Thesis for the Degree of Master of Engineering

Characterization of Anchovy *(Engraulis japonica)* Oil and De-oiled Protein Using Supercritical Carbon Dioxide

by

Seung-Mi Lee

Department of Food Science & Technology

The Graduate School

Pukyong National University

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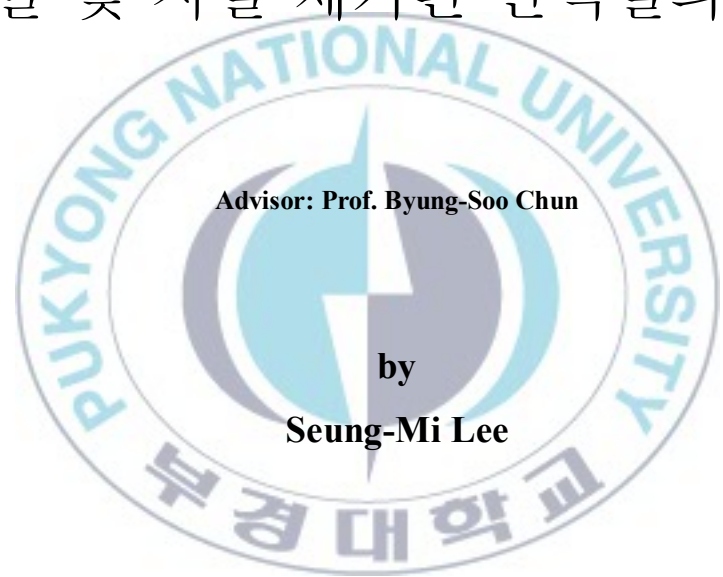
초임계 이산화탄소를 이용한 멸치 추출 오일 및 지질 제거된 단백질의 특성



Advisor: Prof. Byung-Soo Chun

by

Seung-Mi Lee

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Approved by:

_____
Seon-Bong Kim, Ph. D.
(Chairman)

_____
Young-Jae Cho, Ph. D.
(Member)

_____
Byung-Soo Chun, Ph. D.
(Member)

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Contents

List of Tables

List of Figures

초임계 이산화탄소를 이용하여 추출한 멸치 오일 및 지질 제거된 단백질의 특성비교

이 승 미

부 경 대 학 교 대 학 원 식 품 공 학 과

요 약

멸치는 우리나라에서 가장 대중적으로 섭취되고 있는 식품이다. 하지만 자숙멸치를 이용하여 사용되고 있는 것이 대부분이며, 그 이용가치는 높지 않다. 수산가공산업에 있어서 멸치에 함유되어 있는 유용한 물질들의 이용은 경제적으로나 환경적 측면으로 볼 때, 많은 이익을 창출하게 해줄 수 있다. 본 연구에서는 멸치 육에서 친환경 공정인 초임계 이산화탄소 (SCO_2) 추출법과 hexane을 이용한 유기용매 추출법을 이용해 오일을 추출하였다. SCO_2 추출은 온도 범위 40 - 60℃, 압력 15 – 25 MPa 범위에서 실행하였으며 CO_2 유량은 22 g/min로서 총 추출 시간인 1시간 30분동안 지속적으로 흐르게 하였다. 오일의 최대 추출 수율은 온도와 압력이 높을수록 높았다. 오일의 지방산 분석을 위해 gas chromatography (GC)를 이용하였고, 그 결과 SCO_2를 이용하여 추출한 오일에 고도불포화지방산 (PUFAs)의 함량이 높았으며, 그 중 특히 eicosapentaenoic acid (EPA) 와 docosahexaenoic acid (DHA) 의 함량이 우수한 것을 확인하였다. SCO_2를 이용하여 추출한 멸치오일과 hexane을 이용한 유기용매 법으로 추출한 멸치오일의 품질을 비교해 본 결과 SCO_2 를 이용하여 추출한 오일이 유기용매를 이

용하여 추출한 오일보다 높은 산화 안정성을 보였다. 그리고 일반 시중 멸치 분말, SCO_2 추출법으로 지질 제거한 멸치 단백질과 핵산으로 지질을 제거한 멸치 단백질을 이용하여 효소정제체계에 대한 적용가능성과 관능성을 확인해보았다. 회수된 단백질에 함유되어 있는 3가지 주요 소화효소인 프로테아제, 리파아제, 아밀라아제의 특성에 대한 연구를 수행하였다. 가장 많은 오일이 추출된 25 MPa, 45ºC 조건에서 추출 후 회수된 단백질에 함유되어 있는 소화효소들의 특성을 알아본 결과, SCO_2 처리된 추출물 멸치 추출물의 프로테아제, 리파아제, 아밀라아제 활성이 우수하였으며, 이중 아밀라아제의 활성이 가장 높음을 확인하였다. hexane을 이용한 멸치 추출물의 활성은 낮은 수치를 나타냈으며 소화효소의 최적 pH와 pH안정성은 SCO_2와 hexane을 이용한 멸치 추출물에 유의적인 차이가 없었다. 또한 단백질의 이취제거 및 색도를 통한 관능적인 특성을 확인한 결과 SCO_2 를 이용하여 처리한 단백질이 핵산을 이용하여 처리한 단백질 보다 냄새성분 및 색에 대한 관능성이 우수하였다. SDS-PAGE를 이용하여 단백질 변성도를 측정하였을 때, 동결 건조 된 시료와 SCO_2, hexane을 이용하여 추출 후 회수한 단백질에서 단백질 패턴의 큰 차이가 없었고, 이는 단백질 변성이 일어나지 않았음을 입증할 수 있었다. 마지막으로 회수된 단백질의 미생물 안전성과 용해도를 확인해 본 결과 시중의 자숙멸치보다 우수한 품질을 나타내는 것을 확인할 수

있었다.

Introduction

Fermented anchovy is widely used in Korea as a condiment to improve taste and flavor of several traditional foods. Fish provides a high quality protein and a fat, rich in long chain omega-3 fatty acids. Fish is valuable not only for the quantity but also the quality of its protein. By this is meant that the amino acids that make up the protein are present in just the right balance for animal or human nutrition. Not only, the balance of amino acids in fish is suitable for animal feeding, but the availability of the essential amino acids is also greater in fish than for in meat meal. Anchovy is a main source that has functional materials including polyunsaturated fatty acids (PUFAs) and also it is large in number [1, 2]. These fatty acids have health benefits and have been reported to reduce the risk of coronary heart diseases [3, 4], and lower blood pressure and plasma tri acyl glycerol levels [5, 6]. Marine organisms are the unique source of PUFAs. PUFAs, especially ω-3 fatty acids, have been very often subject of scientific studies in the last years. ω-3 fatty acids have proven beneficial effects in the prevention of some diseases. Today it is known that ω-3 fatty acids are essential for normal growth and development and may play an important role in the prevention and treatment of coronary artery disease, hypertension, arthritis, others inflammatory and autoimmune disorders, and cancer[7].

The anchovy contains amino acids, peptides and organic compounds from complete hydrolysis of tissue by enzymes from fish intestine and muscle [8]. In addition, they can improve inflammatory conditions, reduce the symptoms of diabetes [9-11] as well as a range of other disorders [12]. Fish are known to be a rich source of digestive enzymes. In recent years, recovery and characterization of enzymes from fish and aquatic invertebrates has gained importance and this has led to the emergence of some interesting new applications of these enzymes in food processing [13]. Many research works have been carried out during the last few years for investigating the new possibilities offered by enzymes originating from fish and aquatic invertebrates.

The use of supercritical fluid extraction (SFE) offers numerous potential advantages over conventional extraction processes, such as reduced extraction time, reduced organic solvent volume, and more selective extractions [14]. Fig. 1 showed the phase diagram illustrating the supercritical region above the pressure and temperature. Supercritical fluids have a relatively high liquid-like density as well as a relatively low viscosity and high diffusivity [15]. These properties provide a unique solvent that is both effective at dissolving materials as well as penetrating solid matrices.

Supercritical carbon dioxide (SCO_2), in particular, is an attractive supercritical solvent, low critical temperature (31.1℃), and the fact that it is non-flammable,

non-toxic and inert. Because of these properties, SCO_2 can be useful when applied to food and pharmaceutical industries. Supercritical carbon dioxide can achieve extraction yields for nature.

This technology can be extracted high purity materials and economical. In recent years, the use of SFE for the removal of organic compounds from different liquid and solid matrices has attracted much attention. This technique has some advantages over more conventional separation techniques, largely due to the unique physical properties of SFs. SFE using CO_2 is a promising process for the extraction and fractionation of edible oils containing labile PUFAs and lipid soluble bioactive compounds.

The objective of this study was to extract oil from anchovy by SCO_2 with lipid soluble bioactive compounds and the fatty acids compositions of anchovy oil at different extraction conditions were also analyzed. The stability of oil obtained by SCO_2 extraction was also compared to the oil obtained by soxhlet extraction at hexane. In addition after extraction of lipid, the anchovy residues still contain some other useful materials- mainly higher amount of proteins. The antimicrobial activity and sensory characteristics of proteins at different extraction solvent were evaluated. Digestive enzyme activity was also analyzed from anchovy residues. However, other value added materials may be recovered from anchovy residues obtained by SCO_2 extraction.

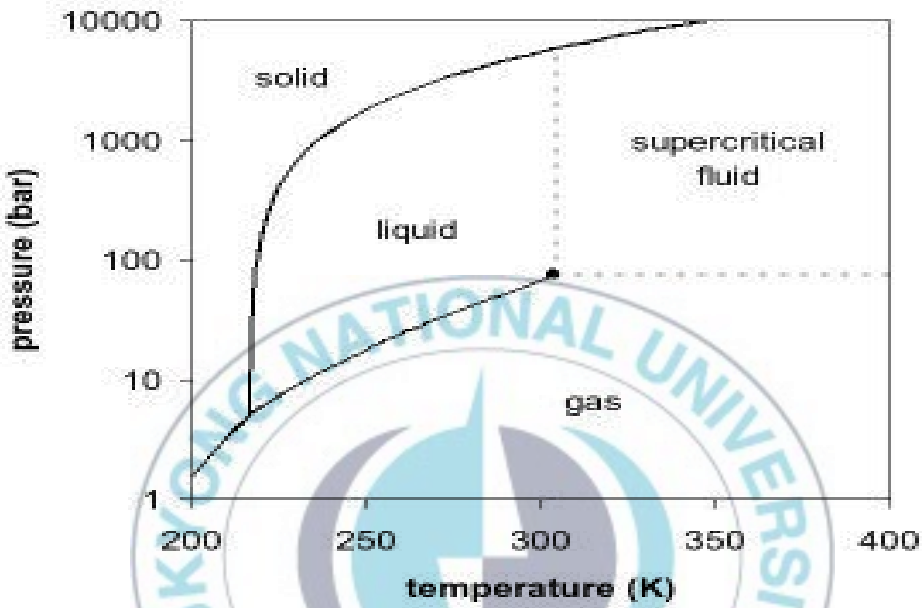


Fig. 1. The phase diagram illustrating the supercritical region above the pressure and temperature.

Materials and Methods

1. Materials

The sample was collected from Dong-kwang Company (Kijang, Republic of Korea). The pure carbon dioxide (99%) was supplied by KOSEM, Korea. All reagents used in this study were of analytical or HPLC grade.

2. Sample preparation

The anchovy samples were dried in a freeze-drier for about 72 hrs. The dried samples were crushed by a mechanical blender and sieved (700 μm) by mesh. It was difficult to make more small size sample because anchovy contained lot of oil that made sticky material by more crushing. These samples called freeze dried anchovy were then stored at -40°C until using for SCO_2 and organic solvent extraction.

3. Method

3.1 Measurement of heavy metal contents in anchovy

Anchovy in the cadmium, lead, mercury, arsenic content was measured. Preparation of samples according to the Korean Food Standards Codex (KFSC)

was used. Heavy metal content analysis ICP-MS (Inductively Coupled Plasma Mass Spectrometry) was used. ICP-MS analysis of the ppt level has possible benefits.

3.2 Supercritical carbon dioxide extraction

The set up of a laboratory scale of SFE process is shown in Fig. 2. This apparatus can be operated at pressure up to 25 MPa. Twenty five gram of freeze dried raw anchovy sample was loaded into the stainless steel extraction vessel which was 200 mL in volume. A thin layer of cotton was placed at the bottom of the extraction vessel. Before plugging with cap another layer of cotton was used at the top of the sample. CO_2 was pumped at constant pressure into the extraction vessel by high pressure pump up to the desired pressure. A back pressure regulator was used to control the pressure of CO_2. The extraction temperature was maintained by connecting the extraction vessel with water bath. Flow rates and accumulated gas volume passing through the apparatus were measured using a gas flow meter. The oil extracted by SCO_2 was collected by a cyclone separating vessel. The amount of extract obtained at regular intervals of time was established by weight using a balance with a precision of ±0.001 g. The extracted oil and anchovy residues were then stored at -20°C until further used and analysis.

The effect of temperature and pressure on lipid extraction from anchovy was studied at 40-60°C and 15-25 MPa at a constant extraction time of 1.5 hrs. The

extractions were performed at low temperature because fish oil is rather in volatile and thermally sensitive [16]. Another cause for selecting low temperature was to retain the high activity of bioactive materials both in extracted oil and residues. The flow rates of CO_2 were kept constant at 22 g/min for all extraction conditions.

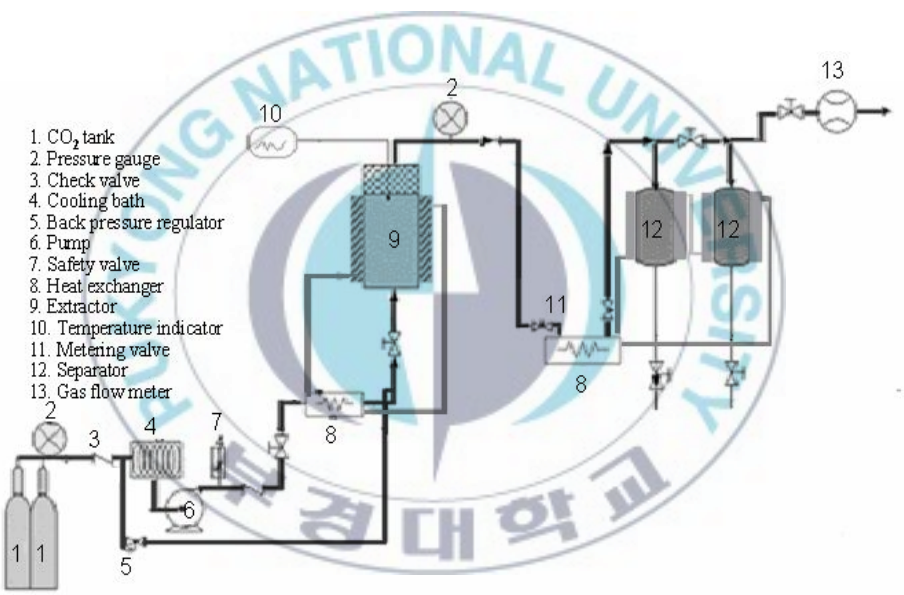


Fig. 2. The diagram of supercritical carbon dioxide extraction.

3.3 Organic solvent extraction

To compare bioactive functionalities of anchovy by SCO_2 extraction and conventional organic solvent extraction, soxhlet extraction of the traditional extraction techniques was applied. Three gram of the sample was placed in a timble holder that is gradually filled with condensed extractant 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.

3.4 Analysis of fatty acids composition by gas chromatograph

The fatty acid compositions of anchovy oil obtained by SCO_2 and organic solvent, hexane extraction were determined by gas chromatography using a Hewlett Packard gas chromatograph (6890 Series II GC system). The fatty acid methyl esters were prepared firstly according to AOCS official method Ce 2-66 (AOCS, 1998) and then separated using an Agilent DB-Wax capillary column (30 m length x 0.250 mm internal diameter, 0.25 μm of film). Nitrogen at a flow rate 1.0 mL/min was used as a carrier gas of fatty acid methyl esters. The split ratio was fixed at 50:1. The oven temperature was programmed starting at a constant temperature of 130ºC for 3 min, and then increased to 240ºC at a rate of 4ºC/min and hold at 240ºC for 10 min. Injector and detector temperatures were 250ºC.

Fatty acid methyl esters were identified by comparison of retention time with standard fatty acid methyl esters mixture (Supleco, USA).

3.5 Measurement of oil stability

Several parameters may use to determine the deterioration of oil. In this study, oil deterioration was monitored by evaluating free fatty acid content and peroxide value.

3.5.1 Free fatty acid content of anchovy oil

Free fatty acids of extracted oil from anchovy were analyzed as described by Bernardez et al. (2005). 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. 3).

Copper reagent was prepared according to Lowry and Tinsley (1976). Briefly, 5% (w/v) aqueous solution of cupric acetate was prepared and filtered. Then the pH of cupric acetate solution was adjusted to 6.1 using pyridine.

3.5.2 Peroxide value

Peroxide value was determined by Y. K. Wang et al. 1 g of sample was

dissolved in 30 mL of chloroform: acetic acid (2:3). Peroxide value of oil was analyzed by titration with 0.1 N Na2S2O3 solution and using an iodometry. The peroxide value was calculated using the following equation.

$$PV = \frac{(A-B) \times F}{C} \times 10 \ (0.01 \times 1000 mg/kg)$$

PV is the peroxide value of the sample, A is the volume of the 0.1 N Na2S2O3 solution of the titration (mL), B is blank, F is the concentration of the 0.1 N Na2S2O3 factor and C is the weight of the sample (g).

3.6 Oxidative stability

To measure the oxidative stability, emulsion of oil in water were oxidized at 37ºC. Three emulsions of oil in water (w/w) (linoleic acid 4%, oil 1%, water 95%; oil 5%, water 95%; astaxanthin 2%, oil 4%, water 94%) were prepared. The deionized and degassed water was used for emulsion preparation. Linoleic acid and standard astaxanthin were used to measure the oxidative stability of anchovy oil. The mixture was properly homogenized by a homogenizer. Oxidative stabilities were checked by thiocaynate [18] and thiobarbituric acid [19] method which were used to measure the antioxidant activity. In this study, these two methods were conducted to measure the quality in terms of oxidative stability.

3.6.1 Thiocyanate method

The peroxide formed by lipid peroxidation reacts with ferrous chloride and form ferric ions. Ferric ions then unite with ammonium thiocyanate and produce ferric thiocyanate. Briefly, 0.1 mL of emulsion solution was added to 4.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red color was measured at 500 nm. The absorbance was recorded at 24 hrs intervals during the incubation.

3.6.2 TBA method

TBA method is used for evaluating the extent of lipid peroxidation. Malonadehyde, the product of lipid breakdown caused by oxidative stress binds with TBA to form a red complex of thiobarbituric acid reactive substance (TBARS). Briefly, 2 mL of 20% trichloroacetic acid and 2 mL of 0.67% 2-thiobarbituric acid were added to 1 mL of emulsion solution. The mixture was placed in a boiling water bath (100ºC) for 10 min. After cooling, the mixture was centrifuged at 3000 rpm for 20 min. Absorbance of supernatant containing TBARS was measured at 532 nm.

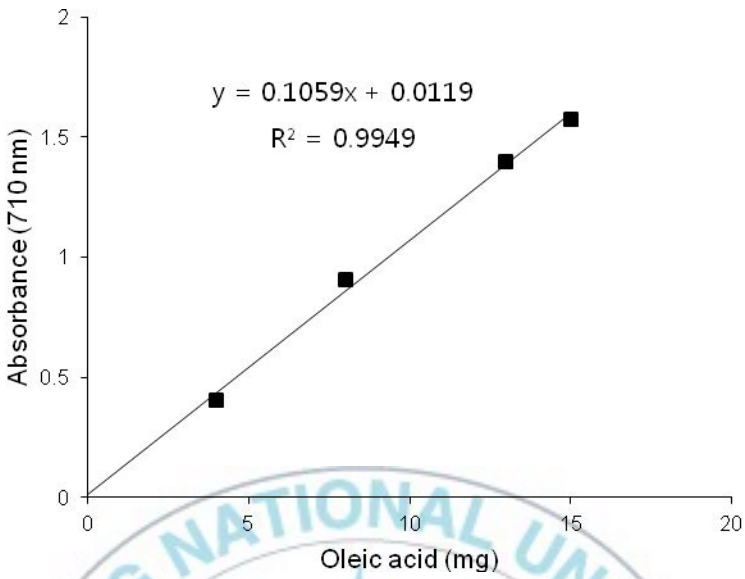


Fig. 3. Calibration curve of oleic acid for estimation of free fatty acids in anchovy oil.

3.7 Color

The color of the extracted oils was measured in triplet by means of means of reflectance spectra in a spectrophotometer. For measurements, samples were placed in a white cup and covered with optical glass. CIE L*a*b* color coordinates (considering stradard illuminant D65 and observer 10) were then calculated.

3.8 Protein characteristic of anchovy residues

3.8.1. Digestive enzyme assay

3.8.1.1. Protease assay

Protease activity was assayed by the casein Folin-Ciocalteau method [20] with slight modification. One percent casein solution in 0.0125 M sodium borate-NaOH buffer (pH 10.5) was used as substrate. Crude enzyme (0.5 mL) was mixed with 2.5 mL of substrate and incubated for 10 min at 37ºC. The reaction was stopped by addition of 2.5 mL of TCA solution and settled for 20 min. The sample was then centrifuged for 10 min at 3000 rpm. The supernatant (2 mL) mixed with 5 mL of 0.55 M Na2CO3 and 1 mL of 1 N Folin-Ciocalteu reagent. The optical density of the color developed at 37°C for 20 min was measured at

660 nm (UVIKON 933, Kontron Instruments). One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine per min from casein. Tyrosine was used to construct a calibration curve.

3.8.1.2. Lipase assay

Lipase activity was assayed using the modified method of Vorderwülbecke et al. (1992) described by Hatzinikolaou et al. (1999). The substrate emulsions were prepared by drop wise addition of 0.2 mL solution A (40 mg of p-nitrophenyl-laurate was dissolved in 12.0 mL of isopropanol) into 3.0 mL solution B (0.4 g Triton X-100 and 0.1 g gum arabic were dissolved in 90 mL of 0.1 M potassium phosphate buffer, pH 7.0) under intense vortexing. These emulsions were stable for 1 hr at room temperature. The crude extract (0.1 mL) was added to 3.2 mL of the substrate emulsion and the mixture was incubated for 20 min in a shaking water bath at 35°C. The reaction was terminated by boiling for 5 min. Following centrifugation (6000 rpm, 10 min) the absorbance of the clear supernatant was measured at 410 nm. The mixture with 0.1 mL of the inactivate enzyme extract (heated at 100°C for 5 min) was used as control. One unit of enzyme activity was defined as the amount of enzyme required to liberate of 1 μmol p-nitrophenol from p-nitrophenyl-laurate per minute under the assay conditions. A calibration curve was constructed using p-nitrophenol.

3.8.1.3. Amylase assay

Amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis. The reaction mixture containing 0.5 mL of enzyme extract and 0.5 mL of 1.0% (w/v) potato starch (Sigma) in 100 mM acetate buffer (pH 6.5) was incubated at 37°C for 10 min. The amount of liberated reducing sugar was determined by the dinitrosalicylic (DNS) acid method [23]. One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per minute. D-Glucose was used to construct a calibration curve.

3.8.2. Effect of pH and pH stability of protease, lipase and amylase

The effects of pH on crude enzyme activity were determined using different buffers of wide range of pH values. The buffers used were 0.1 M citric acid-sodium citrate (pH 4.0-5.5), 0.1 M potassium phosphate (pH 6.0-8.0) and 0.1 M glycine-NaOH (pH 8.5-12). The pH stability was tested by 5 hrs pre-incubation of the crude enzyme extract in buffers that had the same ionic concentrations at different pH values ranging from 3.0 to 12.0 at 0°C. The enzyme activities were measured immediately after this treatment using the standard methods as mentioned above.

3.8.3. Electrophoresis

Sodium dodecyl sulfate-polyacrylamaide gel electrophoresis (SDS-PAGE) of crude extracts were carried out by the method of Laemmli (1970) using 5% (w/v) stacking gel and 12% (w/v) separating gel. Electrophoresis was performed using a Mini-Protein III cell module (Bio-Rad Laboratories, CA, USA) at a constant voltage (100 V for 2 h). The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 for 2 hrs and destained in 50% methanol (v/v) and 10% (v/v) acetic acid solution. Molecular weight markers (Sigma) were used.

3.8.4. Comparative analysis of the microbial safety of residues anchovy

Microbial safety is an important indicator to determine the quality of proteins and storage period. Supercritical carbon dioxide extraction before and after powder anchovies, boiled anchovy, microbial safeties of these three samples are compared. All samples are after homogenization used. To ensure microbiological safety measurement total cell number and E. coli. Measure of the total cell number counts using plate Count Agar medium and according to the Korean Food Standards Codex (KFSC) on the microbiological assay is measured. E. coli was measured using 3M petrifilm the anti-microbial potential of compressed SCO_2.

3.8.5. Analysis of sensory characteristics

3.8.5.1. Analysis of off-flavors

The analysis of off-flavor removal for anchovy powder was conducted by GC/MS system (HP 6890/QP 2010A, Shimadzu, Japan). Samples (untreated anchovy and SCO_2 treated extraction, SCO_2 treated extraction) were volatilized at 50°C in a drying oven during 30min. Volatiles compounds volatilized from each sample were absorbed on absorption tubes [25]. The volatiles compounds absorbed in tubes were desorber into the automatic thermal desorber (ATD; ATD-400, Perkin Elmer, UK) which is directly connected with GC/MS equipped with a AT-1 column (60 m × 0.32 mm i.d., 1.0 μm film thickness). Helium was used as a carrier gas at an inlet pressure of 15.7 psi, flow rate 0.62 mL/min. Temperature gradient used was as follows: 35°C for 10 min, 35-120°C at 8°C /min, 120-180°C at 12°C /min, then 180-230°C at 15°C /min. Ion source temperature was 250°C, ionization voltage was 70 eV and the range of molecular weight scanned was 20-350 m/z [22]. The spectrum of each analyzed off-flavor compounds agreed with that presented in the mass spectrum library (NIST21, NIST107, WILEY229). The percentage of identified off-flavor compounds was presented by peak area %.

3.8.5.2. Comparative analysis of common salt

Common salt is a very important factor sensually. Therefore, supercritical carbon dioxide processing pre-and after anchovy powder and extraction oil

residue using hexane, the three samples is salinity of compared. Common salt measures according to KFSC the assays. Sample containing 1 g of salt evaporate and dry and then dissolved in water by ashing. Then, add water to 500 ml with water, after a filtration with approximately 10ml the sample solution and add 2 drops of potassium chromate Silver nitrate solution titrate with 0.02 N. The salt was calculated using the following equation.

$$\text{salt} = \frac{b}{a} \times f \times 5.85(\text{w/w\%, w/v\%})$$

a : the weight of the sample (g, mL)

b : the volume of the 0.02N Silver nitrate solution titrate (mL)

f : the concentration of the 0.02 N Silver nitrate solution titrate factor

3.8.5.3. Comparative analysis of chromaticity

Color change of the Anchovy protein was measured using a Color Difference Meter. The machine blackboard and Whiteboard were used after standardized Hunter color L * (Brightness), a * (redness) and b * (yellowness) were measured.

3.8.6. Water soluble of anchovy powder

To investigating the influencing factors that solubility of anchovy protein using before and after supercritical extraction residues, organic solvent extraction residues, boiled anchovy powder by changes in temperature and particle size

1 g of powder in each of the anchovy samples and adds 20 ml of distilled water and homogenized by 30 seconds than 30℃, 40℃, 50℃, 60℃ is heated differently and 200 mm, 500 mm 700 mm 1000 mm particle size sample using experiment. The sample is left at low temperature then the supernatant after centrifugation, it is used. The protein content of sample was measured on a calibration curve constructed from bovine serum albumin standard (Fig. 4.)

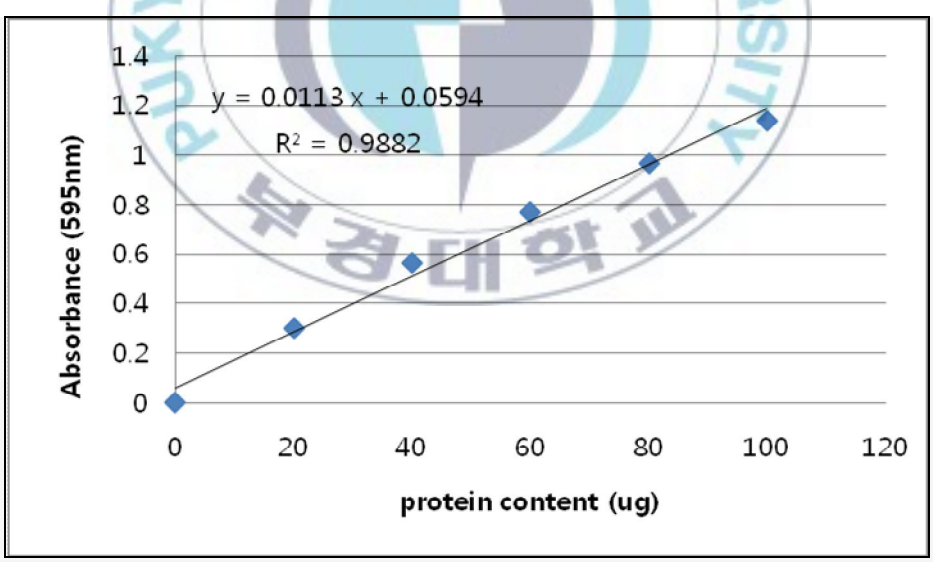


Fig. 4. Calibration curve of bovine serum albumin.

Results and Discussion

1. Measurement of heavy metal contents in anchovy powder

Heavy metals measured value of freeze-dried anchovy shown in table1. As a result, the Cadmium 0.013 ppm, lead 0.011 ppm, mercury 0.001 ppm, arsenic appeared to 0.2 ppm. According to the food and drug administration cadmium and lead in the notice of allowance is 2 ppm, hg allowance 1 ppm, 0.3 ppm arsenic is shown in. As a result of this appears to be safe from heavy metals. Compare squid viscera heavy metal content [26] arsenic and lead is 0.002 ppm and mercury appeared 0.01 ppm. Appeared similar to the content of lead and mercury, arsenic content of the squid viscera showed a slightly higher compared to anchovy.

2. Compositions of freeze dried raw and SCO_2 extracted anchovy

The compositions of freeze dried raw and SCO_2 extracted anchovy are shown in Table 2. The anchovy were dried in a freeze dryer for higher efficiency of SCO_2 extraction of lipid and lipid soluble bioactive materials. The moisture content of freeze dried raw and SCO_2 extracted sample was 3.5±0.2 and 2.51±0.15%, respectively. The moisture content was found to be decreased in SCO_2 extracted

residues. The moisture might be reduced by combining with CO_2. Generally, SCO_2 cannot extract all lipids from the sample. In our study, 2.05±0.21% lipid obtained by soxhlet extraction at hexane from SCO_2 extracted anchovy residues. The protein content in freeze dried raw samples was 65.76±0.64%. After deoiling by SCO_2, the protein content of anchovy increased to 74.32±0.69%. It was also found that the ash and non protein content was high in SCO_2 extracted anchovy residues.

3. Supercritical carbon dioxide extraction

Supercritical fluid offers an alternative mean of selectively extracting lipid components from food [27] Fig. 5.-7 showed the effect of the temperatures (40-60℃) and pressure (15-25 MPa) changes on oil extraction In the range studied, the highest yields were 14.68 wt% at 60℃ 25 MPa, respectively. The higher values of temperature and pressure gave the higher extraction yield. The highest yield was obtained at 25 MPa pressure and 60℃ was 4.2 g from anchovy. As the temperature and pressure change, oil yield was affected. The oil yield is thought to be due to the change of solvent density. At continuous temperature, the amount of oil extracted from anchovy was increased with the increasing pressure. It was caused by an increase in the density of carbon dioxide. So, supercritical carbon dioxide can be peretrated into the sample and the volve of SCO_2 in increased in

the sample increases the solvent. The increased solvating power and the strength of intermolecular physical interactions are considered as belonging to the effect of pressure [28].

3.1. Comparison of oil yield by SCO_2 and hexane extraction

The oil yield obtained by soxhlet extraction at hexane from anchovy was 17.2±1.03% (w/w in freeze dried raw sample) shown in Fig. 8. On the other hand, the highest yield in SCO_2 extraction was 14.8±0.69% for the experiment conducted at 25 MPa and 40ºC. The differences in maximum yield may be occurred due to variation of processing unit, operating conditions, sample size, percentage of lipid in sample etc.

4. Solubility of anchovy oil

The solubility of anchovy oil which is extracted at different conditions were measured and calculated from the slope of the liner sections of the extraction curves. The oil solubility is shown in Table 3. The highest solubility of the extracted anchovy oil was at 25 MPa and 60 ºC. Solubility of oil depends on density of CO_2 and vapour pressure of oil components as described supercritical CO_2 extraction section. At similar temperatures and pressures, some papers reported oil solubility from black currant and apricot seeds. The variation of oil

solubility might have happened due to variation of sample, extraction unit, sample size, flow rate of carbon dioxide and others [29]. The solubility of anchovy oil at supercritical carbon dioxide was correlated by Chrastill model [30]. It was revealed that Chrastil model is useful to correlate oil solubility. This model was based on the direct relationship between solubility of solute and density of a solvent. The correlations based on empirical density are very useful to determine the solubility of solids and liquids in compressed fluids, as they are both simple and do not require physicochemical properties of the solute. Fig.9. shows Chrastil model in which the experimental values and the calculated solubilities were represented by points and lines, respectively. It cleary showed the isotherms and the effects of temperature and solvent density. The correlation of oil solubility with solvent density was obtained from the below equation.

$$y = \rho_{CO_2}^{k} \exp\left(\frac{a}{T} + b\right)$$

Where y is the solubility of anchovy oil (mol/mol), ρ_{CO_2} is the density of CO_2, T is experimental temperature (K) and a, b and k are empirical fitting parameters. The solubility data of anchovy oil obtained by supercritical CO_2 extraction was fitted well in Chrastil model because at a given temperature, almost a linear relation between the solubility of oil and solvent density was obtained.

Table 1. Heavy metals amount analysis of anchovy powder

Heavy metals amount (㎎/㎏)			
Arsenic	Lead	Mercury	Cadmium
0.200	0.011	0.005	0.013

Table 2. Proximate compositions of freeze dried raw and SCO_2 extracted anchovy

Composition (%)	Freeze dried raw anchovy	SCO_2 extracted anchovy
Moisture	3.5±0.2	2.01±0.15
Ash	10.15±0.13	15.57±0.16
Protein	67.76±0.64	74.12±0.69
Lipid	15.24±1.03	5.05±0.21
Non protein	4.35±0.13	3.25±0.53

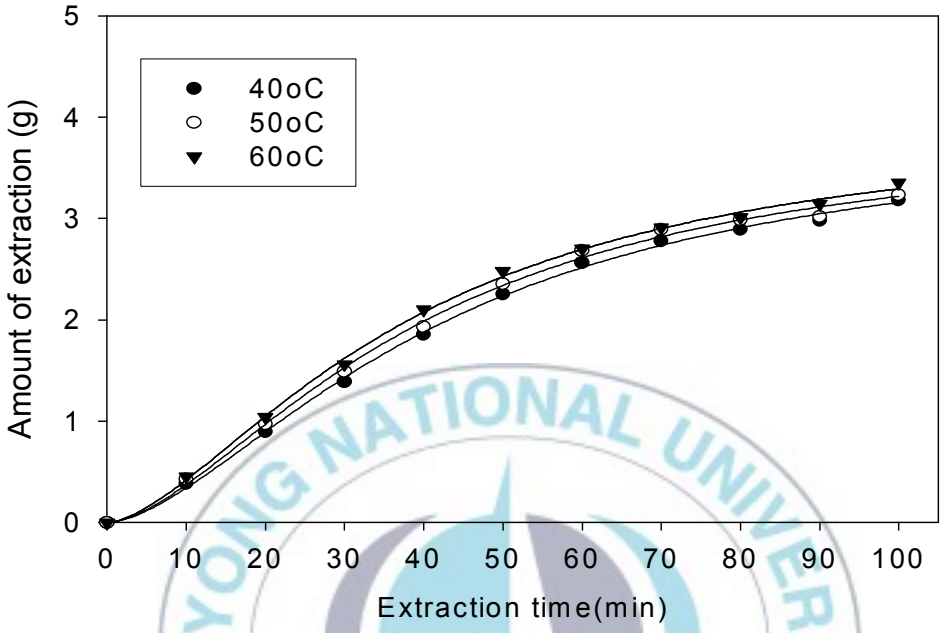


Fig. 5. Anchovy oil extraction curves by SCO_2 at 15 MPa and CO_2 flow rate of 22 g/min.

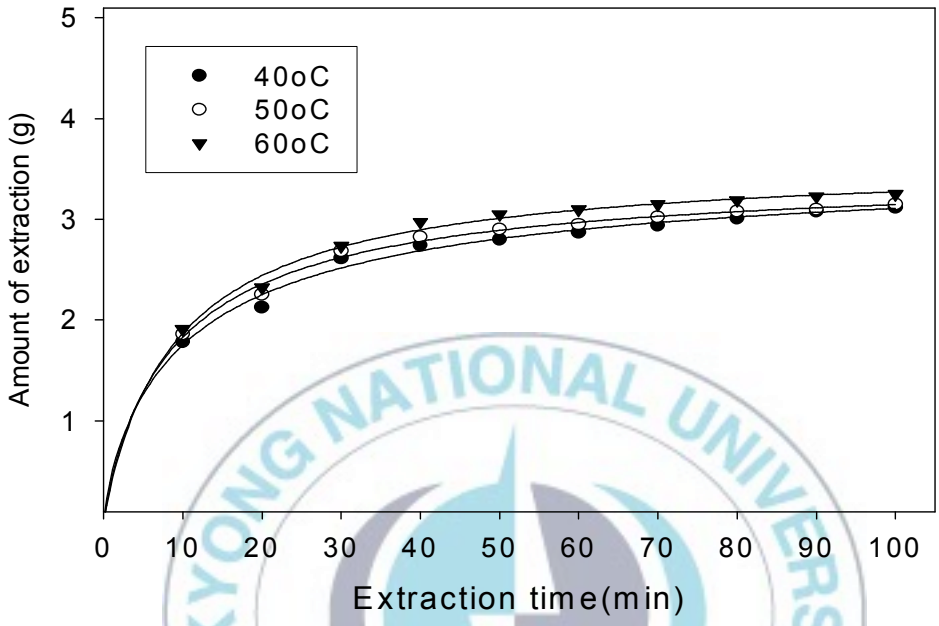


Fig. 6. Anchovy oil extraction curves by SCO_2 at 20 MPa and CO_2 flow rate of 22 g/min.

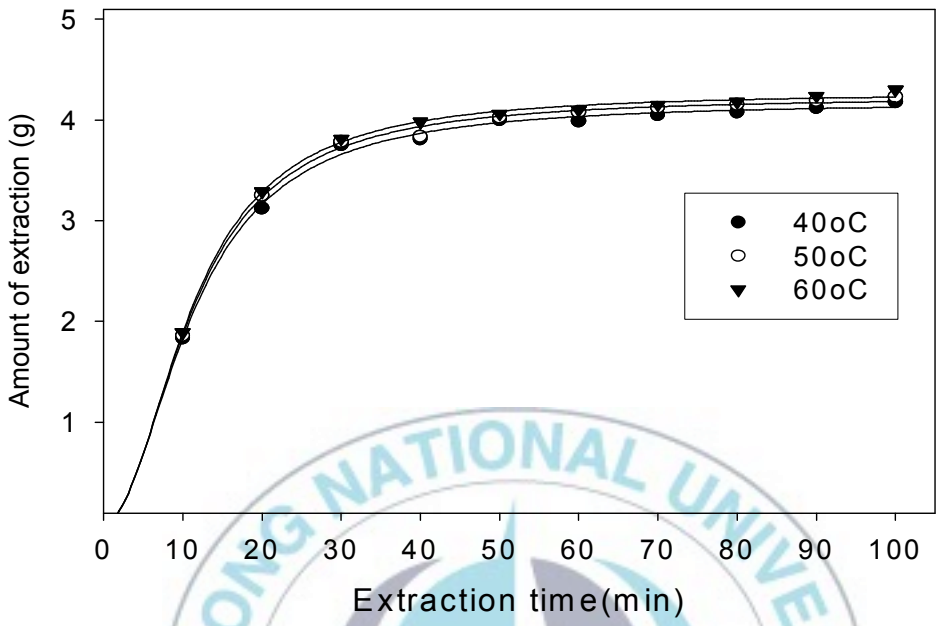


Fig. 7. Anchovy oil extraction curves by SCO_2 at 25 MPa and CO_2 flow rate of 22 g/min.

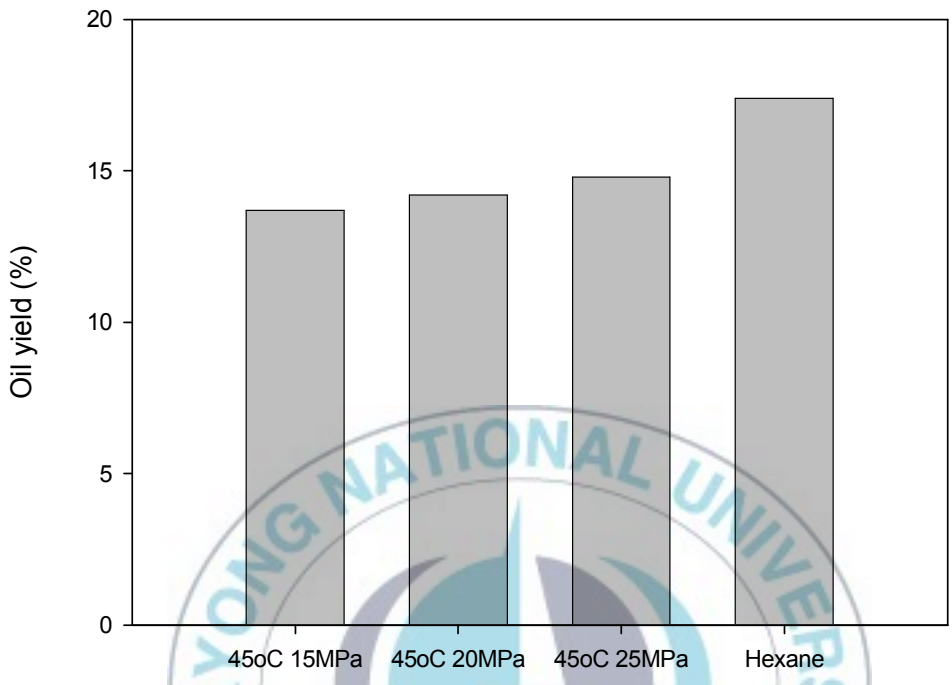


Fig. 8. The percentage of oil yield from anchovy at SCO_2 and hexane extraction.

Table 3. Solubility of anchovy oil in SCO_2 at different temperatures and pressures

Pressure (MPa)	Temperature(℃)		
	40	50	60
15	0.002153	0.002257	0.002295
20	0.004581	0.004688	0.004840
25	0.006506	0.006576	0.006674

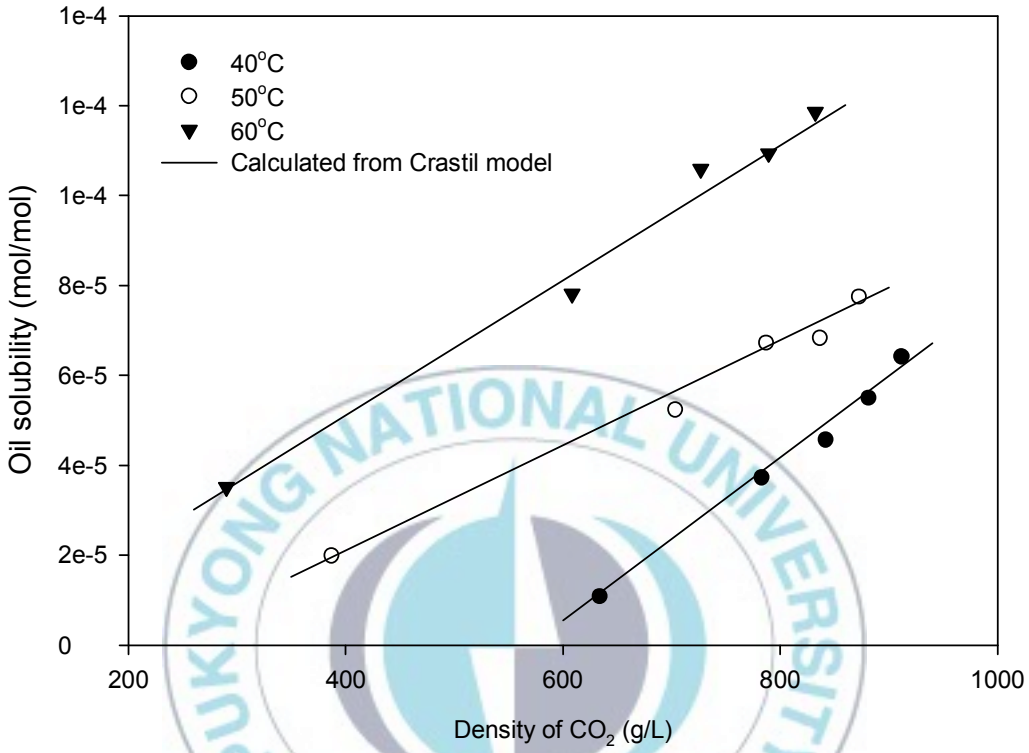


Fig. 9. The experimental values represented by points and the calculated solubilities by lines.

5. Fatty acid compositions

The comparison of the fatty acid composition of the oil obtained by SCO_2 in different conditions and soxhlet extraction with hexane is reported in Table 4. The contents of saturated fatty acid like palmitic acid remained highly compared to unsaturated fatty acid such as EPA (eicosapentaenoic acid), DHA (docosahexaenoic acid) in SCO_2 extraction oil. The main fatty acids are myristic acid, palmitic acid, stearic acid, palmitoleic acid, EPA, and DHA. This result was in accordance with reported fatty acids of boiled- and plain dried anchovy [31]. The major fatty acids classes were PUFAs, monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs). When human continuously takes an excess of SFAs, SFA content in blood will be increase and the cholesterol content will be high. In this work, total SFAs are declined from 49.59% to 37.76% in extracted oil after SCO_2 treatment. But the valuable unsaturated fatty acids (MUFAs, PUFAs) are increased from 24.92% to 31.77% and from 25.49% to 30.48%, respectively. Also contents of MUFAs, PUFAs are increased after organic solvent extraction, but its values are lower than SCO_2 extracted data. All these results suggested that SCO_2 extraction process provides more chance for the marine industry as functional components such as EPA and DHA compared to conventional extraction method

Table 4. Main fatty acid compositions of anchovy oil obtained

Fatty acids	Supercritical Carbon Dioxide									Hexane
	15 MPa			20 MPa			25 MPa			
	40°C	50°C	60°C	40°C	50°C	60°C	40°C	50°C	60°C	
C14:0	10.5	10.1	9.8	8.5	9.8	10.0	9.7	10.7	11.0	12.2
C16:0	25.5	20.3	20.5	17.8	21.0	23.3	23.2	19.4	21.9	26.3
C16:1	8.0	9.0	9.9	4.2	4.4	3.8	4.8	6.1	4.7	4.5
C18:0	4.9	4.1	3.8	3.5	3.8	3.4	4.8	3.4	3.8	8.0
C18:1	14.3	16.0	15.5	21.0	19.7	20.1	19.6	20.4	21.2	15.3
C18:2	2.9	2.2	2.0	1.5	1.4	1.2	1.1	3.3	1.4	3.5
C20:0	0.2	2.7	3.3	2.5	2.9	2.6	2.2	2.4	2.5	3.1
C20:1	3.7	3.3	3.2	4.3	4.4	4.3	3.9	4.6	3.9	3.8
C20:3	3.9	3.4	3.4	2.3	2.9	2.7	1.6	1.7	1.8	1.1
C20:5 EPA	8.9	10.0	10.3	11.1	10.0	10.2	9.2	8.9	9.7	9.9
C24:1	1.1	2.4	3.2	4.1	3.0	3.8	3.0	3.9	3.5	1.3
C22:6 DHA	16.0	16.7	15.2	19.2	16.9	14.6	17.0	15.3	14.6	11.0

6. Measurement of oil stability

High level of PUFAs is present in fish oil. The quality of oil is deteriorated at a varying velocity depending strongly on production and storage conditions [32]. FFAs are responsible for the acidity of oil. Changes of FFA content are mainly related to hydrolytic reactions in the oil. FFA content and peroxide value of the oil extracted by SCO_2 and hexane are given in Table 5. It was found that the amount of FFA and peroxide value were significantly high in hexane extracted oil than SCO_2 extracted. It was also observed that oil obtained at higher extraction temperature contained high amount of FFA and peroxide value. This result agreed with the high FFA content and peroxide value in hexane extracted oil due to higher temperature. Higher temperature and storage time also caused a significant increment of the FFA in the hake byproducts oil [33]. On the other hand, peroxide value of an oil or fat is used as a measurement of rancidity which occurs by auto oxidation. Low exposure of oxygen in SCO_2 extraction caused minimal oxidation. However, the oil extracted by hexane showed lower stability compared to the oil obtained by SCO_2 extraction.

7. Oxidative stability

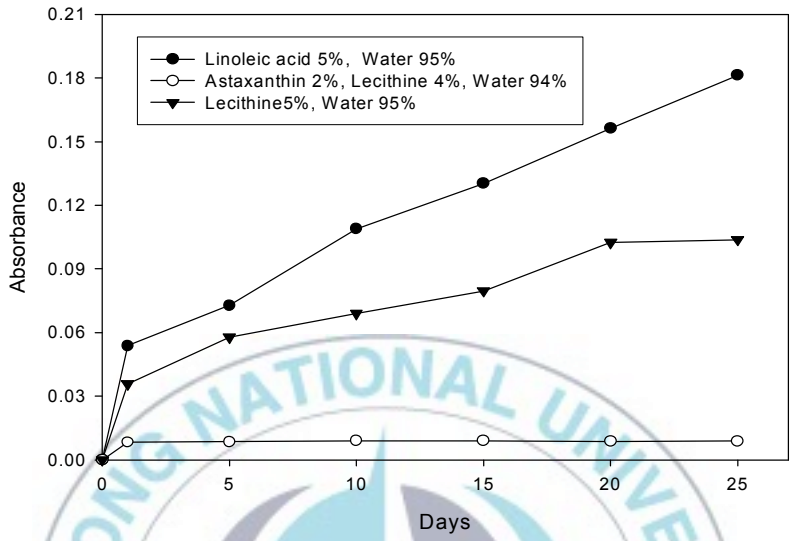
The oxidative stabilities of anchovy oil are shown in Fig. 10. A. and B. In this study oxidation trend was evaluated instead of determining the absolute state of oxidation of incubated sample. The increase in absorbance value was an indicator

of auto oxidation by formation of peroxides during incubation. As in other common oils that contain a high concentration of triglycerides, the most important cause of deterioration in the quality of fish oil, from a flavor and odor standpoint, is oxidation by atmospheric oxygen. Anchovy oil emulsion showed low absorbance value indicating low level of lipid peroxidation. On the other hand, anchovy oil emulsion with Astaxanthin showed high oxidative stability. Astaxanthin inhibited the peroxide formation from lipid by peroxidation in a certain period. Initially, anchovy oil emulsion showed high absorbance comparing to anchovy oil with linoleic acid emulsion. This might be due to the presence of peroxide from the oxidation of anchovy oil. In thiobarbituric acid method, the absorbance measured on 15 day was also similar with oil and oil with Astaxanthin emulsion. However, this value was also high in the oil with linoleic acid emulsion indicating a low absorbance. On the other hand, a significant increase in absorbance was found on 20 day for oil emulsion. EPA and DHA in oil which were the major part of unsaturated fatty acids were the most susceptible to oxidation. The sites of attack by oxygen are the unsaturated portions of the fatty acid moieties of triglycerides (Stansby 1967). Due to its high content of polyunsaturated fatty acids, including EPA and DHA, fish oils are highly susceptible to oxidative spoilage and the rate of fish oil oxidation is significantly different from that of other oils (Boran *et al*. 2006).

Table 5. Free fatty acids and peroxide value of anchovy oil obtained by SCO_2 and hexane extraction

SCO_2 extraction		Free fatty acids (g/100 g oil)	Peroxide value (milliequivalent/1000 g)
Pressure (MPa)	Temperature (℃)		
15	40	6.24	54.20
	50	6.77	55.60
	60	7.19	55.98
20	40	7.96	56.26
	50	7.29	56.89
	60	7.45	57.43
25	40	8.32	58.08
	50	8.54	58.66
	60	8.52	59.52
Hexane extraction		9.45	62.29

A)



B)

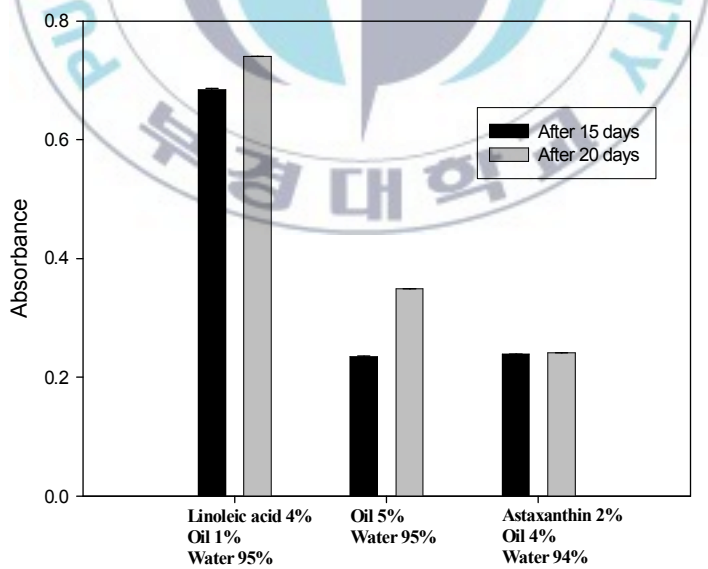


Fig. 10. A-B: Oxidative stability of anchovy oil A) Thiocyanate method and B) Thiobarbituric acid method.

8. Color

The changes in the lightness (L*), redness (a*) and yellowness (b*) over different anchovy oils extracted are shown in Table 7. Lightness does not seen to be affected by extraction conditions, slight difference of L* value was recorded between SCO_2 extracted oil and oil extracted by hexane. The most significant changes are observed in a* values, which are related with the tonality of color, changing from redness (a*) to greenness in hexane extracted oil. The characteristic red color of anchovy oil is denoted by a high value of b* that shows a slight increase at hexane extraction related to other SCO_2 conditions.

Table 7. Color of anchovy oil obtained by SCO_2 and hexane extraction

		SCO_2 extraction									Hexane extraction
		15 MPa			20 MPa			25 MPa			
		40 °C	50 °C	60 °C	40 °C	50 °C	60 °C	40 °C	50 °C	60 °C	
Colour	L*	24.38	24.41	24.25	24.04	25.51	24.21	24.13	24.79	24.38	23.54
	a*	3.21	4.35	4.42	4.32	4.02	4.17	4.26	4.33	4.12	4.34
	b*	9.75	9.18	9.21	9.15	10.11	9.27	9.25	9.29	9.35	10.42

9. Protein characteristic of anchovy residues

9.1. Digestive enzyme activities

Fig. 11. A-C shows the activities of protease, lipase and amylase of crude enzyme extracts of anchovy. Among the three classes of digestive enzymes, the activity of amylase was highest. The activities of protease, lipase and amylase were higher in raw anchovy samples than in SCO_2 extracted samples. This may have resulted from a loss in digestive enzyme activity of anchovy samples by the SCO_2 treatment. The loss of enzyme activity after SCO_2 extraction has been observed in prior studies and this phenomenon has been attributed to interactions between CO_2 and the enzyme. This means CO_2 may form covalent complexes with free amino groups on the surface of the enzyme. Some authors reported similar observation [34]. Due to formation of covalent complex, the activities of enzymes were reduced.

9. 2. Optimum pH of protease, lipase and amylase

The optimum pH of protease, lipase and amylase were shown in Fig. 12.A-C. The highest proteolytic activities of crude extract in both SCO_2 and hexane extracted anchovy were found at alkaline pH 9. High protease activities at pH ranging from 8.0 to 9.0 have also been reported in several fish species [35]. At acidic pH, low protease activities were found in extract. This indicated that the

crude extracts of anchovy contained both the acidic and alkaline proteases. Similar results were found by Salim et al. (2004) for Squid viscera. The enzyme exhibited optimum lipolytic activity at pH 8.5 and stable with in a pH range of 8.0–10.5 were reported from fish and other sources [37]. The activities of amylase in the crude extracts of anchovy were maximal at pH 7-8. It seems to be more similar to that reported for other fishes. Optimal pH values between 7.0 and 8.5 have been extensively reported (2, 3, 6, 9, 12, 13). An optimum pH of 7-7.5 was reported for amylase [38]. Lipase and amylase activities were very low at an acidic pH due to the presence of acidic lipase and amylase. In the crude extracts of SCO_2 and hexane extracted anchovy, the difference in the pH effect on digestive enzyme activities were not significant.

9.3. pH stability

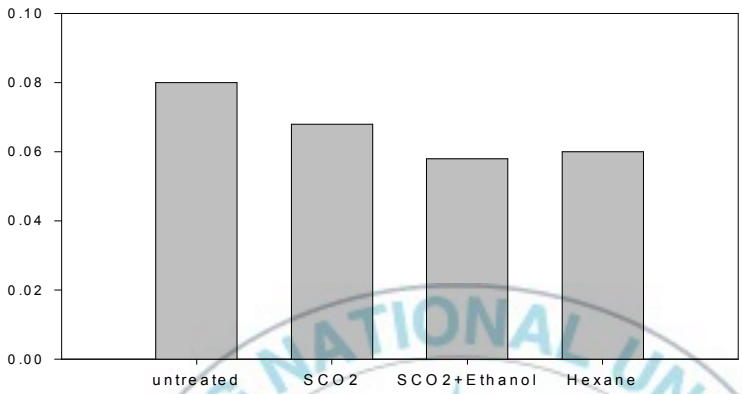
In the pH range of 6-9, the protease activities were more than 90% of its original value and then decreased with increasing pH. The pH stability of crude enzyme extracts of tuna viscera were reported to be in the range of 9-11, where more than 90% activity was retained [39]. The instability of acidic proteases towards the alkaline pH region contrasts with the behavior of gastric proteases obtained from many of the lower vertebrates species [40]. The crude extract lipase was stable within a pH range of 8-9 with 90% activity. The stability of amylase of anchovy extract was in pH range of 6-8.5 (almost 90% activity). Noman et al. (2006)

reported the α-amylase stability at the pH range of 6-8 and the activity was found to decrease gradually at acidic pH, but it decreased rapidly at alkaline pH. Similar optimum pH (7.0) was observed for a-amylase from vine shoot inter-nodes. The enzyme was stable over a narrow range of pH, between 6.0 and 8.0, for 24 h incubation at 4 ^{o}C; the residual activity at pH 9.0 was 35%. The differences in the stable pH range for the crude extract of SCO_2 and hexane extracted anchovy were insignificant.

9.4. Electrophoresis

The gel electrophoresis of marker protein and the crude extract of freeze dried raw, SCO_2 and organic solvent extracted anchovy are shown in Fig. 12. The proteins in freeze dried raw anchovy were very similar in subunit composition to SCO_2 and organic solvent extracted powder. There was no any change in the intensity of protein bands since the gel banding patterns observed were almost identical. From this observation it can be concluded that protein denaturation was not found in SCO_2 extracted anchovy sample [42]. Also reported very little protein denaturation in SCO_2 extracted seed residues.

A)



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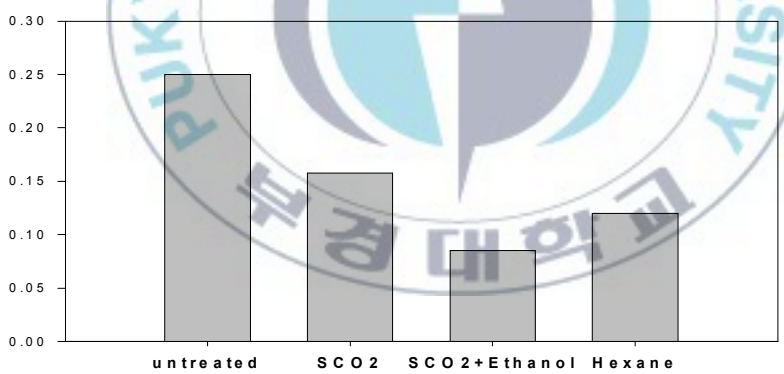


Fig. 11.A-C: Digestive enzyme activities of crude extracts of SCO_2 and hexane extracted anchovy. A) Protease, B) Amylase and C) Lipase

C)

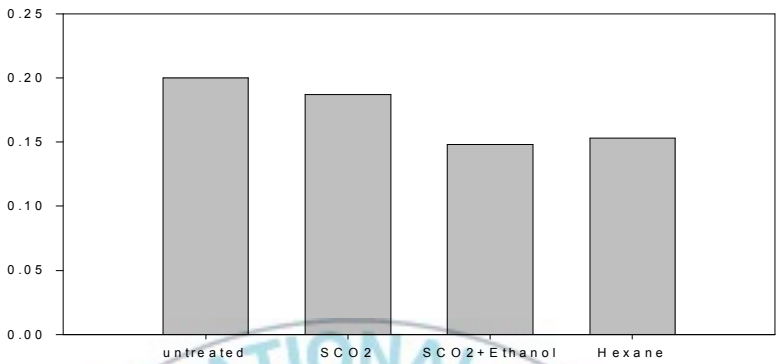
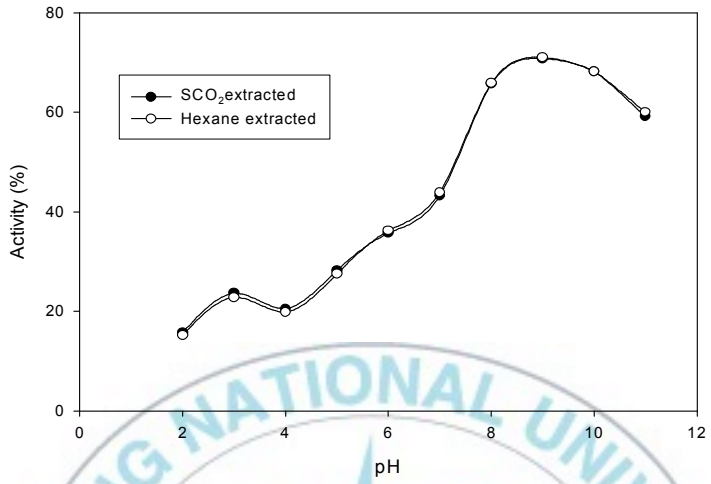


Fig. 10.A-C: Digestive enzyme activities of crude extracts of SCO_2 and hexane extracted anchovy. A) Protease, B) Lipase and C) Amylase

A)



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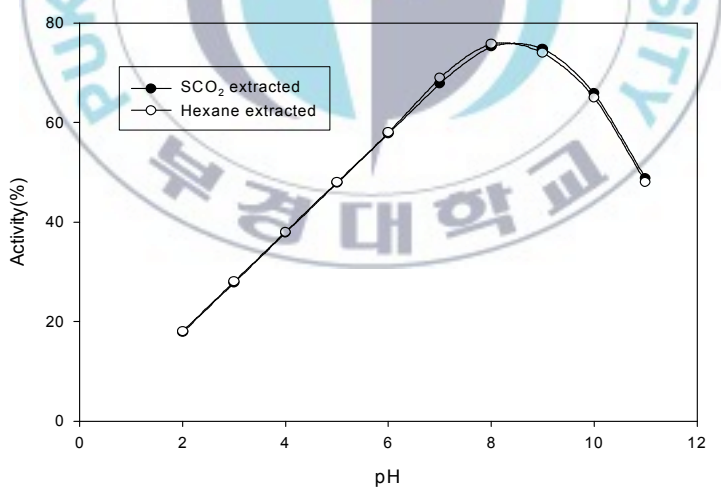


Fig. 11.A-C: Optimum pH of digestive enzymes in crude extracts of SCO_2 and hexane extracted anchovy. A) Protease, B) Lipase and C) Amylase

C)

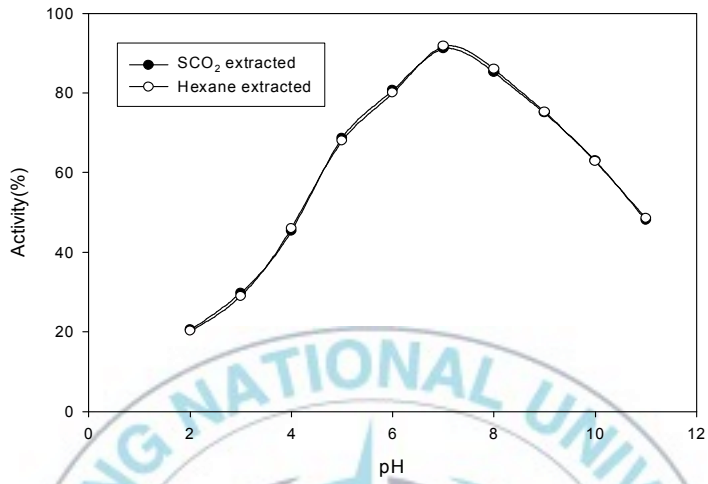


Fig. 11. A-C: Optimum pH of digestive enzymes in crude extracts of SCO_2 and hexane extracted anchovy. A) Protease, B) Lipase and C) Amylase

Fig. 13. SDS-PAGE electrophoresis of crude protein of freeze dried raw and SCO_2 and hexane extracted anchovy.

9.5. Comparative analysis of the microbial safety of residues anchovy

Total Counts, E. coli was measured of Non-consensual sterilization anchovy powder, SCO_2 extraction residue, and hexane extraction residue. Which save in the dark room, during 100 day. Priority, according to lipid extracted powder during storage of anchovy change the total bacteria are shown in Fig. 13. Total counts was an increase of powder according to storage period, treated SCO_2 at 60 ^{o}C powder and anchovy powder prior to processing as compared with total bacteria was lower by about 4 log cycle. E. coli was not detected in 100 days. E. coli is harmful to the body being found in patients with septicemia and the main factor that causes inflammation in the gastrointestinal tract is considered. So an efficient sterilization need is reported. Using supercritical carbon dioxide for disinfection of microorganisms is known by many reports. High pressure (31.5 MPa) and long process time (120-180 min) in the processing of proteins through the crush protein of Staphylococcus aureus has been reported that microbial activity decreased [43], Carbon dioxide at low pressure (6.1 MPa) reacted in a long period of time could be reduced 5 log survival bacteria of the Brocothrix thermosphacta [44]. Such a high-pressure carbon dioxide, various types of microbial activity can reduce the thing have been reported, and this inactivation mechanism of carbon dioxide, the fluidity and permeability with an increase in their essential areas destroyed as easily through the membrane can penetrate and [45], At the same time under pressure, excessive carbon dioxide passing through the changes cell membrane, pH buffering capacity of the cell pool gradient and proton motive force by the failure of excess as well due to lower internal pH [46] is described. In this study the number of total counts is not fully sterilized by

supercritical carbon dioxide processed. This condition is lower than a previous study performed in a short processing time and temperature is thought due [47].

9.6. Analysis of sensory characteristics

9.6.1. Analysis of off-flavors

The chromatogram presented in table 7 in the raw anchovy powder and treated SCO_2 extraction powder, hexane extraction powder. Raw anchovy powder 22peak detected and did entified compounds are classified as many different chemicals such as sorts of alkane compounds, alcohols, aldehydes, and other compounds. And treated SCO_2 extraction powder 16peakd etected and identified compounds are alcohols, aldehydes, and other compounds. Most of the compounds analyzed, alcohols, and highest rate of area% is the ethane was confirmed to have occupied. Also the main volatile compounds in anchovy are tabulated in Table7. Compared to raw anchovy and pre anchovy powder processing by SCO_2 in the volatile compound was decreased. It explained by sugar beverages fragrances extracted uses SCO_2 report [48] in the does not remain residue flavor, extract oils eparation concentrated flavor. This can be explained a fragrance can be easily melted in a reasonable condition carbon dioxide [49].

The strong odor compounds identified were trimethylamine (threshold = 0.000032 ppm, v/v), n-butanal (0.00067 ppm, v/v), dimethyl disulfide (0.0022 ppm, v/v). Those compounds are contained as small amount, while have an

influence on off-flavor due to low odor thresholds. The contents of all flavor compounds are successfully removed or significantly decreased by SCO_2 extraction. Especially methylamine and dimethyldisulfide which are main off-flavor in fishes are completely removed. These results were in accordance with reported isolation of off-flavor from tuna oil using SCO_2 [50].

9.6.2. Comparative analysis of common salt

Aw by varying the concentration of salt useful to grow bacteria for fermentation, optionally [51]. The concentration of salt is more than 29 percent of the growth of microorganisms has been reported to be suppressed. But excessive intake of salt can cause hypertension, heart disease. In recent years, efforts to reduce salt intake has increased. Compare common salt concentration of supercritical carbon dioxide extraction after powder, boiled anchovy and hexane extraction after powder. As a result boiled anchovy's salt concentration was the lowest at 7.5%, the Supercritical Carbon dioxide extraction after powder the salt concentration was approximately 8%. Finally, the highest salt concentration powder is approximately 9.3% of the hexane extraction after powder. This result is considered that as the anchovy to remove oil increased salt concentration relatively.

9.6.3. Comparative analysis of chromaticity

The color is an important factor for consumer's sensory and inherent color is desirable. But color is changed to red during storage gradually. Changing the red color, the factor that worse the quality of the product is known. Chromaticity change was observed according to the extraction method. As a result, did not differ greatly in chromaticity and brightness value was not significantly different from supercritical carbon dioxide extraction residue and raw anchovy. But hexane extraction residue brightness value was slightly reduced. In addition, the hexane extraction residue redness and yellowness value more high significantly than raw anchovy powder.

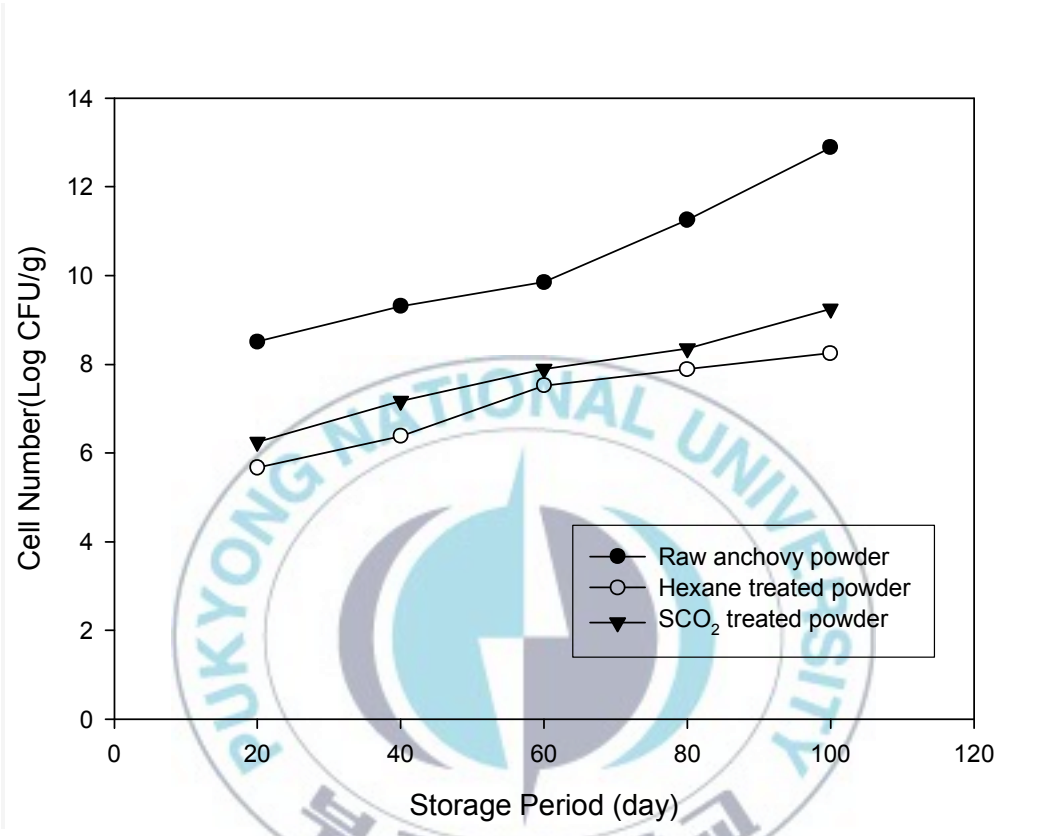


Fig. 14. The change of total counts during storage period for treated anchovy using SCO_2 and hexane.

Table 8. Volatile compounds of raw anchovy powder and SCO_2 treated powder, hexane treated powder

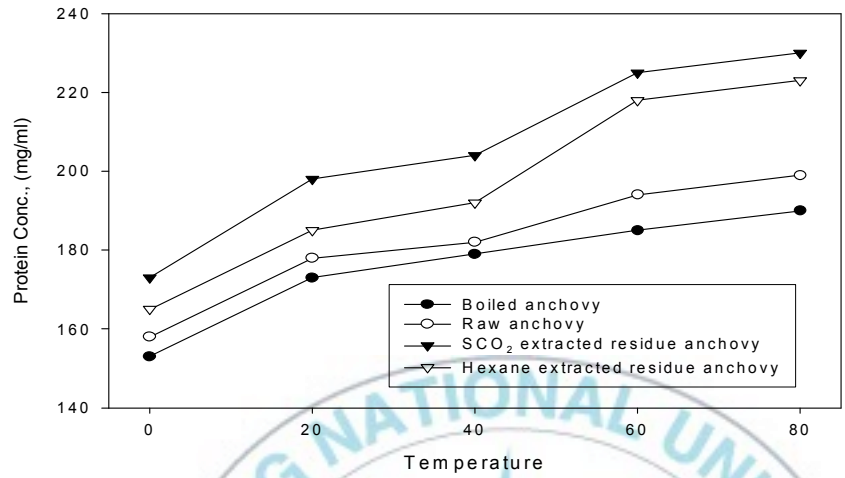
Composition	R.time	Raw Anchovy		SCO_2 treated		Hexane treated	
		Area	%	Area	%	Area	%
Nitrogen	2.814	21758106	2.41	20764317	9.14	9612104	10.87
Carbamic acid	2.863	11918758	1.32	-	-	3876853	4.39
Acetaldehyde	3.243	7008969	0.78	3289045	1.45	5834025	6.60
Ethane	5.275	289940612	32.13	60739919	26.73	26430260	29.90
Isopropyl Alcohol	5.603	317220185	35.15	5648274	2.49	644023	0.73
Methylamine	5.752	4080860	0.45	-		-	
2-Propanol, 2-methyl-	5.987	3182737	0.35	34909455	15.36	24383270	27.58
Ethanol	6.045	8647119	0.96	4614428	2.03	385273	0.44
1-Propanol	6.683	10420142	1.15	7704248	3.39	704248	0.80
Ethyl Acetate	6.782	3286999	0.36	18625177	8.20	-	-
Trimethylamine	7.95	835782	0.09	-	-	35225	0.04
Butanal, 3-methyl-	7.96	57069120	6.32	26644023	11.72	7008969	7.93
3-Methylbutanal	10.43	524837	0.06	65698	0.03	84837	0.10
Paraldehyde	11.349	53315185	5.91	3998934	1.76	-	-
2-	11.38	18286533	2.03	-	-	-	-

Methylene-butanal							
1-Hydroxy-2-butanone	14.686	70834703	7.85	12713215	5.59	4080860	4.62
1-Pentanol	15.137	4448733	0.49	13998436	6.16	4609438	5.21
Dimethyl disulfide	15.41	356662	0.04	-	-	-	-
Propanoic acid	16.066	8643826	0.96	-	-	-	-
Butyrolactone	19.147	2551282	0.28	2608382	1.15	-	-
1-Pentene, 2,4,4-trimethyl-	20.322	4857804	0.54	7983303	3.51	-	-
Hexanoic acid, ethyl ester	22.178	3255883	0.36	2961898	1.30	704248	0.80
Total			100		100.0		100.0

9.7. Water soluble of anchovy powder

The protein content in anchovy sample at different temperatures, particle size is shown in Fig. 13, Fig. 14. It was found that the SCO_2 extracted anchovy and hexane extracted anchovy contained more protein than that of raw anchovy sample and boiled anchovy. The lipid present in the raw materials made them less accessible to water. The protein yield increased with the increase in temperature of SCO_2 anchovy. The protein yields in raw anchovy and SCO_2 extracted anchovy were 228.55±6.25 and 190.58±2.94 mgg^{-1} at 80ºC, respectively. This result suggested that almost all protein could be recovered by SCO_2 extracted sample. Percentage of proteins precipitation was directly related to change in temperature. T. Bourtooma, et al.,) reported increased temperatures of 17 and 30 ^{o}C resulted in increased precipitation. Nishioka and Shimizu (1983) reported similar results in the effect of temperature on the recovery upon washing of minced fish meat. In general, at ambient temperature, protein normally has low solubility in water due to strong aggregation through hydrophobic interactions. The solubility of protein in water increased at higher temperature. Moreover, the protein yield increased at elevated temperature due to the increased rate caused by the increase in dissociation constant or ion product constant of water.

A)



B)

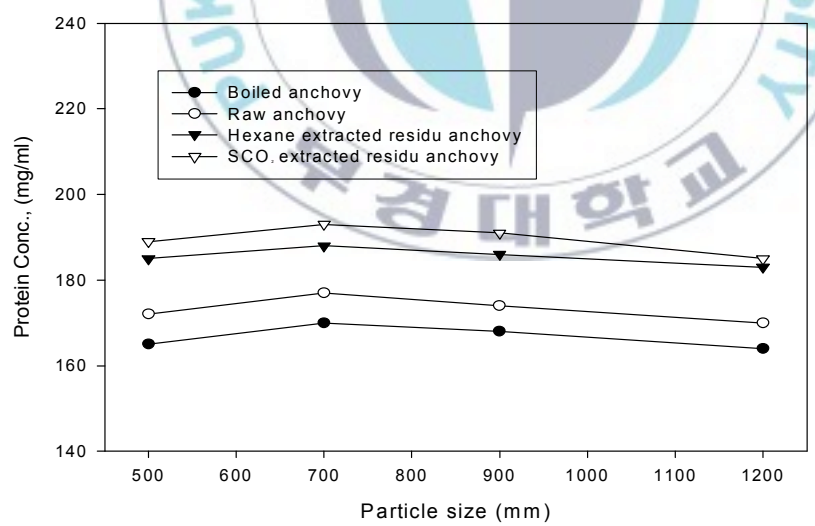


Fig. 15. A-B: The effect of temperature, particle size on water soluble of anchovy powder.

Conclusion

The friendly environmental technology of supercritical carbon dioxide extraction has been widely used to extract oil and bioactive components from materials. Anchovy oil was extracted by supercritical carbon dioxide extraction at different pressures and temperatures. The highest extraction yield of anchovy oil was found at 25 MPa and 60℃. The solubility of anchovy oil calculated from extraction curve was also high at higher pressure and temperature. This state was caused by an increase of the density of carbon dioxide and hence the solvating power of supercritical carbon dioxide. Analysis of fatty acids in anchovy oil by supercritical carbon dioxide was carried out. The major fatty acid components are myristic acid, palmitic acid, stearic acid, palmitoleic acid, EPA, and DHA. Anchovy oil contained a lot of unsaturated fatty acids. The oil obtained by SCO_2 extraction was more stable than organic solvent extraction. Therefore, anchovy oil obtained by SCO_2 extraction may be a good source of PUFAs. SCO_2 extraction oil was more efficient than organic solvent extraction in terms of oil quality and stability. It was found that anchovy contained a natural antioxidant. Anchovy oil may contain small amount of natural antioxidant that might be one of the causes for higher oxidative stability. In this study, microbial safety and sensory characteristics of anchovy residue by SCO_2

process is analyzed. The number of total counts is sterilized by supercritical carbon dioxide processed and E. coli was not detected in storage days. Sensory characteristics also confirmed better than hexane extraction residue. The digestive enzyme activities of SCO_2 extracted anchovy residues were slightly higher compared to organic solvent, hexane extracted residues. Based on the electrophoretic patterns, no protein denaturation was found in SCO_2 extracted samples. The use of carbon dioxide for lipid extraction is environment friendly. Therefore, anchovy after lipid extraction by SCO_2 might be used for the isolation and purification of different digestive enzymes. Thermo stable biocatalysts are highly attractive for economic purposes. Thus, further study using SCO_2 at different extraction conditions may also help to obtain high quality and high thermally stable functional proteins.

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