고 싶으며, 특히 나의 논문 공동저자 뺀질이 윤준현과 (USB 찾아와) 나의 세계를 더 많이 보여주고 싶은 손지선에게 격하게 아낀다는 말을 꼭 하고 싶습니다.

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