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Separation of Protein and Fatty Acids from Tuna Viscera Using Supercritical Carbon Dioxide

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Abstract Supercritical carbon dioxide extraction was investigated as a method for removing lipids and bad flavor from tuna viscera. To find the optimum conditions, different experimental variables, such as pressure, temperature, flow rate of solvent and sample size, were evaluated for the effective removal of lipids and the undesirable smell. Ethanol was used as the entrainer, with a 3% by vol CO_2 flow rate. By increasing the pressure at constant temperature, the efficiency of the lipid removal was improved and the protein was concentrated without denaturalization. The main fatty acids extracted from the tuna viscera were palmitic acid (16:0), heptadecanoic acid (17:1), oleic acid (18:1) and docosahexaenoic acid (22:6). The major amino acids in the tuna viscera treated by supercritical carbon dioxide were glutamic acid, leucine and lysine, and the free amino acids were L-proline, taurine and L-α-aminoadipic acid.

Keywords: supercritical carbon dioxide, ethanol entrainer, tuna viscera, protein, lipid, amino acid

INTRODUCTION

Marine Products, such as fish and seaweeds, have gained increasing interest within the area of food in Korea. In the food industry, the demand for marine products as health food has increased every year as they contain an abundance of useful ingredients. Especially, red fish containing DHA, EPA and proteins have been used for food stuffs. Also, the viscera of red fish were used for feeding animals and as fertilizers, but there have been insufficient previous studies on their development and production. Fish protein concentrate (FPC) has been made of fish meat after the removal of the viscera and gills from the fish. Although this improved the preservation of the fish protein and commercialized its manufacture as an edible form, it was difficult to use as food within a modern system due to the increasing cost of production caused by denaturing of the protein, the unpleasant odor and bitter taste.

Conventional methods for the extraction, fractionation, deodorization and isolation of raw materials include the use of highly flammable or toxic solvents and energy-intensive vacuum distillation. High-temperature processing can result in degradation of thermally labile compounds.

Supercritical fluid technology has been studied intensely over the past few decades as an alternative to a separation process. It is particularly attractive as the more conventional techniques of distillation, solvent extraction, and absorption and membrane separation are impractical, unsuitable or too expensive. The major advantages of near- and supercritical fluid extraction lies in the great variation of the solvating capacity that is achievable near the critical state, with relatively small changes in the system pressure or temperature.

Consideration of these factors has lead investigators to apply supercritical fluid extraction (SFE) techniques to the separation of various components [1,2]. The technology is of special interest to the food and pharmaceutical industries because carbon dioxide, the most common supercritical fluid solvent, is non-toxic and leaves no residues. Recently, SFE and fractionation of fish oils have been the subject of ongoing research, to such an extent that workers have recently published data on the fundamental measurements of solubilities and phase equilibria of polyunsaturated fatty acid fish oils in supercritical fluids [3-7]. Also, oil has been recovered from fish muscle using supercritical carbon dioxide, and the residual protein investigated [8-15] Fujimoto *et al.* [16] studied oils and fishy odor extractions from sardine meat using liquid carbon dioxide, which resulted in a higher quality sardine meat variable, known as Surimi. Esqu vel *et al.* [17] extracted fish oil from sardines using supercritical carbon dioxide. Chun *et al.* [18,19] found that high pressures were preferred for the extraction of lipids from squid internal organs and that the amount of oil extracted decreased with temperature, in line with the lowered solvent density. They was also found little change in the fatty

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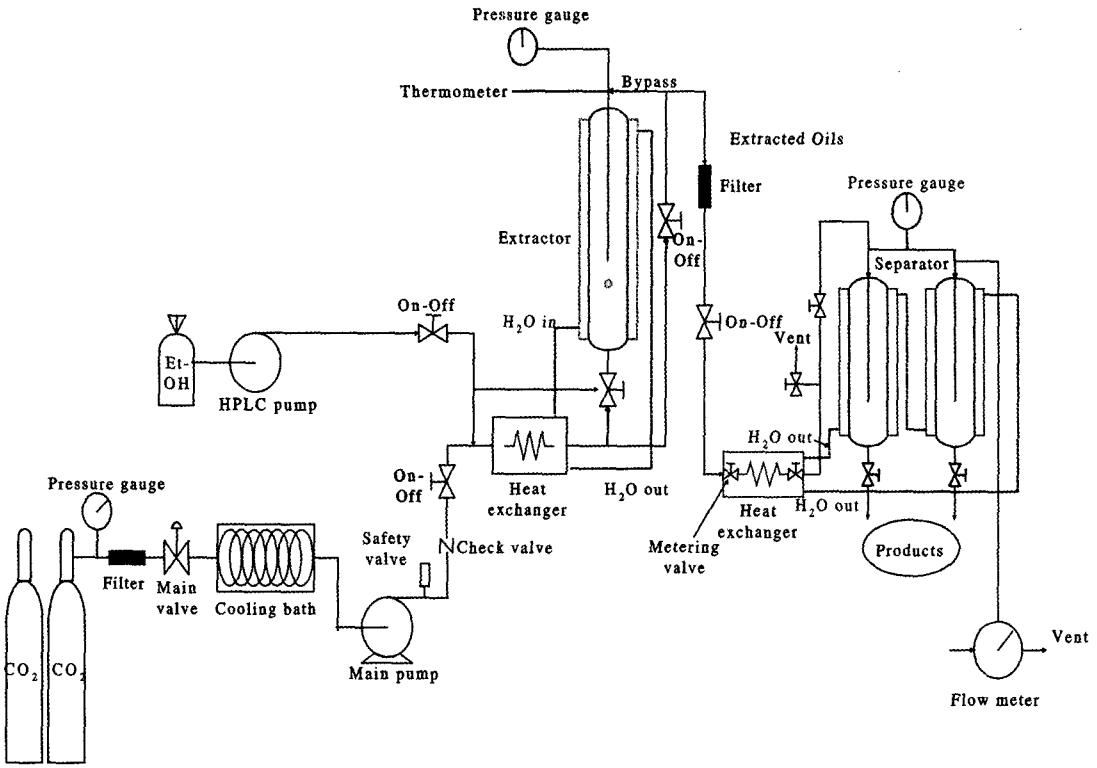

Fig. 1. Schematic diagram of the supercritical fluid process.

acid of oil extracted under different conditions, with the exception of the recovery of DHA appeared to be favored at a pressure of 10.3 MPa and temperature of 35°C.

The objectives of this study were to obtain extractable fatty acid oils, and recover the remaining protein that contains less oil, from the FPC of tuna viscera. To find the optimum conditions for the production of variable compounds from the tuna viscera, experimental variables, such as the pressure, temperature and flow rate of solvent were applied, and the denaturization of protein observed and compared with untreated samples.

MATERIALS AND METHODS

Sample Preparation

The tuna viscera used in these experiments was provided by Dong-Won Ind. Inc. located in Changwon, Korea, and used after being crushed and freeze-dried (-50°C, 5 μHg).

Supercritical Fluid Extract Method

Fig. 1 shows the apparatus used for the supercritical extraction of lipids from the tuna viscera. The extractor was made of stainless steel, with an inner diameter and volume of 2.9 cm and 150 mL, respectively, and 1/4″ and 1/8″ stainless steel pipe used as tubing. For pressurizing the liquid carbon dioxide to supercritical conditions a high pressure pump (Milton Roy MD 93) was used, with a maximum working-pressure of 40 MPa. The carbon dioxide was preheated before entering the extractor, and the temperature and pressure measured using a digital thermometer (Wavetek, model no: 461-112020, Germany) and a Heise Gauge (± 0.14 MPa), respectively.

The supercritical fluid extractor used in this study was a semi-continuous flow type. After filling of the samples, the extractor was charged with carbon dioxide at a constant flow rate. The supercritical carbon dioxide leaving the extractor contained the extracts of the fatty acids and flavors, which were then separated in the cyclone separator. After releasing the pressure, the carbon dioxide was fed into a wet gas meter to measure the flow rate.

This study was performed with a supercritical carbon dioxide flow rate, extraction time, pressure and sample particle size of 50 mL/min, 20~120 min, 10.3~13.8 MPa and 0.25~1.0 mm, respectively. The purity of the carbon dioxide used was 99.9%.

Analysis of Fatty Acids

All reagents were of analytical grade. A lipid standard (fatty acid methyl ester mixture: Sigma Co., 189-19) was used to identify the fatty acids. The extracted sample (0.2 g) was methylated by the AOAC method(Ce 2-66), and aliquots (1 μL) of the methylesters of the fatty acids analyzed by gas chromatography (Hewlett Packard 5890II, USA) using an HP-Innowax column (a fused crosslinked polyethylene glycol capillary column, 30 m × 0.32 mm i.d., 0.15-μm film thickness, Hewlett Packard, USA).

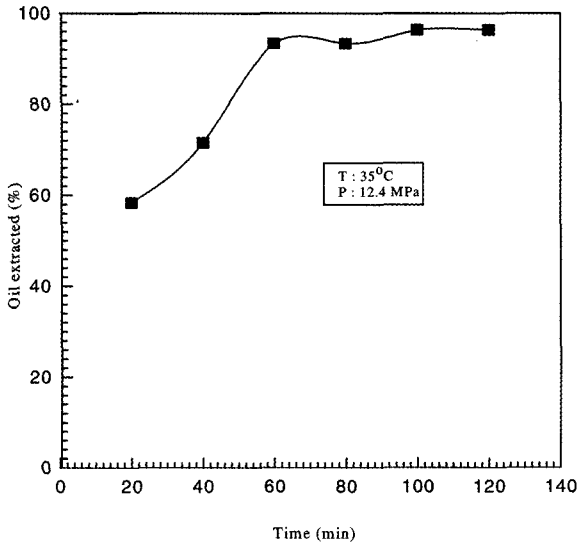

Fig. 2. Effect of time on the extraction efficiency at the supercritical region (Flow rate=50 mL/min, Size=0.25 mm).

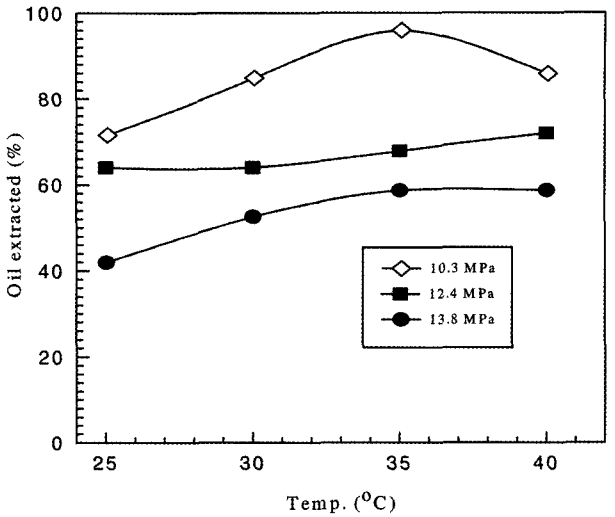

Fig. 3. Effect of temperature on the extraction efficiency at the supercritical region (Flow rate=50 mL/min, Extracted time= 60 min, Size=0.25 mm).

Measuring of Protein Content

The protein content remaining in the extractant was determined by UV spectrometry at 562 nm, using the bicinchoninic acid (BCA) method [20]. The amino acids content was measured using the Sykam Amino Acid Analyzer S433 (SYKAM, Germany).

SDS-PAGE of Proteins in Tuna Viscera

Freeze-dried tuna viscera and that extracted with supercritical carbon dioxide were solubilized in 1% SDS solution (5 mM EDTA, 5 mM Tris-HCl buffer (pH 6.8), 1% SDS, 1% β-mercaptoethanol and 10% glycerol). SDS-PAGE was performed, according to the method of Laemmli' [21] and Fairbank *et al.* [22], with the buffer systems adapted to a 7.5% polyacrylamide slab gel (acrylamide: *N,N'*-methylenebisacrylamide, 37.5:1). Electrophoresis was performed using a Mini-Protein II cell module (Bio-Rad Laboratories, Richmond, CA, USA) at 10 mA/plate for 1 h followed by 20 mA/plate. After the run, the gel was stained with Coomassie brilliant blue R-250, and destained with a methanol/acetic acid solution.

Analysis of Amino Acids in Tuna Viscera

Freeze-dried tuna viscera and that extracted with supercritical carbon dioxide were analyzed for their amino acids composition. The constitutive amino acids were analyzed from the sample, after crushing, which was weighed after adding 50 mL of buffer solution (pH 2.2), and then concentrated using 6 M HCl. To analyze the free amino acids, the sample was prepared in the same way as that centrifuged, filtered the concentrated by 75% ethanol treatment. The amount of free amino acids was found using a S433 A.A. (SYKAM) under the following conditions: column size 4 × 150 mm, Li form resin, analysis cycle time 160 min, flow rates 0.45 mL/min for buffer and 0.25 mL/min for ninhydrin.

RESULTS AND DISCUSSION

Lipid Extraction from Tuna Viscera

Figs. 2~4 showed the effect of the temperature and pressure changes on the lipid extraction using extraction differences at a supercritical carbon dioxide flow rate of 50 mL/min and a particle size of 0.25 mm. The greatest extraction of lipid was achieved at 12.4 MPa, 35°C for 60 min, a particle size of 0.25 mm, with almost 97% of the lipids extracted. As shown in Fig. 2, when the extraction time was extended to 60 min, the amount of lipid extracted remained constant.

As shown in Fig. 3, the lipid extraction efficiency increased with increasing pressure and temperature, but reached a plateau between 35 to 40°C at 12.4 MPa. This trend can be explained by the decreasing of the supercritical carbon dioxide density with increasing temperature at constant pressure. During this process, it appeared remarkable that the lipid extraction efficiency was increased at 12.4 MPa and decreased at 13.8 MPa at constant temperature.

With increasing pressure the supercritical carbon dioxide was observed to form emulsions with the extractants. The lipids in the material were located in the middle of the particles, at a certain distance from the surface. In this situation, the mass transfer coefficients of the solvent play an important role in the extraction efficiency. A higher density of the solvent will then result in lower penetration of the materials [1,2].

Fig. 4 shows the lipid extraction efficiency according to the particle size of the samples. With decreasing particle

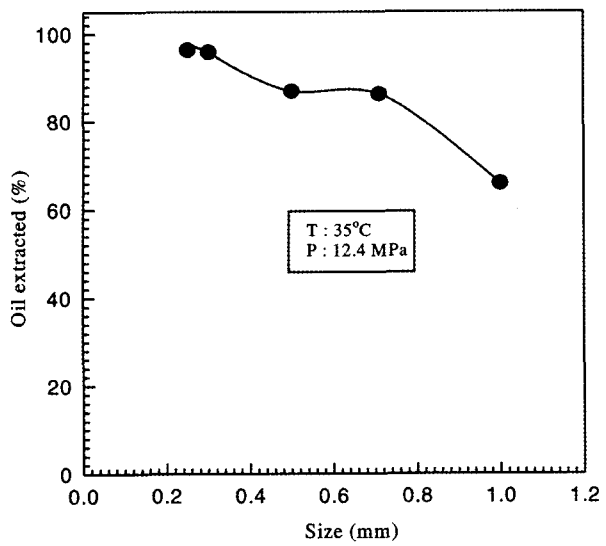

Fig. 4. Effect of size on the extraction efficiency at the supercritical region (Flow rate=50 mL/min, Extracted time=60 min).

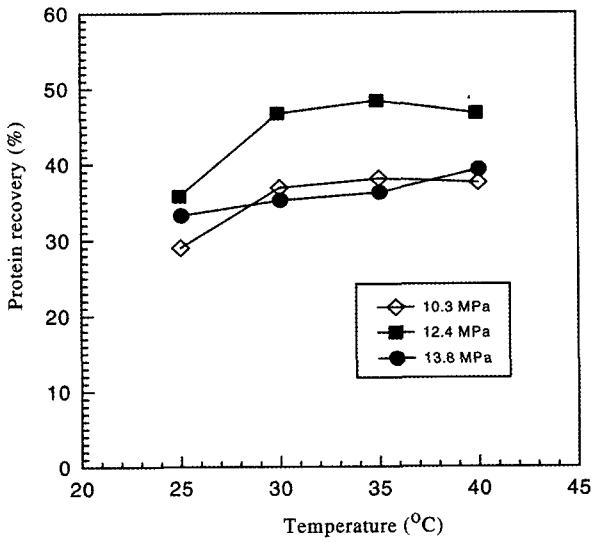

Fig. 6. Effect of temperature on the extraction efficiency at the supercritical region (Flow rate=50 mL/min, Extracted time=60 min, Size=0.25 mm).

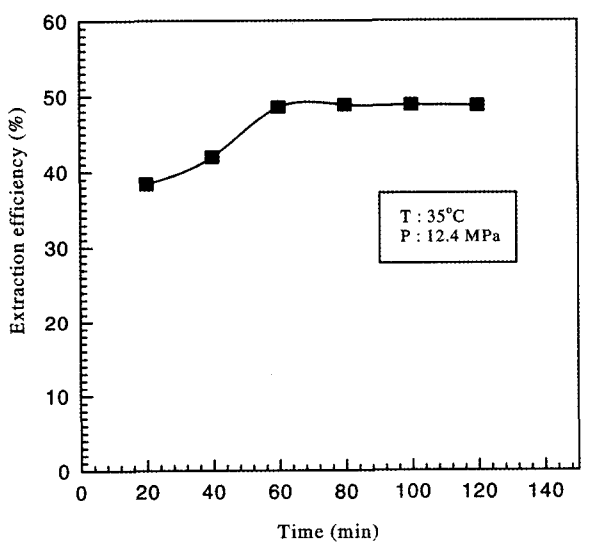

Fig. 5. Effect of time on the extraction efficiency at the supercritical region (Flow rate=50 mL/min, Size=0. 25 mm).

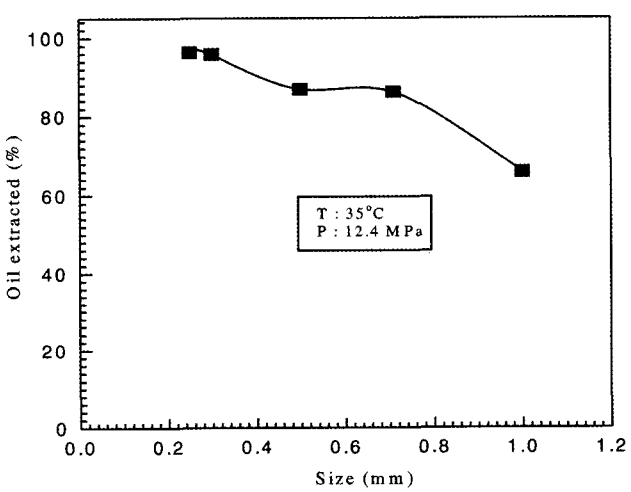

Fig. 7. Effect of size on the extraction efficiency at the supercritical region (Flow rate=50 mL/min, Extracted time=60 min).

size, the extraction efficiency increased. Thus, it can be concluded that the smaller the particle size of the samples, the better they are penetrated, as well as having a larger contact area with the supercritical carbon dioxide.

Determination of the Protein Concentration

Extraction efficiency of protein was determined in relation to the temperature, pressure, particle size and extraction time at a supercritical carbon dioxide flow rate of 50 mL/min. Fig. 5 shows the extraction behavior of protein by the extraction time. As extraction time greater than 20 min, the yield starts to increase then reaches a peak at 60 min (about 49%). At longer extraction time, the yield tends to decrease. Figs. 6 and 7 showed the effects of pressure, temperature and particle size on the protein extraction efficiency, with up to 50% of the initial protein content recovered under experimental conditions of 12.4 MPa, 35°C and 0.25 mm, respectively.

Composition of Lipid Extracts

Table 1 shows the fatty acids composition of the lipid extract under each set of extraction conditions. The major fatty acids found were palmitic acid (16:0), oleic acid (18:1) and palmitoleic acid (18:1), *etc.*

Oleic acid (18:1) had the highest concentration of the unsaturated fatty acids found in the lipid extracts from tuna viscera. Approximately 12.7% of the DHA was ex-

Table 1. Fatty acids composition of the tuna viscera oil extracted by SC-CO_2

P (MPa)	T (°C)	Fatty acid (%)[1]								
		Myristic (14:0)	Palmitic (16:0)	Palmitoleic (16:1)	Heptadecanoic (17:0)	Cis-10-heptadecanoic (17:1)	Stearic (18:0)	Oleic (18:1)	EPA[2] (20:5)	DHA[3] (22:6)
10.3	25	2.0	33.2	4.3	2.0	5.6	10.1	14.5	1.0	4.1
	30	1.8	39.6	3.6	1.8	0.6	11.5	16.8	0.8	2.7
	35	1.6	24.9	3.0	1.6	4.6	8.4	11.0	0.6	1.7
	40	1.0	23.5	1.4	0.9	0.5	0.6	5.7	0.1	1.0
12.4	25	1.5	30.0	2.6	1.7	6.1	0.1	10.1	0.7	0.4
	30	1.4	29.5	2.8	1.4	2.3	0.7	12.5	0.5	1.6
	35	1.1	22.0	2.6	1.9	6.1	0.5	10.8	1.1	0.2
	40	0.6	13.5	1.0	0.8	0.2	1.1	4.2	·	·
13.8	25	1.7	30.2	2.7	1.6	3.6	·	10.2	·	12.7
	30	0.6	16.7	1.4	0.8	0.9	·	7.3	0.2	5.8
	35	1.0	25.4	1.9	1.7	2.6	·	8.6	0.3	0.6
	40	2.0	43.0	3.6	2.5	4.3	13.8	15.3	0.3	0.3

[1]Percentage of gas chromatography peak area, [2]EPA: eicosapentaenoic acid, [3]DHA: docosahexaenoic acid

Fig. 8. SDS-PAGE pattern of tuna viscera treated and untreated supercritical CO_2 (a; untreated treated tuna viscera, and b, that treated by supercritical CO_2).

tracted at a pressure and temperature of 13.8 MPa and 25°C, respectively.

SDS-PAGE

Fig. 8 compares the electrophoretic patterns of the tuna viscera and that extracted by supercritical carbon dioxide, which show the same patterns, demonstrating the tuna viscera protein was not denatured, but remained stable after the supercritical carbon dioxide processing [10,11,23].

Table 2. Amino acids composition of the untreated tuna viscera and of that treated with supercritical CO_2

Amino acids	Untreated tuna viscera (%/100 g sample)	Treated tuna viscera (%/100 g sample)
Aspartic acid	4.91	5.71
Threonine	2.68	3.06
Serine	1.94	2.24
Glutamic acid	7.61	8.62
Proline	2.27	2.93
Glycine	2.41	2.53
Alanine	3.26	3.69
Valine	3.72	4.03
Methionine	1.93	2.70
Isoleucine	3.10	3.60
Leucine	5.21	5.97
Tyrosine	2.29	2.50
Phenylalanine	2.31	2.99
Histidine	2.96	3.03
Lysine	5.55	6.07
NH_3	1.07	1.31
Arginine	4.56	5.04
Total	57.78	66.01

Analysis of Amino Acids

The results of a constitutive amino acids analysis are listed in Table 2. Generally, more constitutive amino acids were found when the sample was treated by supercritical carbon dioxide extraction. The extracted tuna viscera contained more protein compared to the original material. In the remains of the extracted tuna viscera,

Table 3. Free amino acids composition of untreated tuna viscera and of that treated with supercritical CO_2

Free amino acids	Untreated tuna viscera (%/100 g sample)	Treated tuna viscera (%/100 g sample)
Phosphoserine	-	-
Taurine	0.724	1.440
Aspartic acid	0.505	0.466
Threonine	0.578	0.409
Serine	0.560	0.419
Asparagine	-	-
Glutamic acid	1.151	0.792
α-aminoapidic acid	-	-
Proline	0.578	0.440
Glycine	0.357	0.252
Alanine	0.951	0.680
Valine	0.574	0.389
Cystine	-	-
Methionine	0.377	0.213
Isoleucine	0.581	0.425
Leucine	1.204	0.829
Tyrosine	0.578	0.416
NH_3	0.082	0.054
Ornithine	0.066	0.038
Lysine	0.790	0.487
1-methylhistidine	-	-
Histidine	0.569	0.248
3-methylhistidine	-	-
Phenylalanine	0.676	0.447
Carnosine	-	-
Arginine	0.829	0.468
Total	11.731	8.926

glutamic acid, leucine, lysine and other amino acids were found to be equal to these in the original material. Therefore, supercritically treated tuna viscera seem to be a proper source of fish-protein.

Table 3 shows the results of the free amino acids analyses for the untreated tuna viscera and that treated by supercritical fluid extraction, with more free amino acids found in the untreated sample. As an organic solvent was used as the entrainer to support extraction, the tuna viscera protein was partially denaturized.

CONCLUSION

To determine the valuable lipid and protein contents in freeze dried tuna viscera, extraction experiments were carried out at pressures from 10.3 to 13.8 MPa and temperatures from 25 to 40°C, utilizing supercritical carbon dioxide, with 3% by vol of ethanol as the entrainer. The highest extraction efficiency, about 97% of the contained lipids, was found at a flow rate of 50 mL/min, pressure of 12.4 MPa and particle size of 0.25 mm. The protein content found in the residue was about 50%. The free amino acids were L-proline, taurine and L-α-aminoadipic acid. The main components of the extracts were palmitic acid (16:0), heptadecanoic acid (17:1), oleic acid (18:1) and DHA (22:6).

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