Measurement of Stability and Astaxanthin Content of Squid Viscera Oil Extracted by Supercritical Carbon dioxide and Organic Solvent

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Abstract
Supercritical carbon dioxide (SCO_2) and an organic solvent, hexane were used to extract oil from squid viscera. The SCO_2 extraction was carried out at temperatures ranging from 35 to 45ºC and pressures ranging from 15 to 25 MPa. The flow rate of CO_2 was 22 g/min and it was constant entire the extraction period. The extraction time was 2.5 hrs for each extraction conditions. At higher temperature and pressure, the oil yield was the maximum. The extracted oil was analysed by Gas Chromatography (GC) for fatty acids compositions. A high percentage of polyunsaturated fatty acids especially EPA and DHA were found in the squid viscera oil obtained by SCO_2 extraction. The stability of oil extracted by SCO_2 and hexane was tested by measuring the free fatty acids (acidity) and peroxide value. The acidity of squid viscera oil obtained by SCO_2 extraction was lower than that of the oil obtained by soxhlet extraction at hexane. The peroxide value was also high in hexane extracted oil. Therefore, the SCO_2 extracted oil showed more stability than the oil obtained by hexane extraction. The amount of astaxanthin in squid viscera oil was determined by HPLC and compared at different extraction conditions. In SCO_2 extraction, the maximum yield of astaxanthin was found in squid viscera oil extracted at 25 MPa and 45ºC.

INTRODUCTION

Squid are very popular food in Korea. Fish processing industries produce large quantities of squid viscera in Korea as a by-product. These wastes contain a lot of protein, lipid and many kinds of biological active matter. Marine lipids especially polyunsaturated fatty acids and lipid soluble bioactive compounds have been attracted much attention for health benefits. There is commercial interest in obtaining polyunsaturated fatty acids, in particular EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). These ω-3 fatty acids have an important role in the prevention of human diseases, such as in lowering blood cholesterol and thus preventing heart 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 [1].

In addition to ω-3 fatty acids, the oil from marine organisms also contains some natural fat soluble antioxidants including carotenoids. Carotenoid is a generic name used to designate the most common groups of naturally occurring pigments found in the animal and plant kingdoms. These lipid-soluble pigments comprise well over 700 compounds that account for beautiful red, orange, and yellow colors. Due to high antioxidant activity, carotenoids are considered suitable as components of various types of products, e.g. cancer prevention agents, potential life

extenders, inhibiting agents for heart attack and coronary artery disease [2-4]. There are several types of carotenoids which can be obtained from different natural sources. Astaxanthin is one of the most effective carotenoids whose antioxidant activity is 10 times stronger than those of any other carotenoids such as zeaxanthin, lutein, canthaxanthin and β-carotene and was up to 500 times stronger than vitamin E [5]. It is the main carotenoid pigment found in aquatic animals especially in many well known sea foods such as salmon, trout, red seabream, shrimp, lobster, and fish eggs [6,7].

Currently, the most common way for extraction is liquid solvent extraction using toluene, hexane, petroleum ether, chloroform, acetone etc. Decomposition or degradation of thermolabile compounds cannot be avoided in a conventional separation method, since relatively high temperatures are required for these processes. Organic solvents are also harmful to human health as well as the environment. Supercritical fluids extraction (SFE) is an efficient alternative for the extraction of natural substances from foods [4,8]. A supercritical fluid separation process using carbon dioxide as the solvent offers potential advantages because it is non-flammable, non-toxic and inexpensive, and can be used under mild operational conditions. SCO_2 also possesses excellent extractive properties such as high compressibility, liquid-like density, low viscosity, high diffusivity. It has been widely used in many industrial applications, i.e. extraction of fish oil for PUFAs, the decaffeination of coffee, the extraction of hops and carotenoids, the synthesis of polymers, the purification and the formation of nano particles [9-12]. Polar co-solvents such as ethanol is often used to enhance the solute solubility in SCO_2 by interacting with the solute, and thus improving the extraction efficiency. Lim et al. [9] reported that SCO_2 with ethanol as a co-solvent enhanced the yield of astaxathin by 9-24% at different extraction conditions. Astaxanthin molecule is lipid soluble and considered containing no strong polar moieties [2,13]. Therefore, SCO_2 can extract majority of astaxanthin without ethanol from materials containing high lipid. Moreover, heat is required to separate ethanol that can oxidize and reduce the quality of the products. In this study, the use of ethanol as a co-solvent was avoided for also measurement of oil stability at different extraction conditions.

The objective of this study was to measure the amount of astaxanthin of squid viscera oil at different extraction conditions. The stability of oil obtained by SCO_2 extraction was also compared to the oil obtained by soxhlet extraction at hexane.

MATERIALS AND METHODS

Materials

Squid viscera were supplied by F & F Co., Busan, Korea. The visceral waste was washed thoroughly with cold water and brought to the laboratory in iced condition. The carbon dioxide supplied by KOSEM, Korea was 99.99% pure. All reagents used in this study were of analytical or HPLC grade.

Sample preparation

The squid viscera samples were dried in a freeze-drier for about 72 hrs. The dried samples were crushed by a mechanical blender and sieved (700 ㎛) by mesh. These samples were then stored at -80°C until using for SCO_2 and organic solvent extraction.

SCO_2 extraction

A laboratory scale SFE unit was used for extracting oil from squid viscera shown in Fig 1. Twenty five grams of freeze dried squid viscera sample were loaded into 200 mL stainless steel extraction vessel containing cotton at the bottom. Before plugging with cap another layer of cotton was used at the top of the sample. CO_2 was pumped into the vessel by high pressure pump up to the desired pressure, which was regulated by a back pressure regulator. The vessel temperature was maintained by heater. Flow rates and accumulated gas volume passing through the apparatus were measured using a gas flow meter. The extracted oil were collected by a cyclone separating vessel. After SCO_2 extraction, the squid viscera residues remaining in the vessel were stored at -80°C until further analysis.

The effect of temperature and pressure on lipid extraction from squid viscera were studied at 35-45°C and 15-25 MPa at a constant extraction time of 2.5 hrs. The flow rates of CO_2 were kept constant at 22 g/min for all extraction conditions.

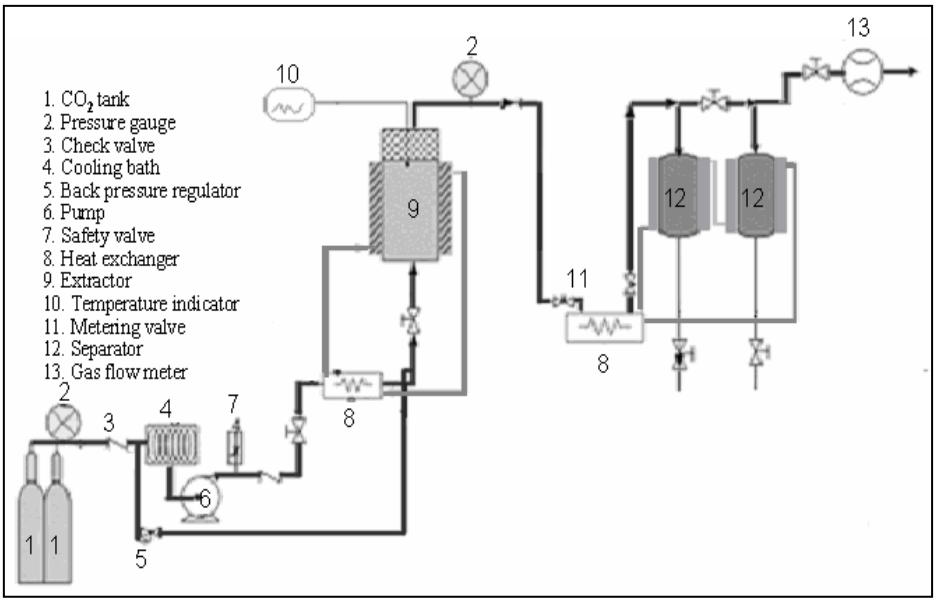

Fig. 1: Schematic diagram of SCO_2 extraction process.

Soxhlet extraction by hexane

The extraction was carried out in a soxhlet apparatus using n-hexane as solvent. Three gram of sample was placed into the extraction thimble and the extraction was run 12 hrs until the colour of the condensed solvent at the top of the apparatus was clear.

GC analysis

The fatty acid compositions of squid viscera oil obtained by SCO_2 and organic solvent, hexane extraction were determined by gas chromatography using a Hewlett Packard gas chromatograph (5890 Series II GC system). The fatty acid methyl esters were prepared firstly according to

AOCS official method Ce 2-66 [14] 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 was used as a carrier gas (1.0 mL/min) of fatty acid methyl esters. 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).

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.

Free fatty acid determination

Free fatty acids of extracted oil from squid viscera were analysed as described by Bernardez et al. [15]. Briefly, 50 mg (Approximately) 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 2000g 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 standards.

Peroxide value

Peroxide value was determined by the AOCS method Cd 8-53 [14]. Peroxide value was expressed as milliequivalents peroxide/1000 g sample.

HPLC analysis for the measurement of astaxanthin

HPLC analysis was carried out using a Waters HPLC equipped with a model 600E system controller, a model 484 UV/VIS detector and a Eclipse Plus C18 column (5µm, 4.6 x 250 mm, Agilent, USA). Astaxanthin was analysed according to the method described by Krichnavaruk et al. [13]. A mobile phase consisting of acetonitrile, dichloromethane and ethanol at the volume ratio of 5:10:85 was eluted 1 mL/min as isocratic method. Astaxanthin was detected at the wavelength of 470 nm. The amount of astaxanthin in the extract was measured based on the peak area of the standard astaxanthin.

RESULTS AND DISCUSSION

SCO_2 extraction

The supercritical fluid extraction of squid viscera oil at different temperatures (35-45°C) and pressures (15-25 MPa) are shown in Fig. 2. The highest oil yield obtained by SCO_2 extraction was 0.3416 g/g squid viscera at temperature, 45°C and pressure, 25 MPa. The applied pressure and temperature variation greatly affected the oil solvating power of SCO_2 and hence the amount of yield. The oil yield was increased with increasing pressure and temperature. Due to increasing pressure, the density of the SCO_2 was increased and hence the solvating power. The effect of pressure can be attributed to the increase in solvent power and by the strengthening of intermolecular physical interactions.

At constant pressure, the oil yield also increased with increasing temperature. Despite of the decreasing of solvent's density, the oil yield was increased with increasing temperature which can be attributed to the increase of the oil components vapour pressure. The effect of the increase of solute vapour pressure seems to have dominated over solvent's density.

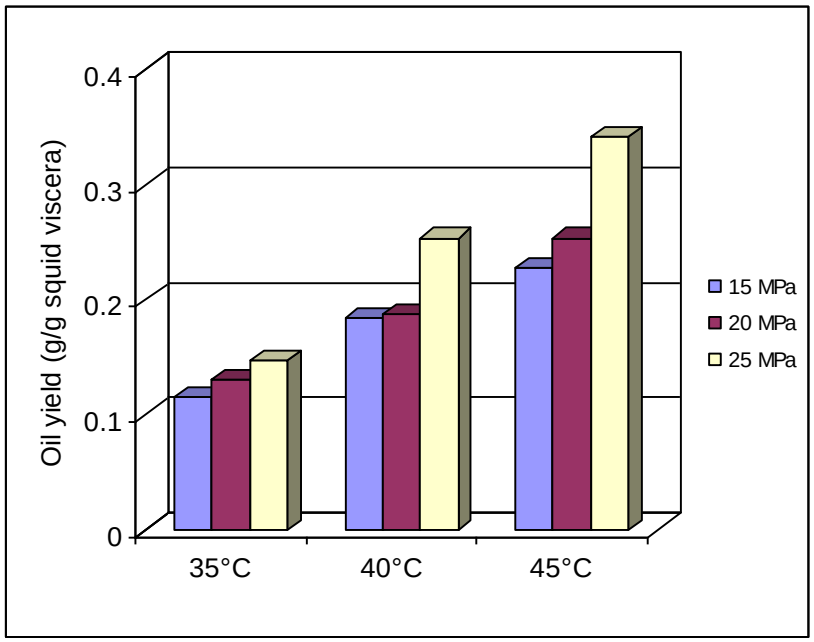

Fig. 2: SCO_2 extraction of squid viscera oil at different temperatures and pressures

Fatty acid compositions

The fatty acid compositions of the oil obtained by SCO_2 and hexane extraction are shown in Table 1. Total of 22 fatty acids were identified in the different extracts analysed. Under certain extraction conditions there were remarkable changes in the fatty acid compositions of the squid viscera oil. Within saturated fatty acids, palmitic acid (C16:0) was present in the highest concentration ranging from 12.43 to 22.65% of total identified fatty acids. Among monounsaturated fatty acids, oleic acid (C18:1) was also found in substantial amounts ranging from 8.56 to 10.90% of total identified fatty acids. The percentage of EPA (C20:5) and DHA (C22:6) in total identified fatty acids were ranged from 5.49 to 10.47 and 10.26 to 18.56, respectively. The composition of total polyunsaturated fatty acids obtained in squid viscera oil was identical with marine fish oils such as cod liver oil and anchovy oil which contained about 14-31% of EPA and DHA.

It was also observed that the oil extracted by SCO_2 showed a higher percentage of polyunsaturated fatty acids than the oil extracted with hexane. This may be happened due to higher temperature applying in soxhlet extraction comparing to SCO_2 extraction. High temperature may lead to thermal degradation of fatty acids, especially unsaturated fatty acids.

Table 1: Fatty acids composition of squid viscera oil obtained by supercritical carbon dioxide and hexane extraction (Fatty acids showed only that were found more than 1%)

Fatty acids (%)	**Supercritical carbon dioxide**									**Hexane**
	25 MPa			20 MPa			15 MPa			
	45ºC	40ºC	35ºC	45ºC	40ºC	35ºC	45ºC	40ºC	35ºC	
C14	3.49	4.29	2.71	3.51	4.53	3.74	2.53	4.39	4.66	4.78
C16	15.90	16.8	13.62	16.77	19.64	17.74	12.43	18.15	20.49	22.65
C16:1	3.39	3.85	2.68	3.91	4.08	3.58	2.59	3.82	4.21	3.86
C17	4.71	5.02	3.92	4.92	5.24	4.84	3.79	4.81	5.31	4.94
C18	2.91	2.86	2.59	3.28	3.55	3.20	2.41	3.18	3.54	2.84
C18:1	9.21	9.12	9.18	10.90	10.68	9.84	8.56	9.63	10.64	9.44
C18:2	1.11	1.09	0.93	1.44	1.28	1.16	1.04	1.17	1.28	1.06
C20	2.37	2.55	2.13	2.30	2.73	2.38	2.14	2.24	2.37	2.86
C20:1	3.51	3.06	3.04	4.08	3.91	3.37	2.92	3.40	3.63	3.58
C20:3	1.82	1.65	1.30	2.18	1.78	1.58	1.53	1.60	1.70	1.06
EPA	9.74	9.30	8.70	10.47	9.33	8.41	8.62	8.28	9.02	5.49
DHA	18.56	15.54	12.39	18.00	15.81	13.64	16.03	12.43	13.59	10.26

Oil stability

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 2. 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. Low exposure of oxygen in SCO_2 extraction caused minimal oxidation. However, the oil extracted by hexane showed lower stability comparing to the oil obtained by SCO_2 extraction.

Table 2: Free fatty acids and peroxide value of squid viscera oil obtained by SCO_2 and hexane extraction

SCO_2 extraction		Free fatty acids (g/100 g oil)	Peroxide value (millieqivalent/1000g)
Pressure(MPa)	Temperature(ºC)		
15	35	2.24	3.77
	40	2.77	4.17
	45	3.19	4.88
20	35	2.96	4.26
	40	3.29	4.89
	45	3.45	5.43
25	35	3.32	5.08
	40	3.56	5.66
	45	4.34	6.52
Hexane extraction		6.57	8.29

Extraction yield of astaxanthin

The SCO_2 extraction of astaxanthin obtained at different pressures and temperatures are shown in Table 3. The highest amount of astaxanthin yield was 6.15 mg/g squid viscera at 25 MPa and 45ºC. It was found that the astaxanthin yield increased with pressure at temperatures of 35 and 45ºC. On the other hand, at 40ºC the maximum value was observed at 20 MPa. This pattern of astaxanthin solubility can be explained by the increase in the SCO_2 density and the decrease in diffusion coefficient. Rising the pressure increases the fluid density which has double effect-an increase in the solvating power of the supercritical fluid, which enhances the extraction process and a reduced interaction between the fluid and the matrix resulting from the decrease in diffusion coefficient, which decreases extraction process. At 35 and 45ºC, the dominant effect was the increase in solvating power of the SCO_2. On the other hand, at 40ºC and 20 MPa, the dominant effect was the decrease in diffusion coefficient.

Solute solubility can also be described by the decrease in the SCO_2 density and the increase in solute vapour pressure. At 15 and 25 MPa the yield of astaxanthin obtained was the maximum while the extraction temperature was 45ºC. Rising the temperature decreases the fluid density, but can increase the solute vapour pressure, which enhances the yield of extraction process. At pressures of 15 and 25 MPa the increase in vapour pressure was dominant over fluid density. On the other hand, a decrease in the yield observed at 20 MPa when the operating temperature was 45ºC. The decrease of astaxanthin yield was due to dominancy of the decrease in the density of SCO_2 than solute vapour pressure.

In hexane extraction, the highest amount of astaxanthin was 7.88 mg/g squid viscera. The highest amount of astaxanthin yield obtained by SCO_2 extraction was almost 78% of the total amount of astaxanthin estimated by soxhlet extraction.

Table 3: Astaxanthin contents in the oil extracted by SCO_2 with different conditions

Pressure (MPa)	Temperature (ºC)	Astaxanthin (mg/g squid viscera)
15	35	2.32
	40	2.41
	45	2.66
20	35	2.99
	40	5.58
	45	4.3
25	35	3.58
	40	4.56
	45	6.15
Hexane		7.88

CONCLUSIONS

Squid viscera oil was extracted both in a high pressure apparatus using SCO_2 and soxhlet extraction using hexane. The lipid compositions of oil showed a high amount of ω-3 fatty acids, especially EPA and DHA. The oil obtained by SCO_2 extraction was more stable than organic solvent extraction. Squid viscera also contained moderate amount of astaxanthin that was extracted in highest amount at 25 MPa and 45ºC. Therefore, squid viscera oil obtained by SCO_2 extraction would be a good source of astaxanthin with polyunsaturated fatty acids. SCO_2 extraction of oil was more efficient than organic solvent extraction in terms of oil quality and stability.

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