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Characterization of Yellowtail Fish Viscera Lipid Extracted Using Supercritical Carbon Dioxide and Organic Solvents

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초임계 이산화탄소 및 유기용매에 의해 추출된 방어 내장의 지질에 대한 특성

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A dissertation

by

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Characterization of Yellowtail Fish Viscera Lipids Extracted Using Supercritical Carbon Dioxide and Organic Solvents

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Abstract

Every year a large number of by-products produced by fish processing industries are dumped or used for less productive purposes. This study prescribes a technique for production of good quality edible lipids from yellowtail fish (*Seriola quinqueradiata*) viscera to valuate this wastes and also serve as a source for omega-3 polyunsaturated fatty acids. The extraction method plays a very strong role in the quality of extracted lipids as they oxidize easily when in contact with atmospheric air, light and high temperature. Conventional method of extraction involves the use of high temperature and solvent which are considered to have unfavorable effects on the environment and consumers health. Supercritical carbon dioxide (SC-CO_2) technique is a promising method for the extraction of edible lipids, as it is a green technology and non-toxic in food.

In the first study, yellowtail fish viscera oil was extracted using SC-CO_2 method and soxhlet method. Oils extracted by SC-CO_2 method showed better physio-chemical properties than oils extracted using soxhlet method. Extracted yellowtail viscera oil

contained a maximum amount of 20.14% of omega-3 polyunsaturated fatty acids. The thermogravimetric analysis showed that yellowtail viscera oil extracted using SC-CO_2 method can be utilized in high-temperature food processing industries.

The second research work aims to study the quality of yellowtail fish viscera phospholipids extracted by SC-CO_2 with ethanol as co-solvent method and organic solvent (ethanol) method. Phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine, and phosphatidylethanolamine (PE) were detected in the extracted phospholipids, with PC accounting 82% of the total phospholipid. Yellowtail fish viscera phospholipids contained a high amount of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Phospholipid extracted by SC-CO_2 with ethanol as co-solvent showed better oxidative stability and free radical properties than organic solvent extracted phospholipid.

In conclusion, SC-CO_2 technique showed to be an excellent method for the extraction of good quality lipids from fish material. Yellowtail fish viscera lipids can serve as a good source of omega-3 polyunsaturated fatty acids and as such, it would help reduce environmental pollution. This research may be helpful in the application of yellowtail fish viscera lipids in food and pharmaceutical industries.

CHAPTER 1

General Introduction

1.1. Background

Yellowtail fish is a specie of jack fish in the family Carangidae. It is native to the western central Pacific Ocean, from Japan and the eastern Korean Peninsular to the Hawaiian Islands but its farming occurs mostly in Japan and Republic of Korea. It spawns along the contour in the East China Sea; juvenile takes three to five years to reach sexual maturity, after which they migrate south for spawning. Yellowtail fish can grow up to a maximum size of 150 cm TL and 40 kg. It is mostly eaten raw as 'sashimi' and a minority of its total production is being consumed after drilling. Recently, the rate of consumption of fresh fish has increased and the consumers are willing to pay good prices for premium products. Therefore, there is growing competition among yellowtail fish producers to supply fresh fish straight to the consumers. Yellowtail fish is one of the economically important finfish for aquaculture in Japan. The total production of yellowtail fish in Japan was about 135,998 tons in 2014, which represent about 53% of the total production of aquaculture marine finfish in Japan. Yellowtail fish can be identified by its compressed elongated body with longitudinal yellow strip [1]

Scientific classification of yellowtail fish

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

Family: Carangidae

Genius: *Seriola*

Species: *S. quinqueradiata*

In the fish processing industry, as high as 60% of the by-products are discarded as waste. After the fillets are obtained the remaining 50 to 60% of the whole fish is discarded as by-product or waste. The waste and by-product of fish processing consist of head, skin, frames, and viscera. Proper utilization of fishery by-products has many advantages; it decreases environmental pollution and also reduces the cost of processing waste disposal. Fish products make wave in the recent trends of food processing industries. Recently, studies suggested that inclusion of these products are essential for the proper functioning of human organs and health improvement. The main reason for this suggestion is because of the presence of bioactive compounds in fish tissues [2] [3] [4]. These bioactive compounds can be separated and purified using various simple and complex technologies for pharmaceutical and biotechnological applications. Some of these compounds are known to possess nutraceutical potentials that have a beneficial effect on human health [5]. Fish viscera has been reported to contain a reasonable amount of omega polyunsaturated fatty acids [6]. The content of oil in fish by-product varies depending on the species and on tissue type. The fat content of viscera is about 33.6% (wet basis) and the viscera can

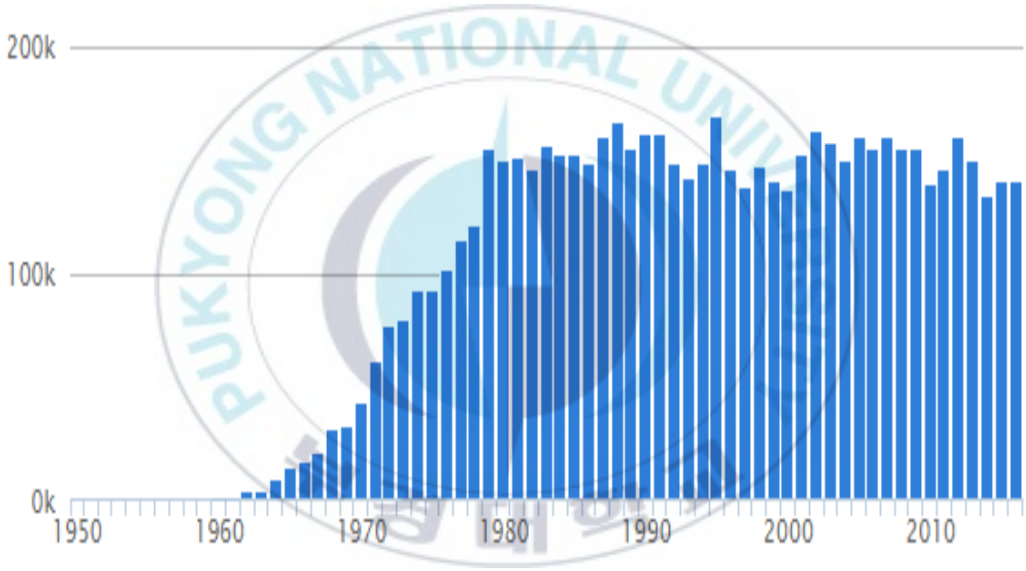

Fig. 1. 1. Global aquaculture production of yellowtail fish (in tonne) [1]

Fig. 1. 2. Main producer countries of yellowtail fish [1]

used for recovering oil that could be converted into an edible product. Producing edible oil from viscera may add value to yellowtail fish viscera. Fish lipids is considered a suitable supplier of long-chain polyunsaturated fatty acids (PUFA); especially omega-3 (Eicosapentaenoic and docosahexaenoic acid).

1.2. Phospholipid

Phospholipid is a class of lipid that contains a glycerol backbone with two fatty acyl chain. The hydroxyl groups at the sn-1 and sn-2 in glycerol phospholipid are esterified with two fatty acids and the hydroxyl group at sn-3 position is esterified with phosphoric acid. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) are formed when the second free hydroxyl group of phosphate ester reacts with other alcohols like choline, ethanolamine, inositol, and serine, respectively. The hydrophobic and hydrophilic phosphate group of the phospholipids are connected via a third molecule, glycerol. When emerged in water, phospholipids form a bilayer with the polar head pointing towards the water and the hydrophobic tail in between the hydrophilic head. This characteristic makes it suitable for the preparations of liposomal, ethosomal, and other nano-formations. Phospholipids can be used in food nanotechnology for the encapsulation, protection and delivering of degradable ingredients like omega-3 fatty acids. Phospholipid is widely used around the world as a food addictive in many products. In pharmaceuticals, phospholipids are mostly used for drug delivery system that helps to transport a drug throughout the body to the area to affect [7]. Phospholipid is known to serve as the building block for cell membranes, furnished with necessary biological and physiological function in about all known living things [8] [9]. More beneficial effects would be annexed from omega-3 PUFA containing

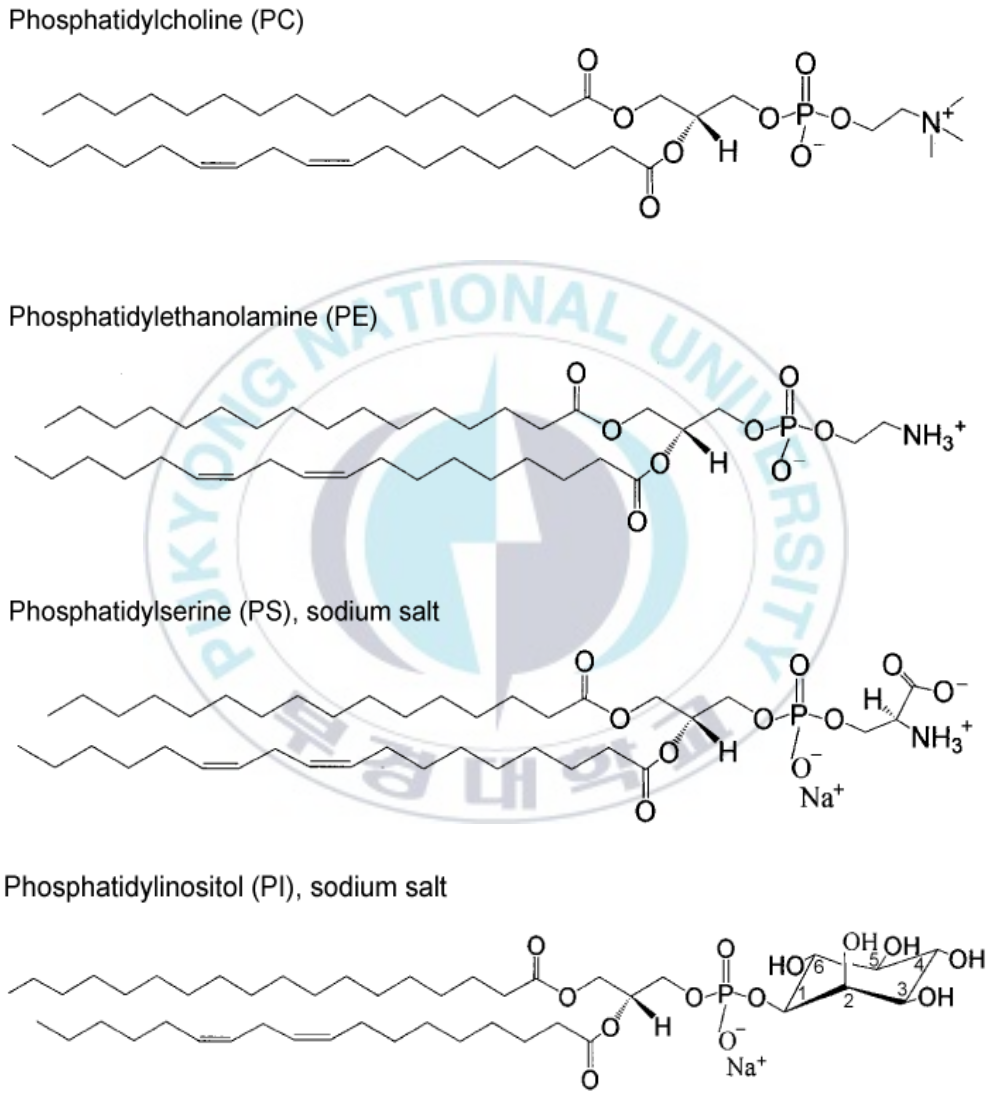

Fig. 1. 3. Structure of major phospholipid groups obtained in yellowtail fish viscera

phospholipid, like regulating liver and blood plasma lipid levels [10] [11]. Phospholipids are commercially extracted from egg yolk which lack omega polyunsaturated fatty acids. Marine phospholipid hold more advantages over traditional phospholipid, in cosmetic and pharmaceutical applications [8]. Recently, extraction of phospholipids from the marine product has attracted so much attention, as this type of phospholipids are rich in omega-3 fatty acids [12].

1.3. Polyunsaturated Fatty Acids (PUFA)

Polyunsaturated fatty acids are those fatty acids that contain more than one double bonds between carbons. This group of fatty acid includes a number of important compounds beneficial for human health such as omega-3 PUFA. Omega-3 refers to the position of the final double bond (C=C) which is third carbon atoms away from the methyl ($-CH_3$) end of the carbon chain [13]. They are considered essential fatty acids because they cannot be synthesized by human metabolism. Since the human body can't produce omega-3 PUFA, the only suitable way it can be introduced in human metabolism is through consummation. Consequently, the World Health Organization (W.H.O) recommends eating oily fish rich in omega-3 PUFA at least two portions in a week [14] [15] [16] [17]. Omega-3 PUFAs differ based on their size and chemical shapes Three types of omega-3 fatty acids are essentially involved in human physiological activities, such as linolenic acid (mostly found in plant seeds oils), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA is an omega-3 fatty acid with 20-carbon chain and five cis double bonds. It produces a chemical called eicosanoid which act as anti-inflammation and also reduces symptoms of depression

[18] [19]. DHA is an omega-3 fatty acid with a 22-carbon chain and six cis double bonds. It is an elementary structural component of the human brain, skin, retina and cerebral cortex. DHA makes up about 8% weight of the human brain and is vital in human brain development and function [20]. Linolenic acid (ALA) is an omega-3 fatty acid abundant in plant seeds oil. This 18-carbon fatty acid with three cis double bonds is mostly used by the body for energy [21]

Recently, there is a number of scientific works that prove the beneficial health effects of omega-3 PUFAs. More than one gram/day of the supplement of omega-3 PUFAs for a minimum of one year has been reported to help protect against cardiac arrest and myocardial infarction. [22]. Omega-3 PUFA is systematic in lowering systolic and diastolic blood pressure in people with hypertension and people of normal bold pressure [23]. Omega-3 PUFA is reported to elevate the good HDL cholesterol and reduces blood pressure, triglycerides, and the formation of arterial plaques [24] [25] [26]. Omega-3 fatty acids are associated with the lowering of the amount of fats in human liver and also aid better bone mineral density [27] [28] [29]. Consumption of omega-3 PUFAs derived from marine organism

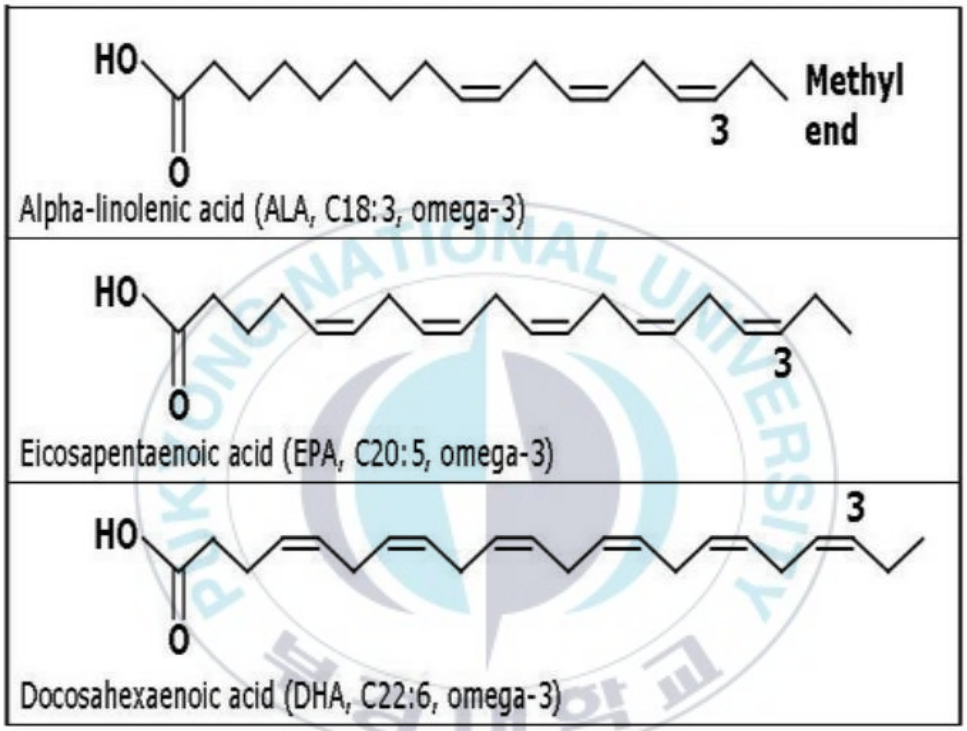

Fig. 1. 4. Chemical structure of omega-3 fatty acids
(source:http://chemistry.tutorvista.com/biochemistry/omega-3-fattyacids.html)

lower blood markers of inflammation like C-reactive protein, TNA alpha, and interleukin 6 [30]. Omega-3 PUFAs are related to healing mental depression associated with bipolar disorder. The association between omega-3 PUFAs and depression attributed to the fact that some of the products of omega-3 PUFAs synthesis pathway play important roles in regulating prostaglandin E3, linked to depression [31]. People who consume high amount of omega-3 fatty acids, tend to possess a slower decline of brain function at old age [32].

1.4. Supercritical fluid extraction

The critical point of a pure substance is defined as the highest temperature and pressure at which the substance can exist in vapor-liquid equilibrium. At temperature and pressure above this point, a single homogenous fluid is formed, which is known as supercritical fluid (SCF). Close to the critical point, small changes in pressure or temperature result in fine-tuning. SCF exhibits physicochemical properties intermediate between liquids and gases. SCF can diffuse through solids like a gas and dissolve materials like a liquid. The phase diagram of supercritical carbon dioxide describe the critical temperature, critical pressure and triple point (Fig.1.4). The critical temperature is the highest temperature at which gas can be converted to liquid with an increase in pressure. The highest pressure at which liquid can be converted to gas is referred to as critical pressure. The triple point refers to the point at which gas, liquid, and solid phases exist in equilibrium [33]. Carbon dioxide is the most widely used supercritical fluid because it possesses a lower critical temperature (31.10°C) and pressure (7.39MPa), which make it suitable for extraction of thermos-labile compounds like omega polyunsaturated fatty acids. Supercritical carbon dioxide (SC-CO_2) has the capability to dissolve nonpolar material, but lack the ability to dissolve polar

compounds. Therefore, when working with SC-CO_2, polar solvent like ethanol should be used as a co-solvent/modifier. Addition of polar co-solvent modifies the properties of SC-CO_2 to solubilize polar solutes [34] [35]

Conventional method, such as hydraulic pressing, vacuum distillation, urea crystallization, hexane extraction, and conventional crystallization involves the use of solvents which have unfavorable effects on environment and consumers health [36] [37] [38] [39]. To avoid these hindrances, new methods that use supercritical fluid are replacing the conventional method of lipid extraction. SC-CO_2 is a promising method for obtaining high-quality oil from fish by-product because it can be used under mild operational conditions which aim to reduce the omega3 fatty acids oxidation during the extraction process [40] [41] [42]

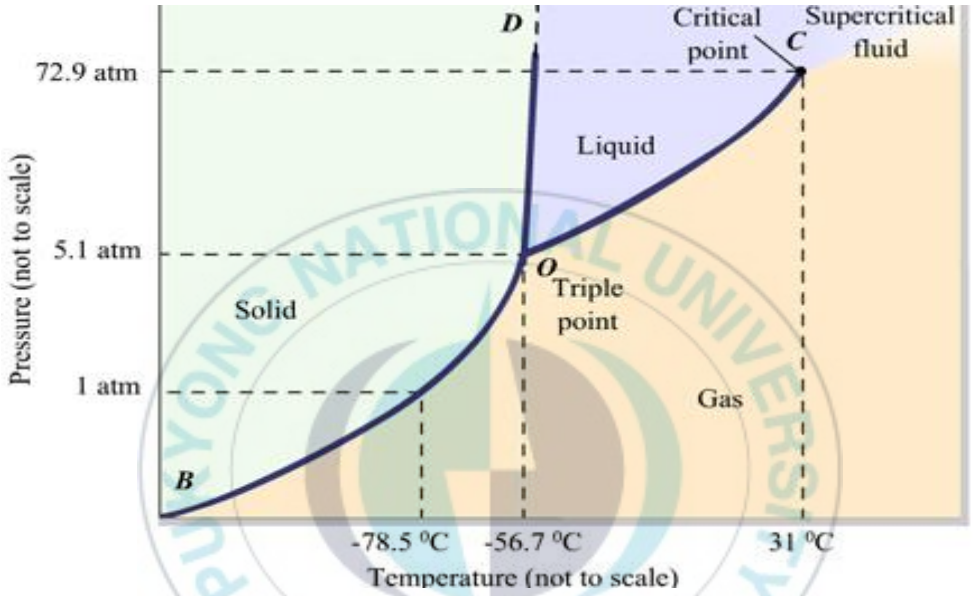

Fig. 1. 5. Phase diagram of carbon dioxide

1.4.1. Properties of supercritical fluid

The properties of supercritical fluid depend mostly on temperature and pressure. The density of supercritical fluid increases with increase in pressure at a constant temperature and decreases with increase in temperature at low pressure. The density of supercritical fluid is between that of gas and liquid but closer to liquid. Density is an essential parameter because it determines the dissolving effect of supercritical fluid [43]. At high pressure, the solvating power of supercritical fluid increases with increased temperature. Also, the solvating power of supercritical fluid decreases with an increase in temperature at low pressure. Diffusivity of supercritical fluid is more than that of a liquid and less than that of a gas. The viscosity is almost the same as a gas and less than that of a liquid (Table. 1.1). Consequently, solid matrix show better diffusivity in supercritical fluid than in liquid.

Table 1. 1 Physical properties of gases, supercritical fluids and liquids [44]

State of compound	Density (g/ml)	Dynamic viscosity (g/cm-sec)	Diffusion coefficient (cm^2/sec)
Gas (ambient)	6.0×10^{-4}- 2.0×10^{-3}	1.0×10^{-4}-3.0×10^{-3}	0.1-0.4
Supercritical fluid	0.2–0.5	1×10^{-4}-3.0×10^{-4}	0.0007
Liquid (ambient)	0.6-1.6	2.0×10^{-3}-3.0×10^{-2}	2.0×10^{-6}-2.0×10^{-5}

Table 1. 2 Critical properties of solvents [45]

Solvent	Molecular weight (g/mol)	Critical temperature (K)	Critical pressure (MPa)	Critical density (g/cm^3)
Carbon dioxide	44.01	304.1	7.38	0.469
Water	18.02	647.3	22.12	0.348
Methane	16.04	190.4	4.6	0.162
Ethane	30.07	305.3	4.87	0.203
Propane	44.09	369.8	4.25	0.217
Ethylene	28.05	282.4	5.04	0.215
Propylene	42.08	364.9	4.6	0.232
Methanol	32.04	512.6	8.09	0.272
Ethanol	46.07	513.9	6.14	0.276
Acetone	58.08	508.1	4.7	0.278

CHAPTER 2

Characterization of yellowtail fish viscera oil extracted using supercritical carbon dioxide and organic solvents

2.1. Introduction

Yellowtail fish is native to the western, central Pacific Ocean, from Japan and the eastern Korean Peninsular to the Hawaiian Islands. However, its farming occurs mostly in Japan and the Republic of Korea (ROK). Yellowtail fish is one of the economically important finfish for aquaculture in Japan. The total production of yellowtail in Japan was approximately 135,998 tons in 2014, which represents about 53% of the total production of aquaculture marine finfish in Japan [1].

In the fish processing industry, as high as 60% of the by-products is dumped as waste. After the fillets are obtained the remaining 50 to 60% of the whole fish is discarded as by-product or waste [46]. Proper application of fishery by-products has numerous benefits; it decreases environmental pollution and also reduces the cost of processing waste disposal[47].

The content of oil in fish by-product varies depending on the species and on tissue type[48]. Fish tissues are considered to contain some bioactive compounds especially omega polyunsaturated fatty acids (PUFA) which cannot be synthesized by human metabolism. These bioactive compounds are labeled as essential fatty acids because the only suitable way it can be introduced in human metabolism is through consummation [14] [15] [16]. The long chain PUFA has been beneficial in the effectual hindrance and

treatment of many inflammatory diseases like heart diseases and cancer [49] [50] [51] as well as in various mental illnesses [52]. Fish oil, rich in both EPA and DHA, is essential for both physical and mental health and also play a relevant role in infant brain and eye development [53]. In the general populace, the amount of omega3 PUFA required to annex a beneficial effect is not being met, therefore, an emphasis on increasing the consumed levels exist. Currently fish oil and oily fish is the predominant dietary sources of long chain omega-3 PUFA.

Numerous methods of extraction and purification of fish oil have been reported. Conventional method, such as hydraulic pressing, vacuum distillation, urea crystallization, hexane extraction, and conventional crystallization involves high-temperature processing that may lead to decomposition or denaturation of the thermally labile compounds or use of toxic solvents that have unfavorable health effect [38] [39]. In order to avoid these unfavorable conditions, new methods that use supercritical fluid are replacing conventional method of lipid extraction.

Supercritical carbon dioxide extraction (SC-CO_2) and fractionation of fish viscera oil have been studied by many researchers and emphasized on the excellent solubility of fish oil in SC-CO_2, and possible prevention of polyunsaturated fatty acids degradation during extraction process [54] [15] [55]. SC-CO_2 has numerous ecological and practical benefits more than conventional extraction methods. This promising extraction technique is suitable for obtaining high-quality oil from fish by-product because it can be used under moderate operation conditions which aim to reduce the omega3 oxidation during the extraction process [40] [41] [42] [56]. The use of CO_2 as solvent tenders new opportunity for solving separation problem because it is inflammable, nontoxic, green solvent and inexpensive. Furthermore, the solvating

power of SC-CO_2 can be regulated by tuning temperature and/or pressure to permit fitting selectivity [57]. The SC-CO_2 extraction has demonstrated to be an efficient separation method in the area of functional foods and nutraceutical supplements [14] [58]

The aim of this work was to evaluate the oil yield in yellowtail fish viscera and to compare the efficiency of different procedures for extracting oil from yellowtail fish viscera.

2.2. Materials and Methods

2.2.1. Chemicals and reagents

Carbon dioxide (CO_2) gas with 99.9% purity was supplied by KOSEM (Yangsan, Korea). p-Anisidine and fatty acid methyl ester (FAME) were obtained from Sigma-Aldrich Co., St. Louis, Missouri, USA. All reagents and solvent used in this study were of analytical or HPLC grade.

2.2.2. Sample preparation

Yellowtail fish viscera was collected from Heechang Trading Co. Ltd. Seo-gu. Busan, Republic of Korea. The sample was washed thoroughly with cold water (4°C) and lyophilized for 72h using EYELA FDV-2100, Rikakikai Co. Ltd. Tokyo, Japan. After drying, the sample was ground using an electric blender (Hanil HMF_3260S, 2000 ml. Seoul, Korea) and screened using 1.7 mm sieve and stored at -2°C until extraction

2.2.3. Supercritical carbon dioxide (SC-CO_2) extraction

SC-CO_2 extraction of lipids was performed using a laboratory scale apparatus (Fig. 2.1.) described in a previous work [54]. An amount of 60 g of ground sample was charged into 200 mL steel extraction vessel. The top and bottom of the vessel were covered with a thin layer of cotton before closing to avoid the sample from entering through the gas line. A high-pressure pump (Mirloyal, Milton, Roy, USA) was used to pump CO_2 into the extraction vessel at the desired pressure. To attain the desired pressure, a back pressure regulator (BPR) was used to control the pressure. Water baths were connected to the extraction vessel and separator to maintain the temperature. During the extraction, a gas flow meter (Shinagawa, DC-1, Tokyo, Japan) was used to measure the CO_2 consumed. The oil from the sample was extracted at 40°C, 45°C and 50 °C and pressure of 15 MPa, 20MPa, 25 MPa, and 30 MPa. The flow rate of CO_2 was maintained constant at 27 g/min, and the extraction time was fixed to 3 h. The extracted oil was collected from the separating vessel every 30 mins to study the influence of extraction time. Extracted oils were kept at -20°C for analysis.

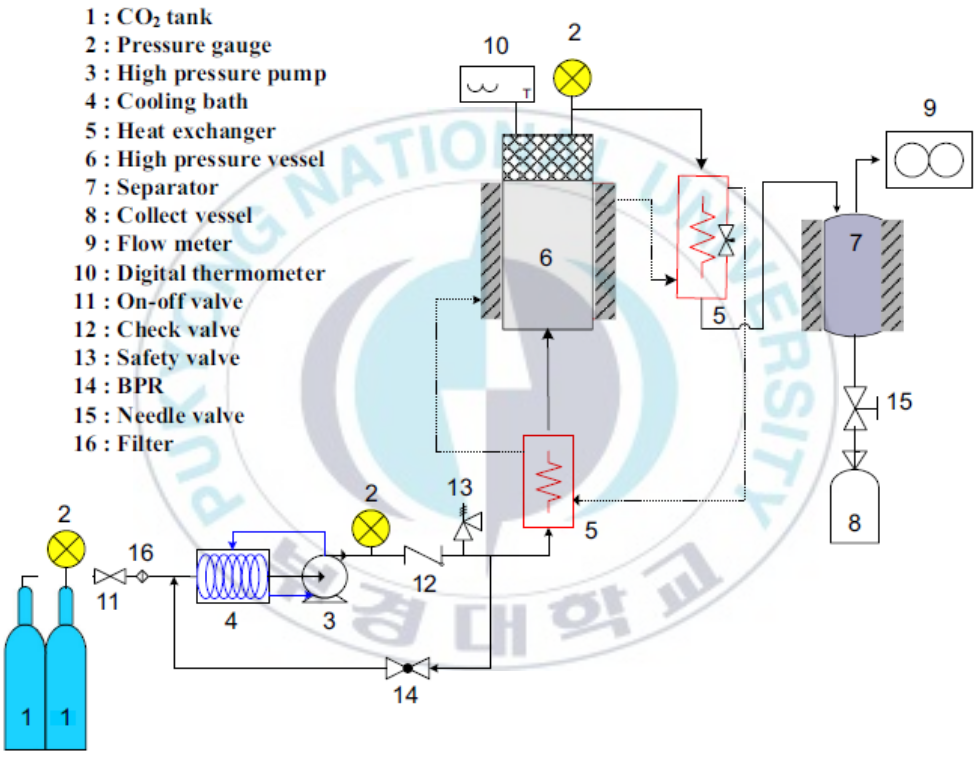

Fig. 2. 1. Schematic diagram of SC-CO_2 extraction system used in this study [54]

2.2.4. Soxhlet extraction

Oil was extracted using method of Ahmed et al [59] Approximately 5 g of ground sample was put into a thimble and put into a soxhlet extractor. A round bottom extraction flask was filled with 100 ml n-hexane or ethanol (97.8% purity, SK chemicals, Gyeonggi-do, Korea). The flask was heated at 65 °C using a heating mantle (Wisd Daihan Scientific Co., Ltd, Gangwon-do, Korea) for 16 h. After the extraction was completed, the solvent was removed using rotary evaporator (Eyela N-1100. Tokyo, Japan) at 50 °C and then placed in an oven at 40 °C for 1 h.

2.2.5. Oil stability tests

Peroxide value (meq/kg) was determined in agreement with the American Oil Chemist' Society (AOCS) official method. Free fatty acid value (%) was determined using the AOCS official method. [60] [61]

P-Anisidine value was determined according to the AOCS official method. In order to remove moisture and impurities, oil sample was filtered through a Whatman No. 40 filter paper. About 1g sample was weighed in a 50 ml volumetric flask. After dissolving and diluting the oil sample with 25 mL 2, 2, 4-trimethylpentane (iso-octane), a spectrophotometer (UVmini-1240 UV-Vis spectrophotometer, USA) was used to measure the absorbance of the oil at 350nm. Approximately 1 ml of p-Anisidine reagent was added into a test tube containing 5 ml oil sample. The blank was prepared by measuring 5 ml of iso-octane into another test tube and 1 ml of p-Anisidine reagent was added into it. The p-Anisidine reagent was made by adding 0.25 g of p-Anisidine to 100 mL of glacial acetic acid. The absorbance of the oil sample with p-Anisidine

reagent was measured at 350 nm after 10 min. The following equation was used to determine the value of p-Anisidine

$$\text{p-Anisidine value} = \frac{25*(1.2*As-Ab)}{Sample\ weight\ (g)} \quad (2\text{-}1)$$

As= Absorbance of oil after reaction with p-Anisidine

Ab= Absorbance of oil before reaction

Sample weight=1 g

TOTOX value was considered as p-Anisidine value plus twice the peroxide value.

2.2.6 Color

The color of the extracted oils was accessed according to a previous method with the L*a*b* system[54] using a Portable Reflectance Spectrophotometer (Lovibond RT Solstice Park, Amesbury, UK). The instrument was standardized each time with white and black references. L* expressed the lightness of the sample from 0 to 100, with 100 being pure white; the a* value represented red (+) to green (-), and b* value represents yellow (+) to blue (-).

2.2.7. Thermogravimetric analysis

Yellowtail fish viscera oil extracted using SC-CO_2 and with soxhlet was subjected to thermal stability analysis using a Thermogravimetric Analyzer (Perkine Elmer Model, USA, TGA 7). About 5 mg of sample was placed in the furnace and the temperature was increased from 50 °C to 700 °C at the rate of 10 °C/min. O_2 was provided at the rate of 50 cm^3/min to aid proper oxidation conditions as the sample purge gas. The plotter (ColorPro, Hewlett-Packard) automatically records the reduction of sample weight every 6 s.

2.2.8. Fatty acid composition analysis

Gas chromatography (GC) was used to determine the fatty acids composition of yellowtail fish viscera oil extracted using SC-CO_2 and soxhlet methods [62]. The analysis was carried out using a 6890 Agilent (Agilent Technologies, Wilmington, NC, USA) gas chromatography with a fused silica capillary column (100 m length x 0.25 mm internal diameter, 0.25 um of film) (Supelco, Bellefonte, USA). Fatty acid methyl esters (FAMEs) of extracted oil were carried out in accordance with the Official Method of the AOCS [63] . The oven temperature was maintained at a constant temperature of 130 °C for 3 mins, afterward increased to 240 °C at a rate of 4 °C/min and then retained at 240 °C for 10 mins. The temperature for the injector and detector was held at 250 °C. Fatty acids were identified by comparing the retention time with a standard FAMEs mixture and quantified by the area % of total fatty acids

2.2.9. Statistical analyses

The multiple and repeat sets of data were analyzed as an ANOVA from IBM SPSS version 20 (SPSS Inc., Chicago, USA). The differences within groups were defined using the Tukey HSD $p < 0.05$.

2.3. Results and Discussion

2.3.1 Oil yield

Fig. 2.2. show the extraction yield of yellowtail fish viscera oil extracted by SC-CO_2 extraction method at different conditions and soxhlet method. The yield of oil extracted using n-hexane (48.48%) or ethanol (56.13%) soxhlet extraction method was significantly higher than that of SC-CO_2 extraction (11.03% - 40.87%). The extent of

selectivity of SC-CO_2 plays a big role in its lower yield. SC-CO_2 is highly selective that it can only extract non-polar compounds. And n-hexane has poor selectivity and thus makes it efficient at extracting non-polar and amphiphilic compounds, e.g., phospholipids [54] Extraction yield is reasonably reliant on fish species and part used for extraction [64]. Rabio-Rodriguez [65] reported that approximately 10 g of oil/100 g of dry raw material was recovered from processing scrap of hake but the fatty fish species e.g. salmon and orange roughly offcut yield greater quantities of 40g and 50g of oil respectively at 100 g raw material. And also Indian mackerel provided 52.3 g oil/100 g dry sample [66], African catfish 67 g oil/100 g dry sample [67], Tuna 36.2 g [68]

2.3.2. *Influence of extraction time, pressure and temperature*

During SC-CO_2 extraction process, sample was collected from the separating vessel every 30 mins and weighed to ascertain the influence of extraction time. Based on the obtained results, it is observed that the yield of oil entered in equilibrium stage after 180 mins (Fig.2.3). More than 78% of total oil was extracted within 90 mins of the extraction period.

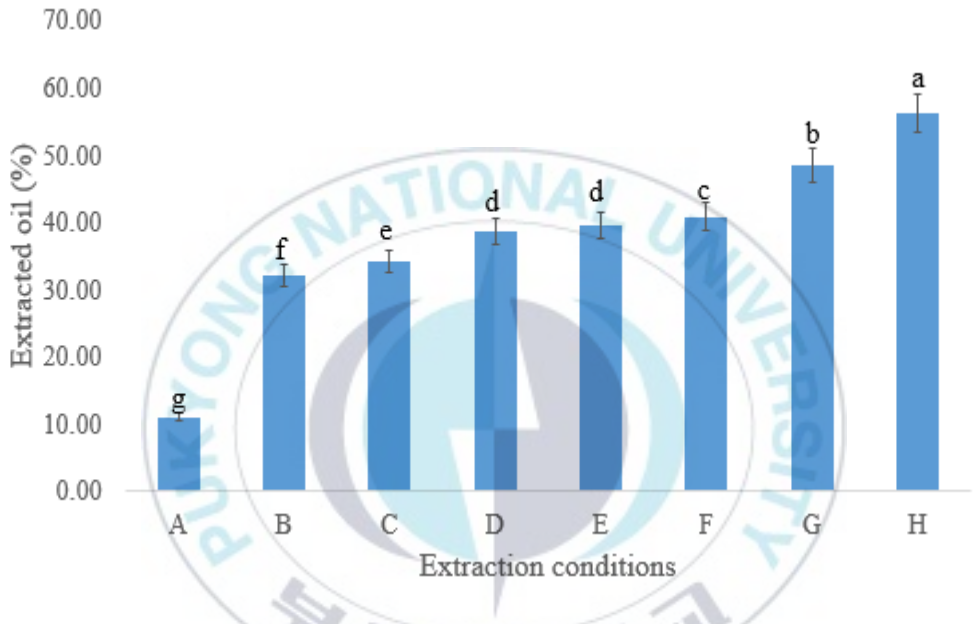

Fig. 2. 2. Yield of yellowtail fish viscera oils extracted by SC-CO_2 and Soxhlet appara tus

(A= 45℃, 15MPa; B= 45℃, 20MPa; C= 45℃, 25MPa; D= 40℃, 30MPa; E=45℃, 30MPa; F= 50℃, 30MPa; G= Hexane; H= Ethanol)

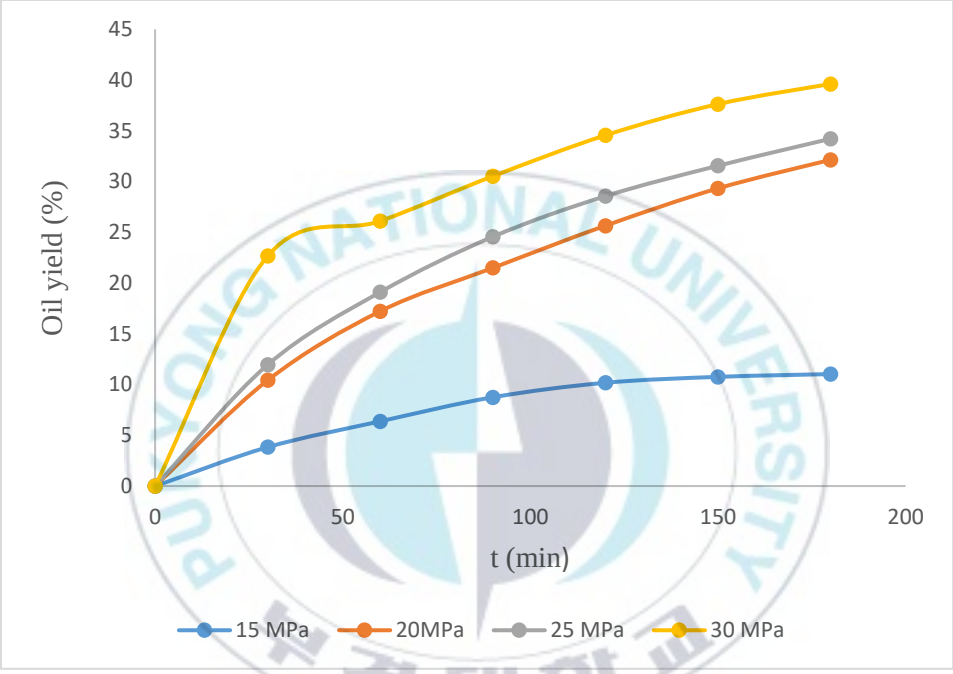

Fig. 2. 3. The influence of extraction time on oil yield

(Temperature: 45°C; CO_2 flow rate: 27 g/min)

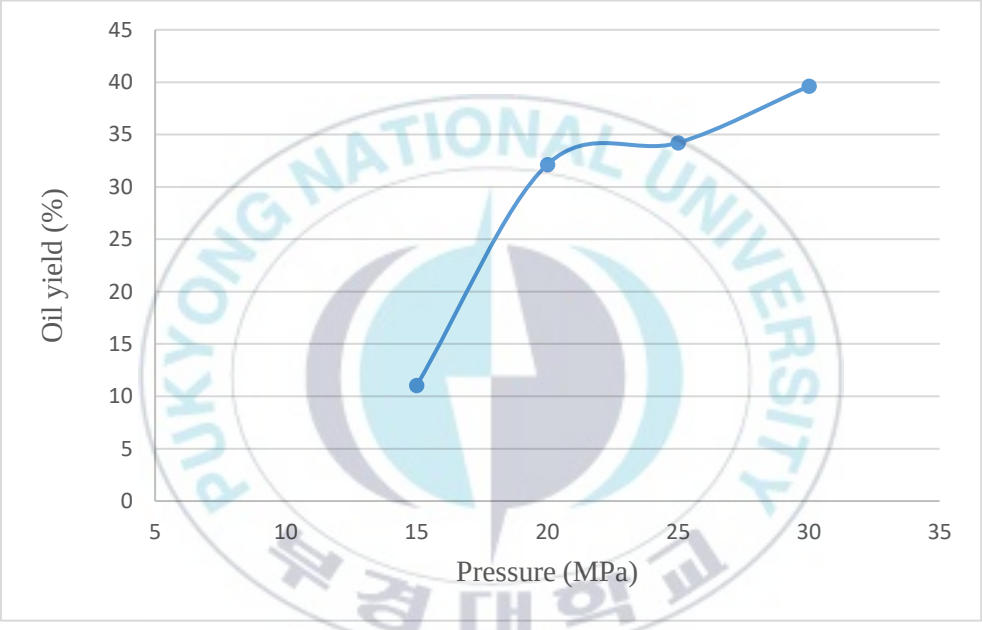

Fig. 2. 4. Influence of pressure on oil yield

(Temperature: 45°C, CO_2 flow rate: 27g/min)

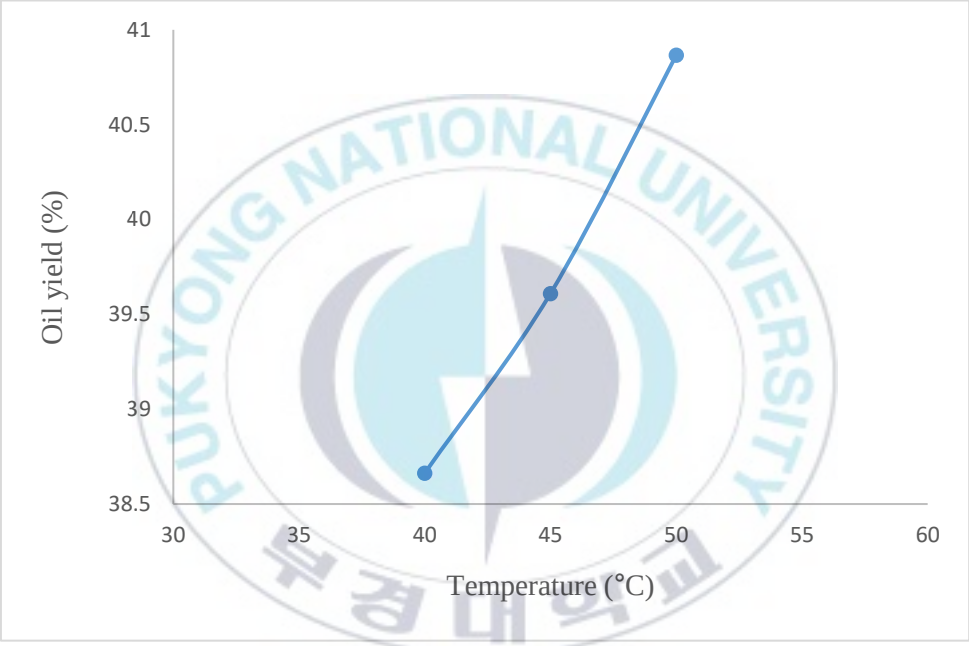

Fig. 2. 5. Influence of temperature on oil yield

(Pressure: 30 MPa; CO_2 flow rate: 27 g/min)

The influence of pressure at a constant temperature of 45°C was studied. It was noted that increase in pressure resulted in an increase in oil yield The maximum oil produced was 40.87% (72.81% of the total oil) at 30 MPa and the lowest yield was 11.03% (19.65% of the total oil) at 15 MPa. The total oil content of yellowtail viscera was 56.13% which was determined using soxhlet extraction method (ethanol).

The density and solubility of CO_2 increase with an increase in pressure, thereby enhancing its solvating power. Bulgarevicg et al., [69] reported that by raising the pressure, the solvent power increases, hence strengthening intermolecular physical interactions. Lee et al[70] also recorded a similar result during extraction of oyster oil. Similarly, increased temperature at a constant pressure of 30 MPa increases the oil yield (Fig.2.5). The highest yield of oil was found at 50°C while 40°C produced the lowest oil yield. The mass of CO_2 increased at high pressure and temperature leading to high extracted oil yield.

Meanwhile, the effect of temperature on omega 3 fatty acids was also observed. Fig.2.6. shows that at a constant pressure of 30 MPa, the omega-3 content decrease with increased temperature. DHA can easily degrade at high temperature due to the thermal instability of the 6 double bonds[71]. Therefore a decrease in omega-3 was observed as the temperature increases.

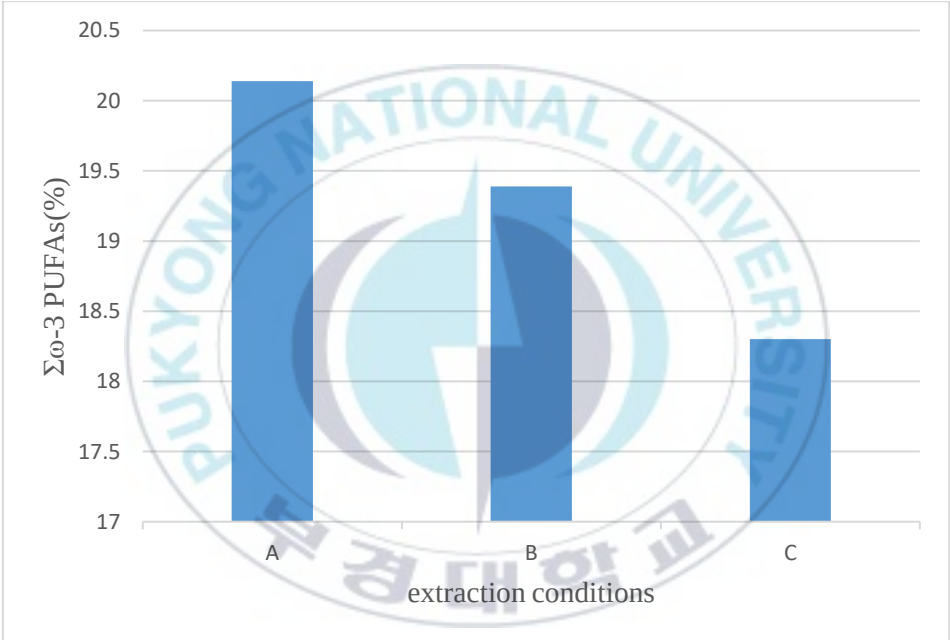

Fig. 2. 6. Effect of temperature on Σω-3 PUFAs

(A: 40°C, 30MPa; B: 45°C, 30MPa; C: 50°C, 30MPa)

2.3.3. Fatty acids composition

Table 2.1. represents the fatty acids composition of yellowtail viscera oil extracted by SC-CO_2 and soxhlet apparatus using organic solvents n-hexane or ethanol. The fatty acids composition of oil extracted comprises of 18 fatty acids in total. The FA of yellowtail fish viscera oil extracted by n-hexane and ethanol contain 38.63% and 41.42% total saturated fatty acids (SFA; C14:0, C15:0, C16:0, C18:0, C20:0, and C23:0) respectively. The total unsaturated fatty acids (UFA; C16:1, C17:1, C18:1, C20:1, C22:1, C24:1 C18:2, C18:3, C20:2, C22:2, C20:5, and C22:6) of oil extracted using n-hexane and ethanol comprised 61.37% and 58.58% of total fatty acids respectively.The greatest recovery of SFA (C14:0, C15:0, C16:0, C18:0, C20:0, and C23:0) constituents in yellowtail fish viscera oil extracted by SC-CO_2 were achieved at 45°C, 25 MPa (41.3%), 45°C 20MPa (41.47%), 50°C 30 MPa (41.3%), 45°C 30MPa (41.17%), 40°C 30MPa (41.16%), and 45°C 15MPa (40.43%). The total amount of UFA extracted were also similar to that of oils extracted using n-hexane: 59.57%, 58.4%, 58.83%, 58.7%, 58.53%, 58.16% at 45°C 15MPa, 40°C 30MPa, 45°C 30MPa, 50°C 30MPa, 45°C 20MPa, and 45°C 25MPa respectively. Among the SFA, Palmitic (C16:0) was present at the highest concentration ranging from 25.65% to 22.4% among the extracted oils. Among the UFA of extracted oil, Oleic (C18:1n9c) was found to be the highest constituent ranging from 26.63% to 23.87%. The total omega-3 fatty acids content of n-hexane and ethanol extracted oils was higher at 21.38% and 20.37% respectively, than the SC- CO_2 extracted oils (18.13%-20.14%). SC-CO_2 is highly selective in extracting only non-polar lipids whereas n-hexane have very high ability in extracting

amphiphilic and non-polar compounds [59].A substantial amount of DHA and EPA are present in phospholipids [12]

2.3.4. Physico-chemical properties

The physicochemical properties of yellowtail fish viscera oil extracted by SC-CO_2 at different conditions and soxhlet (n-hexane and ethanol) are shown in Table.2.2. In instrumental color measurement, the most frequently used systems are the L*, a*, b*, with primary parameters of lightness (L*), yellowness (b*) and redness (a*). Lightness was significantly affected by the extraction conditions and method. The highest value of L* was found in SC- CO_2 extracted oil at 45°C 30MPa (51.73±0.44). While the lowest value was found in oil extracted by ethanol (24.10±0.34), followed by n-hexane extracted oil (25.03±0.06). Oils extracted by n-hexane and ethanol are lower due to the oxidation process that occur during extraction which leads to the formation of dark compounds. The color is contributed by impurities, pigment content, oxidation compound, and the processing conditions[72] The highest value of redness was found in SC- CO_2 extracted oil of 45°C 20MPa and the lowest value was found in soxhlet extracted oils.

Peroxide value analysis determines the primary oxidation of oil and the measurement of hydroperoxide [73]. The acceptable limit of peroxide value set by the Global Organization for EPA and DHA (GOED) and Food and Agricultural Organization of the United Nations (FAO) for quality and approval of fish oils for human consumption is ≤5 meq/kg [74]. The findings from the present study indicated that all oil samples extracted at different temperature and pressure have peroxide value between 2.07-3.60

meq/kg with the exception of oil samples extracted by n-hexane and ethanol which has a value above the acceptable value of ≤5 meq/kg (Table 2.2).

The *p*-anisidine value is used to determine the measurement of aldehydes with α- and β-unsaturation and secondary oxidation. In this study the soxhlet extracted oils have the highest *p*-anisidine value (Table 2.2), this is due to prolonged exposure of oil to heat and atmospheric oxygen during soxhlet extraction. The *p*-anisidine values of SC-CO_2 extracted oil at different temperature and pressure were between 9.73 and 13.65 which is within the allowed limit of ≤20. Factors such as lipid class compositions, light, the concentration of oxygen and the presence of antioxidant can impact the formation of hydroperoxide and degradation into secondary oxidation products [75] [76]. Pak [77] studied the stability and quality of fish oil and reported the *p*-anisidine value to be 19.8 Honold [78] reported an anisidine value of 0.60-1.21 from fish fillet by-product.

In order to determine the quality of fats and oil, free fatty acids (FFA) content is regarded as one of the main criteria. Also, FFA was used to evaluate fish degradation during frozen storage [79] and an increase in storage time result to increase in FFA conten

Table 2. 1. Fatty acids composition of yellowtail fish oil extracted by SC-CO_2 and soxhlet

Fatty acids	SC-CO_2 extraction						soxhlet extraction	
	45°C, 15 MPa	45°C, 20MPa	45°C, 25MPa	40°C, 30 MPa	45°C, 30 MPa	50°C, 30 MPa	Hexane	Ethanol
Mystric Acid (C14:0)	5.81^{a}±0.06	5.29^{b}±0.03	5.12^{bc}±0.01	5.01^{bc}±0.01	5.07^{bc}±0.34	4.8^{c}±0.11	5.02^{bc}±0.20	5.31^{b}±0.49
Pentadaecanoic Acid (C15:0)	1.14^{a}±0.1	1.06^{b}±0.20	1.03^{cd}±0.01	1.01^{cd}±0.00	1^{d}±0.01	1.01^{cd}±0.0	1.03^{cd}±0.03	0.97^{e}±0.01
Palmitic Acid (C16:0)	26.09^{a}±0.23	25.92^{a}±0.04	26.2^{a}±0.05	25.74^{a}±0.07	25.65^{a}±0.1	25.9^{a}±0.03	22.4^{b}±3.08	25.40^{a}±0.12
Palmitoleic Acid (C16:1)	8.65^{a}±0.02	7.54^{b}±0.06	7.27^{c}±0.01	7.16^{c}±0.01	6.83^{d}±0.05	6.81^{d}±0.00	7.21^{c}±0.28	6.94^{d}±0.05
cis-10-HeptadecanoicAcid (C17:1)	0.7^{a}±0.01	0.62^{b}±0.01	0.63^{b}±0.01	0.62^{b}±0.02	0.61^{bc}±0.01	0.63^{b}±0.01	0.62^{b}±0.02	0.59^{c}±0.00
Stearic Acid (C18:0)	6.77^{e}±0.06	7.39^{d}±0.01	7.7^{bc}±0.01	7.61^{c}±0.02	7.71^{bc}±0.02	7.88^{b}±0.01	8.25^{a}±0.34	7.89^{b}±0.04
Oleic Acid (C18:1n9C)	24.21^{d}±0.21	24.08^{d}±0.01	24.48^{cd}±0.07	24.15^{d}±0.01	25.71^{b}±0.07	26.63^{a}±0.03	24.97^{c}±1.01	23.87^{d}±0.13
Linoleic Acid (C18:2n6c)	1.8^{a}±0.01	1.68^{bc}±0.02	1.64^{bc}±0.02	1.65^{bc}±0.01	1.54^{d}±0.01	1.54^{d}±0.01	1.71^{bc}±0.11	1.62^{c}±0.01
Arachidic Acid (C20:0)	0.31^{e}±0.5	0.36^{d}±0.02	0.43^{b}±0.03	0.4^{bc}±0.00	0.38^{cd}±0.00	0.39^{cd}±0.01	0.47^{a}±0.02	0.43^{b}±0.01
cis-11-Eicosenoic Acid (C20:1)	0.83^{f}±0.01	1.11^{e}±0.02	1.20^{c}±0.01	1.22^{c}±0.01	1.16^{d}±0.00	1.22^{c}±0.02	1.31^{a}±0.06	1.27^{b}±0.01

Linolenic Acid (C18:3n3)/ ALA	1.37^{a}±0.02	1.28^{b}±0.02	1.23^{cd}±0.01	1.26^{bc}±0.01	1.17^{e}±0.01	1.14^{e}±0.01	1.26^{bc}±0.01	1.21^{d}±0.01
Eicosadienoic Acid (C20:2)	2.33^{a}±0.72	2.09±0.1	1.93±0.07	2±0.02	1.84±0.01	1.76±0.01	1.94±0.08	1.85±0.02
Euric Acid (C22:1n9)	0.4^{g}±0.01	0.62^{ef}±0.01	0.69^{d}±0.00	0.72^{c}±0.02	0.61^{f}±0.00	0.63^{e}±0.01	0.82^{a}±0.03	0.78^{b}±0.01
Tricosanoic Acid (C23:0)	1.60^{a}±0.01	1.45^{b}±0.00	1.36^{d}±0.00	1.39^{cd}±0.01	1.36^{d}±0.01	1.31^{e}±0.01	1.46^{b}±0.07	1.42^{bc}±0.01
Docosadienoic Acid (C22:2)	0.59^{b}±0.00	0.59^{b}±0.00	0.57^{b}±0.01	0.59^{b}±0.00	0.54^{c}±0.00	0.52^{d}±0.00	0.62^{a}±0.03	0.58^{b}±0.01
Eicosapentanoic Acid(C20:5n3)/ EPA	7.82^{a}±0.07	7.26^{b}±0.01	6.82^{e}±0.01	7.06^{cd}±0.02	6.25^{f}±0.02	5.8^{g}±0.01	7.23^{bc}±0.28	6.9^{de}±0.03
Nervonic Acid (C24:1)	0.30^{g}±0.01	0.5^{f}±0.01	0.55^{d}±0.01	0.59^{c}±0.01	0.58^{c}±0.00	0.6^{c}±0.01	0.79^{a}±0.04	0.71^{b}±0.01
Docosahexanoic Acid (C22:6n3)/ DHA	9.28^{e}±0.07	11.16^{d}±0.03	11.15^{d}±0.07	11.82^{c}±0.04	11.97^{bc}±0.02	11.43^{d}±0.01	12.89^{a}±0.46	12.26^{b}±0.01
Σω-3 PUFAs	18.49±0.16	19.7±0.04	19.2±0.1	20.14±0.07	19.39±0.05	18.37±0.02	21.38±0.75	20.37±0.05
Σω-6 PUFAs	1.8±0.1	1.68±0.00	1.64±0.02	1.65±0.01	1.54±0.01	1.54±0.01	1.71±0.11	1.62±0.01
ΣUFAs	59.57±0.38	58.53±0.34	58.16±0.22	58.84±0.34	58.83±0.26	58.7±0.26	61.37±0.78	58.58±0.61
ΣSFAs	40.43±0.38	41.47±0.34	41.84±0.22	41.16±0.34	41.17±0.26	41.3±0.26	38.63±0.78	41.42±0.61

N.B.: values are mean ± SD of three determinations. Means with the different superscript letter in each row differ significantly (P<0.05)

Table 2. 2. Physio-chemical properties of SC- CO_2 and soxhlet extracted yellowtail fish viscera oil

extraction		SC- CO_2 extraction						Soxhlet	
Parameters		45℃, 15MPa	45 ℃, 20MPa	45℃, 25MPa	40℃,30MPa	45℃,30MPa	50℃,30MPa	Hexane	Ethanol
color	L*	41.74^{d}±0.13	46.67^{c}±0.25	43.91^{d}±0.25	46.54^{c}±0.41	51.73^{a}±0.44	50.57^{b}±0.06	25.03^{f}±0.39	24.10^{g}±0.34
	a*	4.61^{e}±0.08	16.17^{a}±0.65	12.92^{c}±0.51	15.23^{b}±0.62	6.83^{d}±0.25	12.91^{c}±0.15	1.12^{f}±0.06	0.40^{g}±0.04
	b*	21.11^{e}±0.47	31.28^{c}±0.24	26.86^{d}±0.58	30.68^{c}±0.68	36.83^{b}±0.71	37.87^{a}±0.37	1.70^{f}±0.27	0.38^{g}±0.02
Peroxide value (meq/kg)		3.20^{cd}±0.20	2.60^{de}±0.20	2.07^{e}±0.12	2.50^{de}±0.10	3.60^{c}±0.20	2.73^{de}±0.32	6.17^{b}±0.76	8.33^{a}±0.58
FFA value (%)		4.28^{d}±0.29	4.37^{d}±0.16	5.04^{c}±0.16	4.56^{d}±0.29	4.56^{d}±0.29	4.47^{d}±0.16	6.75^{b}±0.16	7.41^{a}±0.29
p-Anisidine value		9.73^{e}±1.36	10.87^{de}±0.68	12.68^{cd}±1.68	13.65^{c}±0.56	12.08^{cd}±0.39	11.47^{cde}±1.16	17.12^{b}±1.08	20.91^{a}±1.69
TOTOX value		16.13^{f}±0.13	16.07^{f}±0.07	16.82^{e}±0.10	18.65^{d}±0.20	19.28^{c}±0.20	16.93^{e}±0.10	$29..46^{b}$±0.20	37.57^{a}±0.57

N.B.: values are mean ± SD of three determinations. Means with the different superscript letter in each row differ significantly ($P<0.05$)

According to Aryee et al., [80] lipids extracted from fish tissues possess relatively high autolytic activities which are highly sensitive to both oxidation and lipolysis. In this work, SC- CO_2 extracted oil at 45°C and 150 MPa had the lowest value of FFA of 4.28%, whereas the FFA content of ethanol extracted oil and n-hexane extracted oil has the highest values of 7.41% and 6.75% respectively. At higher temperature the hydrolysis of ester bonds of triglycerides is greater, therefore oil extracted at higher temperature contain higher FFA [81] The yellowtail fish viscera oils extracted by SC-CO_2 at different temperature and pressure showed FFA values within the recommended limits of 2-5% [82] The existence of high FFA contents in the gut samples was due to the presence of endogenous enzyme causing autolysis of viscera tissues during oil extraction and processing [83]. Huang and Sathivel [84] reported that the FFA content of unpurified salmon oil was 3.5%.

TOTOX value of SC- CO_2 extracted oils (16.07-19.28) shows better quality over soxhlet extracted oils (29.46-37.570). The TOTOX values of SC- CO_2 extracted oils were within the range of $\leq$26 which is the recommended acceptability of fish oil for human consumption. The SC-CO_2 method operates under lower oxidizing conditions (mild temperatures, non-oxidant atmosphere, and darkness) than the non-SFE procedures, made possible to reduce significantly the TOTOX value [65]. Aidos [85] reported TOTOX value of 14.9 in oil extracted from herring by-product. Garfish, golden mullet, shad and mackerel were also reported to have TOTOX value ranging from 8.04 to 35.29 [86]

2.3.5. Thermogravimetric analysis

Thermogravimetric analysis is used to determine the weight degradation pattern of yellowtail fish viscera oil for thermal application based use. The thermogravimetric degradation of yellowtail fish viscera oil is shown in Fig.2.7. Yellowtail fish viscera oil extracted using SC-CO_2 present better oxidation degradation than soxhlet extracted oil. SC-CO_2 extracted oil was stable until 200°C and it starts to decline gradually. At 200°C, the obtained weight of oil extracted using SC-CO_2 at 45 °C 15 MPa and 50 °C 30 MPa was 81.99 % and 80.47 % respectively while soxhlet (ethanol) extracted oil was 63.87%. Notable decrease of sample weight was recorded at 300 °C, and at this temperature, the recorded weight of oil extracted using SC-CO_2 at 45 °C 15 MPa, 50 °C 30 MPa and soxhlet (ethanol) were 40.91 %, 37.49 %, and 18.75 %, respectively. Usually, in the presence of atmospheric oxygen, lipids are oxidized resulting in the formation of peroxides, involving in the increase of sample weight [87]. No increase in the weight was sighted in the thermogravimetric curve of yellowtail fish viscera oil, which indicates that there is no relationship between oxygen absorption and thermal degradation of oil. The better stability of SC-CO_2 extracted oil at high temperature indicates that they are suitable for use in thermal application base food. Sathivel [72] reported that red and pink salmon oil weight was drastically reduced at 200°C and 450°C

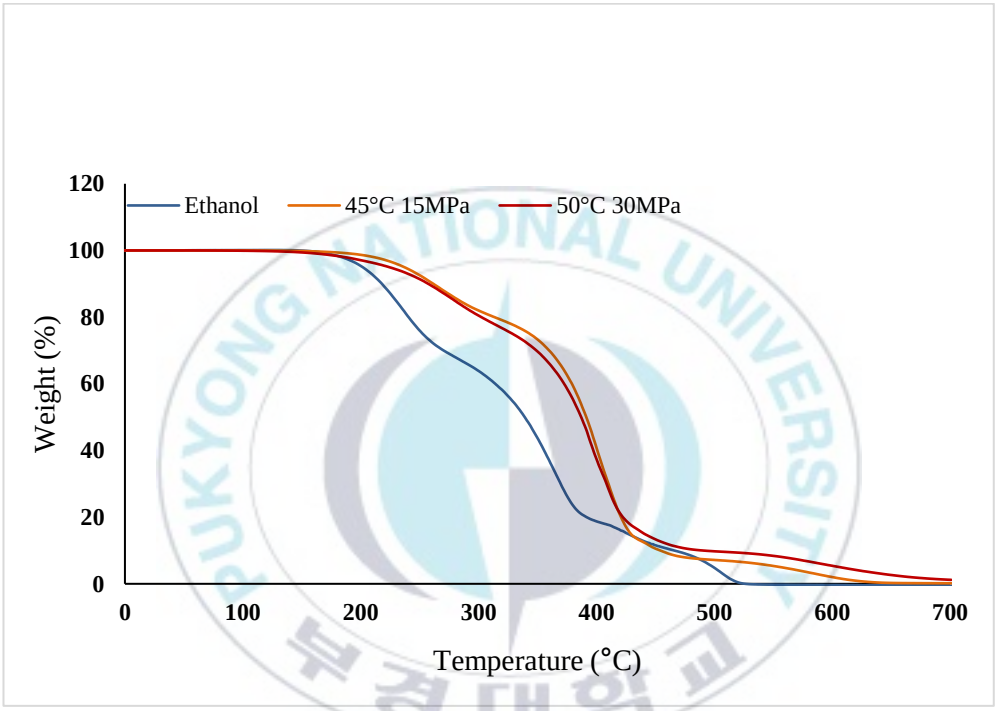

Fig. 2. 7. Thermogravimetric analysis of yellowtail fish viscera oil

2.4. Conclusion

A study on the yield of yellowtail fish viscera oil extracted using SC-CO_2 technique was carried out through determination of the influence of different extraction conditions. In addition, the quality of extracted oil was determined by analyzing the physical and chemical properties. The SC-CO_2 extraction of yellowtail fish viscera oil at 50°C and 30 MPa was found suitable for oil yield and stability. Oil extracted by ethanol and n-hexane exhibited a higher yield than SC-CO_2 extraction but has inferior physicochemical properties. Yellowtail fish viscera oil contained a reasonable amount of omega polyunsaturated fatty acids, with the highest content of omega-3 fatty acids recovered at 40°C and 30 MPa. The generated results showed that yellowtail fish viscera which is considered as a waste product can be utilized as a potential source of omega polyunsaturated fatty acids. And by so, it would help reduce environmental pollution. Furthermore, the obtained results indicated that supercritical carbon dioxide extraction method can be considered a suitable technique for extraction of high-quality oil from fish matrix.

CHAPTER 3

Characterization of phospholipids extracted from yellowtail fish viscera using supercritical carbon dioxide with ethanol as co-solvent

3.1. Introduction

Yellowtail fish is an important marine commercial fish in Asia and it is native to the central Pacific Ocean. The major fishing area is in Japan and the Republic of Korea. In 2014, the total production of yellowtail fish in Japan was approximately 135,998 ton, which represents about 53% of the total production of aquaculture marine finfish in Japan (FAO 2018). Our previous work revealed that lipids from yellowtail fish viscera (YTV) contain a reasonable amount of omega-3 fatty acids mostly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are known to possess the functions of preventing the rate of cancer, heart diseases, inflammation and so on [88]. Most of the omega-3 fatty acids are present in the phospholipid form, where phosphatidylcholine (PC) is the predominant lipid class [89] [7]. Phospholipid (PL) is known to serve as the building block for cell membranes, furnished with necessary biological and physiological in about all known living things [8] [9]. More beneficial effects would be annexed from omega-3 fatty acids containing phospholipid, like regulating liver and blood plasma lipid levels [10] [11]. Commercially available phospholipids are extracted from egg yolk which lacks omega-3 fatty acids. Marine

phospholipid hold more advantages over traditional phospholipid, in cosmetic and pharmaceutical applications [8]. Recently, extraction of phospholipids from marine product has attracted so much attention, as this type of phospholipids is rich in omega-3 fatty acids [12].

Generally, phospholipids are extracted with organic solvents like hexane, ethanol, diethyl ether, and chloroform. Some of these solvents are considered to have unfavorable effects on the environment and consumers health [36] [37]. Recently, supercritical carbon dioxide (SC-CO_2) extraction technique is widely applied in the extraction of lipids and other organic compounds from different solid and liquid matrixes. The SC-CO_2 technique is a promising method for the extraction of edible lipids, as it is a green technology and non-toxic in food [90] [42]. SC-CO_2 extraction process occur in a closed chamber with no presence of outside air. Consequently, there is lesser oxidation of recovered compounds and the residues compared to organic solvent extraction [91]. Phospholipid, being a polar compound is relatively less soluble in pure SC-CO_2 [92]. Hence, SC-CO_2 extracts primarily non-polar lipids but the remaining residue contains some amphipathic and polar lipids like phospholipid. Since SC-CO_2 cannot effectively dissolve phospholipids, addition of a modifier to SC-CO_2 can attain recovery of phospholipid [93]. Addition of co-solvent can fix the relatively low extraction of SC-CO_2 for polar compounds [94] because co-solvent enhances the viscosity, density, and solubility of lipids in SC-CO_2 hence improving the mass transfer rate [95] [34]. The suitable co-solvent must be chosen based on thermodynamic considerations and food safety. In respect to this, ethanol is considered a notable solvent for extraction of food grade phospholipid (Prosise, 1985). Ethanol showed a positive effect on the extraction of phospholipid from canola meal [96]. [35] reported that

extraction of phospholipid from soya lecithin was maximized by use of SC-CO_2 and ethanol as co-solvent.

Many research work has been done on organic solvent extraction of phospholipid from de-oiled residues [36] [91] but there is a limited report on phospholipid extraction by SC-CO_2 with ethanol as co-solvent. Therefore, the aim of this work was (1) to isolate phospholipids from YTV using SC-CO_2 with ethanol as co-solvent, and using a traditional organic solvent (ethanol). (2) To analyze the quality characteristics of extracted phospholipids.

3.2. Materials and Methods

3.2.1. Chemicals and reagents

Pure CO_2 (99.9%0 was provided by KOSEM in Yangsan, Korea. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine, fatty acids ethyl ester (FAMEs), Trolox, 2,2 Diphenyl-1-picrylhydrazyl (DPPH), and 2,2'azino-di, 3 ethylbenzthiazoline-6-sulphonic acids (ABTS) standard were purchased from Sigma-Aldrich, ST. Louis, MO, USA. All solvents and reagents were of HPLC or analytical grade.

3.2.2. Sample preparation

The sample was prepared according to the method in chapter 1.

3.2.3. Phospholipid extraction using SC-CO_2 and ethanol as co-solvent

Phospholipids extraction was carried out using SC-CO_2 laboratory scale described in the previous chapter. Precisely 60g of dried, ground YTV sample was filled into a 200 ml stainless extractor. The sample was de-oiled using SC-CO_2 extraction at 45°C

and 25 MPa for 3 hours. The extracted oil was collected and stored at -6°C for analysis. Afterward, the de-oiled residue was kept in the extractor for extraction of phospholipid by SC-CO_2 and ethanol as co-solvent. Phospholipid was extracted at a temperature of 45°C and pressure of 27.5 MPa for 2.5 hours. CO_2 and ethanol flow rate were kept constant at 27 g/min and 3 ml/min respectively. The phospholipid extraction condition was suggested by some researchers [97]. Finally, the extracted phospholipids-ethanol mixture was collected in a glass tube. Ethanol was evaporated using rotary evaporator set at 45°C. The extracted Phospholipids was stored at -20°C for further analysis.

3.2.4. Total organic solvent extraction of phospholipid

Organic solvent extraction of phospholipid from YTV was carried out following the method of Lee et al. [98]. About 40g of YTV SC-CO_2 extracted residue was placed in a beaker containing 400 ml ethanol (94%) and stirred for 12 hours using a magnetic stirrer. The resulting mixture was filtered using a filter paper, and the filtrate was stored at 4°C, and the remaining residue was extracted again using the same method. The two recovered ethanol extract solution was mixed together and twice the volume of n-hexane was added to fractionate the polar lipids. The rotary evaporator was used to evaporate the ethanol extract containing phospholipid. Afterward, the nonpolar lipid extract was dissolved by the addition of a small amount of hexane. A one-fifth volume of acetone (4°C) was added and the mixture was kept in an ice bath and gently shaken for 20 mins for phospholipids precipitation. Then, the solvent was removed by filtration, and the phospholipid was stored at -20°C for analysis.

3.2.5. YTV phospholipid yield and purity measurement

Phospholipid yield was calculated as % of de-oiled YTV residue. The purity of phospholipid was measured according to a method used by Stewart [99]. An amount of 1mg of extracted phospholipid was dissolved in chloroform, followed by addition of 200 µL of a solution containing the equal volume of ferric chloride (27 g/L) and ammonium thiocyanate (30g/L). Afterward, the mixture was vortex for 30s and was subjected to centrifugation at 3500 rpm for 15 mins. The lower phase absorbance was ascertained using a microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) at 488nm. A calibration curve was prepared using a standard PC (0.25-1.5mg). The calculation of obtained net phospholipid was based on the purity and yield of extracted phospholipid using this equation

$$\text{Obtained net phospholipid} = (\text{Yield} \times \text{Purity})/100 \quad (3.1)$$

3.2.6. Thin layer chromatography (TLC) analysis of phospholipid

TLC was used to determine the major phospholipid groups following a slight modification of the method used by Miniadis-Meimaroglou et al [100]. The static phase was a silica plate 60(10 x 10 cm 0.2mm thick, Machery-Nazel, Duren, Germany). Chloroform, methanol, acetic acid glacial and water (50:8:8:2 v/v) were used as mobile phase. Chloroform was used to dissolve the phospholipid samples. Iodine vapor was used to visualize the spots.

3.2.7. Analysis of major phospholipid groups using HPLC

The major groups of phospholipid extracted from YTV were identified and quantified using HPLC with evaporating light scattering detection (ELSD) (Model 400,

Softa Corporation, London, UK), a silica column (Sunfire Prep Silica Column, 250 x 4.6 mm, 5 μm, Waters Corporation, MA, USA), and a pump (PU-2080 Plus). Phospholipid was dissolved in chloroform and 20 μL of the solution was injected into the injector. The gradient mobile phase was Isopropanol, Hexane, and Water as phase A, phase B, and phase C respectively. The multi-step linear gradient was set at: 0-9 mins, 58% A, 40% B, 2% C; 10-24 mins, 48% A, 50% B, 2% C; 25-54 mins, 42% A, 50% B, 8% C; 55-60 mins, 48% A, 50% B, 2% C. The flow rate was 1 mL/min and the temperature of spray and drift tube were fixed at 70°C and 60°C respectively. The nebulizer gas (N_2) pressure was maintained at 50 psi. Major groups of phospholipid were identified by comparing with the retention time of phospholipid standard. The obtained data from HPLC were analyzed by millennium software.

3.2.8. Phospholipid stability analysis

The Free Fatty Acids value (FFA), Peroxide Value (PV), and Acid Value (AV) were determined according to AOCS official method Ca 5a-40 , AOCS official method Cd 8-53, and AOCS official method Cd 3d-63 respectively [61] [101] [60].

3.2.9. Thermogravimetric analysis of phospholipid

The extracted phospholipids were subjected to thermogravimetric analysis using a thermogravimetric analyzer (Perkine Elmer Model, USA, TGA 7). The thermal stability of extracted phospholipids were compared with that of phospholipid standard. An amount of 5 mg was placed in the aluminum pan and inserted into the furnace. The temperature of the furnace was heated from 50°C to 700°C at the rate of 10°C/min. In other to provide a suitable condition for oxidation, O_2 was provided at a rate of 50

cm^3/min. Decrease in sample weight was recorded automatically every 6s on a plotter (ColorPro Hewlett-Packard).

3.2.10. Fatty acid composition

Fatty acid composition of YTV phospholipid was determined with a gas chromatography system (Model: 6890, Agilent Technologies, Wilmington, USA) with a fused silica capillary column and a flame ionization detector. The extracted phospholipid fatty acid methyl esters (FAMEs) were carried out in accordance with the official method of AOCS [63]. The temperature of the oven was initially maintained at a constant temperature of 130°C for 3 mins, later increased to 240°C at the rate of 4°C/min and finally held at 240°C for 10 mins. The injector and detector temperature were kept at 250°C. Fatty acids were identified by comparing with FAMEs of standards and quantified by percentage area of the total fatty acids.

3.2.11. DPPH radical scavenging activities of YTV phospholipids

The DPPH scavenging effect of extracted phospholipids was determined following the method of [102] with slight modifications. DPPH solution (0.2mM) was prepared in methanol. The extracted phospholipids was diluted to different concentrations (20, 40, 60, 80, 100 mg/ml) and Trolox (20, 40, 60, 80 100 μg/ml) in methanol. Exactly 200 μL of the sample was mixed with 1 mL of DPPH solution, vortexed for 30s and kept in the dark for 30 mins. Afterward, a microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT USA) was used to record the sample absorbance at 517 nm.

3.2.12. *ABTS radical scavenging activity*

ABTS assay was determined by the method of [103] with some modifications. To prepare ABTS stock solution, 7 mM/L of ABTS (prepared in distilled water) and 2.45mM/L of potassium persulphate (prepared in distilled water) were mixed at a ratio of 1:1 (v/v). The mixture was kept in dark at room temperature for 16 h. Next, the ABTS working solution was prepared by dilution of stock solution with methanol to absorbance value of 0.74 ± 0.02 at 734 nm. Afterward, different concentrations of YTV extracted phospholipids (20, 40, 60, 80, 100 mg/ml) and Trolox (20, 40, 60, 80, 100 µg/ml) were prepared using methanol. An amount of 1 mL of ABTS solution was added into a test tube containing 100 µL of sample, vortexed for 30 s and kept for 10 mins in the dark. Then, a microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) was used to measure the absorbance value at 734nm.

3.2.13. Statistical analyses

The multiple and repeat sets of data were analyzed as an ANOVA from IBM SPSS version 20 (SPSS Inc., Chicago, USA). The differences within groups were defined using the Tukey HSD $p < 0.05$.

3.3. Results and Discussion

3.3.1. Yield and purity of phospholipids from YTV

In this study, 7.4 % phospholipids were extracted from YTV using organic solvent method and 6% phospholipids was extracted using SC-CO_2. The higher yield of phospholipids found in organic solvent extraction was due to the extraction of impurity.

YTV phospholipids extracted using SC-CO_2 method has a higher purity of 81.5% and organic solvent extracted phospholipids has a lower purity of 63.08%. The net phospholipid obtained was found to be 4.9% for SC-CO_2 extracted phospholipids, and 4.64% for organic solvent extracted phospholipids. [97] studied phospholipids extracted from salmon frame bone and reported a purity ranging from 66.1% to 80.9% and yield between 5.0% and 7.6% depending on extraction conditions. [91] reported that the yield of phospholipids extracted from squid viscera using SC-CO_2 method was 3.8%. The use of SC-CO_2 with ethanol as co-solvent in the extraction of phospholipids increases the concentration of phospholipids [35].

3.3.2. TLC analysis of major phospholipid groups

Fig.3.1. presents the TLC profile of YTV phospholipid. TLC identified three major phospholipids which are PC, PS, and PI. PC was found to be the largest quantity followed by PI, PS, and PE accordingly. [98] also reported that TLC analysis identified PC, PE, and PI in lecithin extracted from anchovy using SC-CO_2 and ethanol as co-solvent

Table 3. 1. Yield and purity of YTV phospholipid

	parameters		
Extraction method	Yield (%)	Purity (%)	Net PL obtained (%)
SC-CO_2 PL	6.00 ± 0.	81.53 ± 0.	4.90 ± 0.
Organic solvent PL	7.35 ± 0.	63.08 ± 0.	4.64 ± 0.

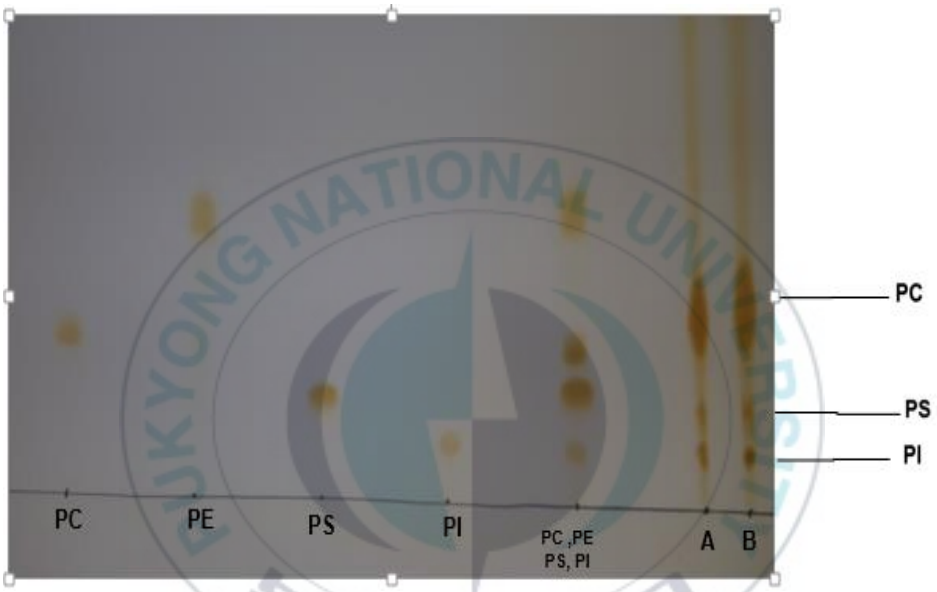

Fig. 3. 1. TLC analysis of phospholipid groups

(A: SC-CO_2/ethanol co-solvent extracted phospholipid; B: organic solvent extracted phospholipid)

3.3.3. Analysis of major YTV phospholipid groups using HPLC

Analysis of different major groups of phospholipids was performed using an HPLC-EID system. Four phospholipid classes, PC, PI, PS, and PE were identified in YTV phospholipid corresponding to phospholipid standard chromatogram. In SC-CO_2 extracted phospholipid, PC with a composition of 82.90% was found to be the most abundant phospholipid class. Followed were PI, PS, and PE with the content of 7.73%, 7.04%, and 2.33% respectively. YTV phospholipid extracted using organic solvent contain 67.29% PC, 16.11% PS, 13.70% PI, and 2.90% PE. Zhang, jing, et al. also detected PC, PI, PS, PE, LPC, and LPE in pacific saury viscera using HPLC-EID and found that PC and PI have the highest content [104]. Similarly, Wang et al. reported that the major phospholipid components in egg/gonad from several aquatic products were PC and PI [105]. Wang et al. found PC, PE, PI, SM CL, and LPC in squid egg using HPLC-EID [106]. The composition of phospholipid may vary depending on species, season, habitat and so on [98]. The discovery designated that YTV could be a beneficial source of marine phospholipid PC and PI levels

3.3.4. Phospholipid stability analysis

Table.3.2. presents the oxidation stability parameters of YTV phospholipid. Peroxide value is used to determine the rancidity that occurred due to autoxidation [107]. The POV of YTV phospholipid extracted using SC-CO_2 and ethanol as co-solvent was 4.80 meq/1000g. The POV of YTV organic solvent extract was found to be 8.53 meq/1000g, which shows the oxidation extent during the extraction process. The recommended POV for food quality phospholipid is 10 meq/1000g [108]. Ali-

Nehari et al. reported a POV of 4.66 meq/100g in purified phospholipid from krill residue de-oiled by SC-CO_2 [109].

The FFA content of SC-CO_2 with ethanol as co-solvent was found to be 4.14% and that of the organic solvent extract was 7.72%. Hydrolytic rancidity which occurred in the presence of moisture during extraction process may cause the formation of FFA [98]. Therefore, determination of FFA content provides an oxidative index of marine phospholipid.

The AV is used to determine the fish lipids acidity generated by FFA from oxidation and the presence of acidic compounds that are non-lipid [54]. AV of phospholipids extracted from YTV were 18.39 mg KOH/g and 32.52 mg KOH/g for SC-CO_2 with ethanol as co-solvent and organic solvent respectively. The FAO/WHO recommended AV for food grade phospholipids is up to 36 mg KOH/g [108]. The AV of phospholipids extracted from mackerel muscle were reported to be 21.32 ± 0.21 mg KOH/g and 37.60 ± 0.42 mg KOH/g for SC-CO_2 with ethanol as co-solvent extract and ethanol extract respectively [12]. High AV in lipids indicates low oxidative stability [110].

Table 3. 2. YTV phospholipids stability index

Extraction Methods	Quality index		
	Peroxide value (meq/1000g)	Free fatty acids (%)	Acid value (mg KOH/g)
SC-CO_2 /ethanol co-solvent	4.80 ± 0.68	4.14 ± 0.19	18.39 ± 0.44
Organic solvent	8.53 ± 0.82	7.72± 0.34	32.52 ± 0.67

3.3.5. Fatty acid composition of YTV phospholipids

The fatty acids composition of YTV phospholipids is presented in Table 3.3. The main fatty acids were palmitic acid with the percentage of 26.56 % in SC-CO_2 extract and 24.47 % in the organic solvent extract, followed by oleic acid, DHA, stearic acid, palmitoleic acid, and EPA. Polyunsaturated fatty acids (PUFA) account for 28.11% of the total phospholipid. DHA and EPA contents were the highest among the PUFAs with values ranging from 17.56% to 17.82 % and 3.70% to 4.07% respectively. YTV phospholipid is rich in monounsaturated fatty acids, which account for 32% of the total phospholipid, with oleic acid having the major content. [91] reported a DHA content of 14.5% in lecithin extracted from squid viscera. [12] reported that DHA was found to be 17% in lecithin extracted from mackerel muscle.

Table 3. 3. Fatty acids composition of YTV phospholipids

Fatty acids composition	$SC\text{-}CO_2$	Organic solvent
	Contents (%)	
Mystric acid (C14:0)	$3.08^{a} \pm 0.41$	$2.56^{a} \pm 0.6$
Palmitic acid (C16:0)	$26.56^{a} \pm 0.58$	$24.47^{b} \pm 0.04$
Palmitoleic acid (C16:1)	$4.73^{a} \pm 0.57$	$3.95^{a} \pm 0.5$
Stearic acid (C18:0)	$8.88^{a} \pm 0.38$	$9.77^{a} \pm 0.17$
Oleic acid (C18:1n9C)	$23.85^{a} \pm 0.74$	$23.79^{a} \pm 0.61$
Linoleic acid (C18:2n6c)	$1.88^{a} \pm 0.08$	$1.39^{b} \pm 0.4$
Eicosenoic Acid (C20:1)	$3.42^{a} \pm 0.36$	$2.11^{b} \pm 0.08$
Eicosatrienoic Acid (C20:3n3)	$3.48^{a} \pm 1.64$	$2.40^{a} \pm 0.17$
Arachidonic acid (C20:4n6)	$1.12^{b} \pm 0.67$	$3.70^{a} \pm 0.3$
Tricosanoic Acid (C23:0)	$1.37^{b} \pm 0.6$	$4.02^{a} \pm 0.37$
Eicosapentaenoic Acid (C20:5n3)/EPA	$4.07^{a} \pm 0.15$	$3.70^{a} \pm 0.33$
Docosahexaenoic Acid (C22:6n3)/ DHA	$17.56^{a} \pm 0.35$	$17.82^{a} \pm 0.83$
EPA + DHA	21.63	21.52
ΣSFA	39.89	40.82
ΣMUFA	32.00	29.85
ΣPUFA	28.11	29.01

N.B.: values are mean ± SD of three determinations. Means with the different superscript letter in each row differ significantly ($P<0.05$)

3.3.6. Thermogravimetric analysis

Thermogravimetric analysis was used to determine the weight degradation pattern of yellowtail fish viscera phospholipid for thermal application based use. Fig. 3.2. represents the thermogravimetric degradation of yellowtail fish viscera phospholipid. SC-CO_2 extracted phospholipid showed better oxidation degradation than organic solvent extracted phospholipid. SC-CO_2 extracted oil was stable until 300°C and it start to decline gradually. At 300°C, the obtained weight of phospholipid extracted by SC-CO_2/ethanol co-solvent was greater than 80%, while the obtained weight for organic solvent extracted phospholipid was 56.76%. At 400°C, a notable weight lost was recorded for phospholipid extracted by SC-CO_2 with ethanol co-solvent. Usually, in the presence of atmospheric oxygen, lipids are oxidized resulting in the formation of peroxide, involving in the increase of sample weight [87]. This result indicates that yellowtail fish viscera phospholipids extracted by SC-CO_2 with ethanol co-solvent is suitable for use in thermal application base food. [97] reported that weight of phospholipids extracted from salmon by-product using SC-CO_2/ethanol co-solvent was drastically reduced at 450°C.

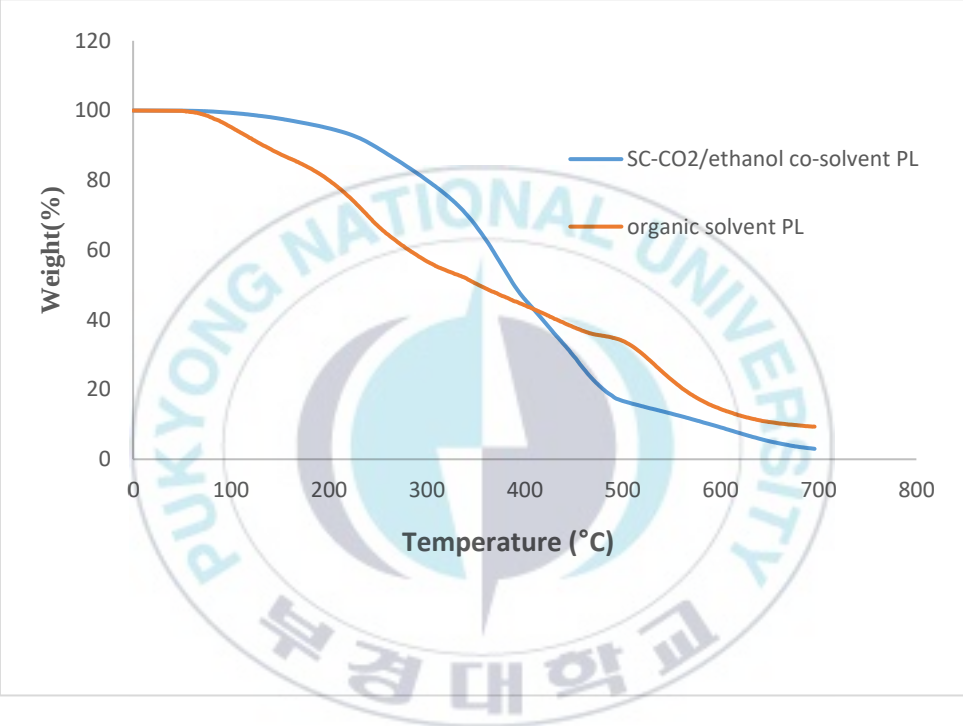

Fig. 3. 2. Thermogravimetric analysis of yellowtail fish viscera phospholipid

3.3.7. Antioxidant activity of YTV phospholipid

DPPH and ABTS free radical scavenging activity of YTV phospholipid were examined to determine the antioxidant activity. Fig.3.2. present a dose-dependent manner at which YTV phospholipids exhibited DPPH and ABTS free radical scavenging activity. Phospholipids are considered as emulsifying agents with both hydrophilic and hydrophobic properties [12]. Also, DPPH is known to be specifically hydrophobic antioxidant, whereas ABTS is considered to be hydrophilic [111]. The highest exhibition of DPPH and ABTS free radical scavenging activity was found in Trolox, followed by SC-CO_2 with ethanol co-solvent extracted phospholipid and organic solvent extracted phospholipid. The DPPH solution absorbance value was 1.082, which decreased linearly with increasing sample concentration, and the values for Trolox, SC-CO_2 with ethanol co-solvent extract, and organic solvent extract were 0.092, 3.000 and 0.438 respectively, at the maximum concentration used. Likewise, the ABTS solution absorbance value was 0.739, which gradually decreased linearly with increasing concentration of the sample. At the maximum concentration used, the absorbance value of Trolox, SC-CO_2 with ethanol co-solvent extract, and organic solvent extract were to be 0.079, 0.141 and 0.195 respectively. The presence of some amount of tocopherol in phospholipids can be attributed to the antioxidant activity [112]. The existence of highly unsaturated fatty acids in the extracted phospholipids together with the polar head is responsible for their antioxidant activity [113]. [97] reported a similar result of antioxidant activity in Atlantic salmon Phospholipid. Phospholipid antioxidant activity may vary based on the functional groups, structures, fatty acids pattern, and chemical composition of lipids [114] [115].

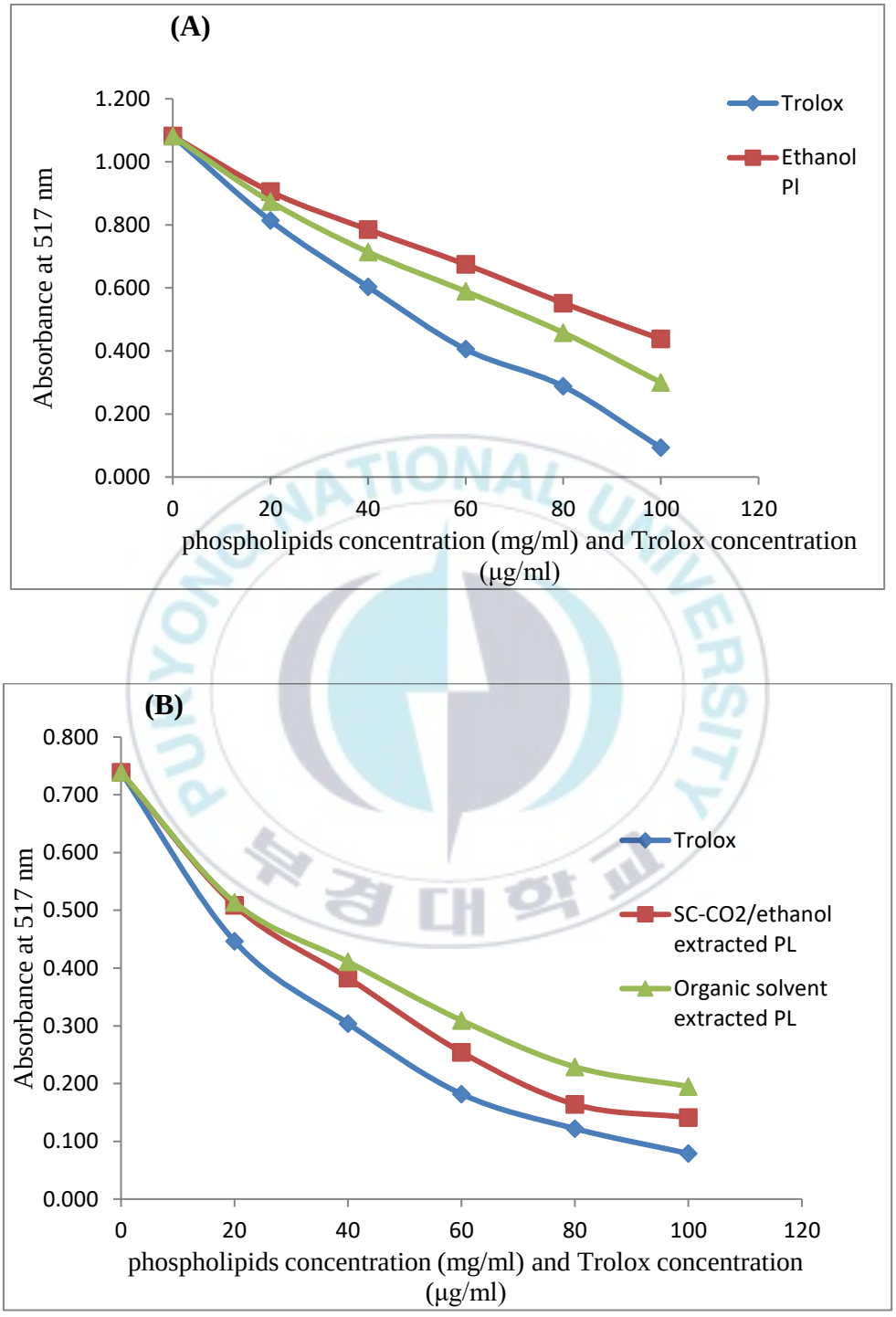

Fig. 3. 3. Antioxidant activity of YTV phospholipids; (A) DPPH free radical scavenging effect and (B) ABTS free radical scavenging effect

3.4. Conclusion

Yellowtail viscera phospholipid was shown to be rich in polyunsaturated fatty acids especially DHA and EPA, and contain a large amount of PC which was ascertained by HPLC-ELSD analysis. Stability parameters such as PV, FFA, and AV of YTV phospholipids were determined and values were lower in SC-CO_2 with ethanol co-solvent extract than organic solvent extract. SC-CO_2 with ethanol co-solvent extract exhibited higher free radical scavenging activities than organic solvent extract. SC-CO_2 technique showed to be an excellent method for the extraction of good quality phospholipid from fish material. Yellowtail fish viscera phospholipid can serve as a good source of omega polyunsaturated fatty acids and as such, it would help reduce environmental pollution. This research may be helpful in the application of yellowtail fish viscera phospholipids in food and pharmaceutical industries.

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Abstract (in Korean)

Characterization of Yellowtail Fish Viscera Lipids Extracted Using Supercritical Carbon Dioxide and Organic Solvents

초임계 이산화탄소 및 유기용매에 의해 추출된 방어 내장의 지질에 대한 특성

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Abstract

매년 수산 가공 산업에서 생산된 많은 부산물들이 버려지거나 낮은 생산적인 방법으로 사용되고 있다. 본 연구는 방어(*Seriola quinqueradiata*)의 내장에서 양질의 식용 지질 생산 방법 및 오메가-3 다가불포화 지방산의 공급원으로서의 역할 제시를 통해 부산물의 가치를 평가한다. 지질은 대기, 빛, 고온과 접촉할 때 쉽게 산화되기 때문에 추출방법은 추출된 지질의 품질에 매우 중요한 역할을 한다. 기존의 추출 방법은 고온 및 용매의 사용을 포함하기 때문에 환경 및 소비자의 건강에 바람직하지 않다. 초임계 이산화탄소(SC-CO_2)기술은 친환경 기술이며 식품에 무해하기 때문에 식용 지질을 추출에 유망한 방법이다.

첫 번째 연구에서는 SC-CO_2와 속슬렛을 이용하여 방어 내장으로부터 지질을 추출하였다. SC-CO_2로부터 추출한 지질은 속슬렛으로부터 추출한 지질보다 우수한 물리화학적 특성을 보였다. 추출된 방어 내장 지질에는 최대 20.14%의 오메가-3 다가불포화 지방산을 함유하고 있었다. 열중량 분석을 통해 SC-CO_2로부터 추출한 방어 내장 지질이 고온의 식품가공 산업에 활용될 수 있는 것으로 나타났다.

두 번째 연구는 에탄올을 보조용매로 사용한 SC-CO2 및 유기용매(에탄올)에 의해 추출된 방어 내장 인지질의 특성을 파악하는 것을 목표로 하였다. 추출된 인지질에서 Phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine, and phosphatidylethanolamine (PE)이 검출되었으며, PC 는 전체 인지질의 82%를 차지하였다. 방어 내장의 인지질은 4.07%의 Docosahexaenoic acid(DHA)와 17.82%의 Eicosapentaenoic acid(EPA)를 함유하고 있다. 에탄올을 보조용매로 사용한 SC-CO_2에 의해 추출된 인지질은 유기용매로 추출된 인지질보다 우수한 산화 안정성 및 free radical 소거능 활성이 나타났다.

결론적으로, SC-CO_2 추출 기술은 어류 소재에서 양질의 지질 및 인지질을 추출하는 우수한 방법으로 나타났다. 방어 내장의 지질은 오메가-3 다가불포화 지방산이 함유되어 우수한 공급원이 될 수 있으며, 환경오염을 줄이는 데 기여할 수 있다. 본 연구에서 확보된 방어 내장 지질은 식품 및 의약품 산업분야의 응용에 도움이 될 수 있을 것으로 기대된다.

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