Fatty Acids and Protein Recovery of Squid Viscera with Supercritical Carbon Dioxide

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Abstract Supercritical carbon dioxide (SCO_2) extraction was investigated as a method for protein-sourcing material from squid viscera. To find the optimum conditions, the extraction of squid viscera using SCO_2 was performed under the conditions of temperature range from 35 to 45°C and constant pressure 25 MPa using Hewlett-Packard 7680T. Also from result of SDS-PAGE, the protein denaturation was minimized when using SCO_2 extraction. And the major amino acids in the squid viscera were glutamic acid, aspartic acid, lysine, leucine, arginine, alanine, glycine, isoleucine, and valine. The main fatty acids from squid viscera were myristic acid, palmitic acid, stearic acid, heneicosanoic acid, palmitoleic acid, elaidic acid, oleic acid, eicosenoic acid, EPA (eicosapentaenoic acid), and DHA (docosahexaenoic acid).

Key words : Squid viscera, supercritical carbon dioxide, SDS-PAGE, amino acids, fatty acids

Introduction

Squid is popular seafood that is used in various ways as part of the human diet. It is widely consumed in Southeast Asia and other parts of the world. In southern Europe squid is known as calamari. Not only is squid consumed fresh, but it is also processed into other forms of food in huge quantities [2]. Squid inhabit all the world's major oceans and seas. The viscera are usually discarded during processing, yet the by-product oil obtainable from this waste is high in polyunsaturated fatty acids (PUFAs), particularly the ω-3 fatty acid oils [15].

About 40% of squid in Korea are used as dried squid, seasoned squid, seasoned-frozen food, salted fish (jeotgal) and 60% are used as living body type. The viscera excluding the edible portion such as skin, trunk, fin, fish meat were removed as the non-edible portion in processing. This removal amounts were occupied over 20% and most of the non-edible portion are used as feed for animal, squid liver oil for feed [16].

The application of supercritical fluid extraction (SFE) to biomaterials has been recognized for several years [7-9,11,12,19,24]. Conventional methods for the extraction, fractionation and isolation of PUFAs include the use of highly flammable or even toxic solvents or energy-intensive vacuum distillation, as normal near atmospheric pressure high-temperature distillation can result in degradation of thermally labile compounds. Consideration of such factors has led investigators to apply SFE techniques to the separation [20].

In the supercritical state, the distinction between the liquid and the gas phase has disappeared and the fluid can no longer be liquefied by raising the pressure nor can gas be formed on increasing the temperature. Thus, the physicochemical properties of a given fluid, such as density, diffusivity, dielectric constant and viscosity can be easily controlled by changing the pressure or the temperature without ever crossing phase boundaries [23]. The main advantages of using supercritical fluids instead of conventional organic solvents are the minimal consumption of organic solvents, the exclusion of oxygen, and the reduction of heat. Modern SFE offers shorter extraction times, potentially higher selectivity and increased sample throughput (due to available automated instruments) compared to conventional solvent

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extraction techniques [27]. Several advantages are obtained when using carbon dioxide in SFE: selectivity, speed and efficiency, oxygen-free environment, minimal post-extraction manipulation, low operating temperature, and low toxicity [21]. SCO_2 extraction and fractionation of fish oils has been the subject of ongoing research, where a lot of information has been published on fundamental measurements of solubility and phase equilibria of polyunsaturated ω-3 fatty acid fish oil compounds in supercritical fluids [4,5,17,25,26].

Therefore, the purpose of this work was to obtain extraction data of squid viscera using SCO_2, determined at various conditions (from 35 to 45°C and at 25 MPa). At the optimal condition, also we determined protein denaturation by SDS-PAGE and found constitutive amino acids in the squid viscera. Another aim of this study was to identify the fatty acids in the oil and powder of squid viscera.

Materials and Methods

Materials

The squid viscera used in these experiments was produced from the East Sea in Korea. And used after vacuum freeze-dried (SFDSM 24L, SamWon Freezing Engineering Co.), crushed (Philips, HR1727) and sieved (710 μm, Chung gye sang gong SA). The samples were stored at -60°C in deep freezer (Samwon Freezing Engineering Co., SW-UF-200). Carbon dioxide with a purity of 99.9999% was supplied by Air Liquid (Australia). Also CO_2 (Liquid, Australia), required for cooling different zones in the SFE apparatus, was used as cryo gas. Also all other reagents are analytical grade and HPLC grade supplied by Sigma Co..

Supercritical carbon dioxide extraction

A HP 7680T supercritical fluid extractor (Hewlett-Packard, USA) was used for SFE. A schematic diagram for SFE was shown in Fig. 1. One gram squid viscera samples were filled into the 7 mL stainless steel extraction vessels. This thimble was plugged first with filter paper. Then it was filled with sample and again plugged with filter paper. The caps at each end contain porous frits to hold the sample in place and form high-pressure seals when the extraction chamber closes. The CO_2 was pumped and allowed to pass through the vessel with various oven temperatures at the constant pressure (35°C, 24.7 MPa; 40°C, 25.0 MPa; 45°C, 25.3 MPa). The flow rate of carbon dioxide was set to 3 mL/min. The 30 seconds equilibrium time and 40 min dynamic time with 3 min interval were selected. The modifier is not used in this work. The components extracted were collected on an octadecylsilane (ODS) (Hewlett-Packard) trap and were rinsed out to collection vials with 1.5 mL using *n*-hexane. The nozzle temperature was kept constant at 50°C and the trap temperature was kept at 45°C.

SDS-PAGE analysis

SDS-PAGE analysis was performed. The untreated and treated SCO_2 samples of squid viscera were used, respectively. Protein solutions were initially prepared by mixing 0.1 g of sample with 1 mL of distilled water. And centrifuged and take supernatant and mix 500 μL 1:1 (v:v) with SDS-PAGE disruption mix: this is 0.5 M Tris-HCl (pH 6.8) / 2-mercaptoethanol / 10% SDS / 50% glycerol, containing a little bromophenol blue. Then sample incubated for 5 min at 100°C and analyzed by reducing SDS-PAGE electrophoresis in 12% polyacrylamide gels. Electrophoresis was performed using a Mini-Protein III cell module (Bio-Rad Laboratories, CA, USA) at a constant voltage (100 V for 2 hr). The gels were stained with 0.1% Coomassie Brilliant blue R-250. The destaining was performed in methanol/acetic acid solution. SigmaMark™ Wide Molecular Weight Range, purchased from Sigma, was used as molecular weight standards from 6.5 to 205 kDa for SDS-PAGE electrophoresis.

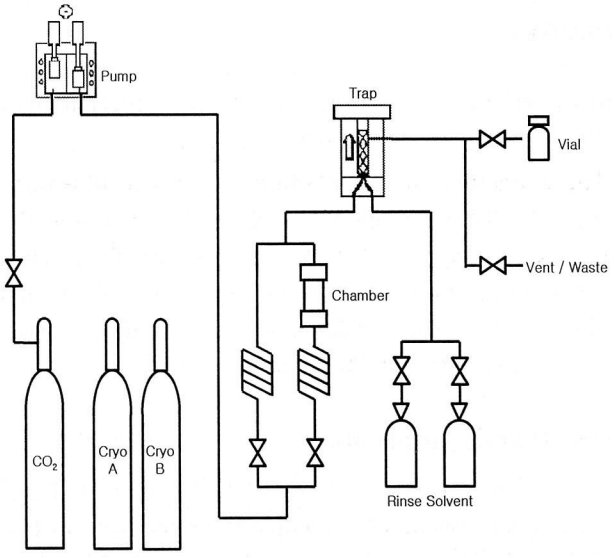

Fig. 1. A schematic diagram of SFE process.

Analysis of amino acids

The untreated and treated SCO_2 samples of squid viscera were used for their amino acids composition, respectively. 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. The amount of constitutive amino acids was found using a S433 amino acid analyser (Sykam, Gilching, Germany) under the following conditions: column size 4 mm i.d. × 150 mm, lithium form resin, analysis cycle time 160 min, reactor temperature 130°C, reactor size 15 m, flow rates 0.45 mL/min for buffer and 0.25 mL/min for ninhydrin.

Analysis of fatty acids

The samples (0.3 g) were methylated by the AOAC method (996.06 standard) [1], and the methyl esters of fatty acid compounds in the squid viscera were determined by gas chromatography-flame ionization detector (GC-FID) (HP5890II, USA). The column used was a DB-wax column (Agilent, 30 m × 0.25 mm i.d., 0.25 μm film thickness). The GC conditions were: the initial temperature of oven was 40°C, and programmed from 40 to 180°C at 10°C/min, and then to 260°C at 5°C/min, finally at 260°C for 5 min, injector temperature 250°C, injection volume was 1 μL, the split ratio was 100:1, total carrier gas (nitrogen) flow rate was 1.52 mL/sec. A lipid standard (fatty acid methyl ester mixture, Supelco 37 Component FAME Mix) was used to identify the fatty acids.

Results

Supercritical carbon dioxide extraction curve

The extraction curves of squid viscera are illustrated in Fig. 2. These curves are obtained by summing the extracted oil magnitude of the samples over time. They were acquired during extractions from 35 to 45°C and at the 25 MPa. The extracted amount of squid viscera was recorded the maximum at the 40°C.

SDS-PAGE analysis

The electrophoretic patterns of SCO_2 untreated and treated squid viscera were compared respectively in Fig. 3. Lane M shows the pattern of standard. Also lane a and b show the protein band of squid viscera. A main single band of squid viscera was estimated to be approximately 29 kDa and shows no change in intensity after treatment with SCO_2.

Analysis of amino acids

The results of constitutive amino acids were listed in Table 1. Among the 15 amino acids of squid viscera, eight essential (arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, and valine) and seven

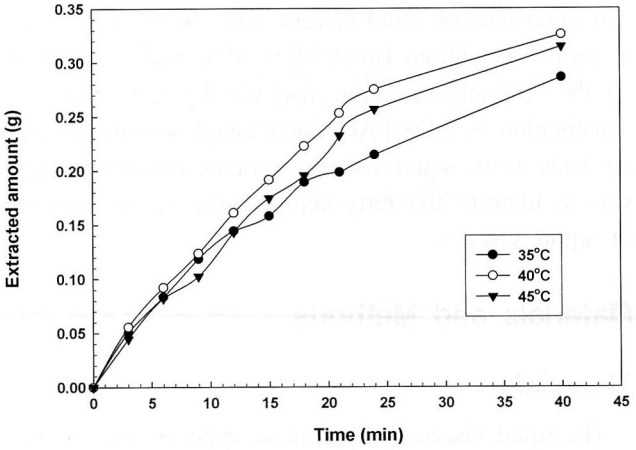

Fig. 2. Effect of temperatures on the amount of extracted oil from squid viscera (pressure = 25 MPa).

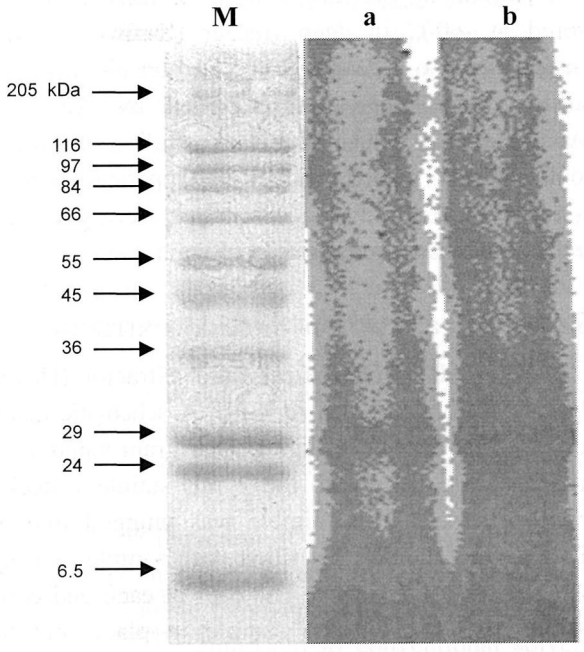

Fig. 3. SDS-PAGE pattern of squid viscera untreated and treated SCO_2 (M; Molecular weight standard, a; untreated squid viscera, b; treated squid viscera).

Table 1. Amino acid composition of squid viscera untreated and treated SCO_2

	Untreated	Treated
Essential amino acids	19.81	29.86
Arginine	2.90	4.60
Histidine	1.25	1.93
Isoleucine	2.36	4.12
Leucine	3.35	4.70
Lysine	3.43	4.63
Phenylalanine	2.14	3.96
Threonine	2.06	2.86
Valine	2.32	3.06
Non-essential amino acids	21.06	30.57
Alanine	2.64	3.68
Aspartic acid	4.96	8.14
Glycine	2.55	3.26
Glutamic acid	5.82	8.52
Proline	1.93	2.41
Serine	2.06	2.88
Tyrosine	1.10	1.68
Total	40.87	60.43

non-essential (alanine, aspartic acid, glycine, glutamic acid, proline, serine, and tyrosine) amino acids were determined. Glutamic acid was the highest contained component in squid viscera. The following main contents were aspartic acid, lysine, leucine, arginine, alanine, glycine, isoleucine, and valine. Essential amino acids contain 19.81% in constitutive amino acids of squid viscera. After SCO_2 extraction, essential amino acids composition increased to 29.86%. Moreover constitutive amino acids amounts of squid viscera were increased from 40.87% to 60.43%.

Analysis of fatty acids

The fatty acid composition of squid viscera was presented in Table 2. This experiment was performed at 40°C and 25.0 MPa as the optimal condition from the extraction data. The total contents of fatty acids are 477.86 mg/g in untreated squid viscera, 414.03 mg/g in SCO_2 treated powder, 1061.72 mg/g in SCO_2 extracted oil, respectively. From these results, we obtained the oil contains a plenty of PUFAs. The contents of saturated fatty acid like palmitic acid remained highly compared to unsaturated fatty acid such as EPA, DHA in SCO_2 treated sample. In the contrast, the contents of PUFAs are highly contained in the oil after SCO_2 extraction. The main fatty acids from squid viscera were myristic acid, palmitic acid, stearic acid, heneicosanoic acid, palmitoleic acid, elaidic acid, oleic acid, eicosenoic acid, EPA and DHA.

The major fatty acids classes were PUFAs, monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs). In this work, total SFAs are declined from 33.80% to 22.79% in extracted oil after SCO_2 treatment. But the valuable unsaturated fatty acids (MUFAs, PUFAs) are increased from 22.79% to 24.57% and from 43.41% to 52.63%, respectively.

Discussion

From the extraction data, we suggested that 40°C temperature condition of squid viscera is optimal at 25 MPa. And from the SDS-PAGE data, there is no change in intensity after SCO_2 extraction. Also due to no use of a high temperature heating in SCO_2 extraction technology, it seems to be denaturation of protein was minimized. Therefore, we extracted using SCO_2 from throwing-away squid viscera, the protein was minimized denaturation.

The major amino acids were glutamic acid, aspartic acid, lysine, leucine, arginine, alanine, glycine, isoleucine, and valine. These results were in accordance with reported total amino acids of squid *Loligo vulgaris* [28]. In particular, glutamic acid and glycine were recognized commonly to be taste-active in seafood [10]. Also aspartic acid, glycine and glutamic acid are known to play an important role in the process of wound healing [6]. Especially, lysine which is easy to lack for Korean who is eating cereals as a staple food, was contained in squid viscera [18]. Moreover amino acids are increased after SCO_2 extraction.

The main fatty acids from squid viscera in this study, the result was in accordance with reported fatty acids of squid oil [13,22]. In general, when human continuously takes an excess of SFAs, SFA content in blood will be increase and the cholesterol content will be high. As this result, it comes as cardiovascular disease such as atherosclerosis and hypertension [3]. 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 [14,22].

Table 2. Fatty acid composition of the squid viscera untreated and treated with SCO_2

Compounds	R.T. (min)	Weight % (Weight mg/g sample)		
		Untreated	Powder	Extracted oil
Butyric acid ($C_{4:0}$)	3.55	ND[7]	ND	ND
Capric acid ($C_{10:0}$)	9.03	1.31 (0.27)	7.32 (1.77)	ND
Undecanoic acid ($C_{11:0}$)	9.83	ND	5.45 (1.32)	ND
Tridecanoic acid ($C_{13:0}$)	11.62	ND	8.46 (2.04)	ND
Myristic acid ($C_{14:0}$)	12.84	17.03 (3.56)	9.36 (2.26)	36.66 (3.45)
Pentadecanoic acid ($C_{15:0}$)	14.21	2.91 (0.61)	ND	6.31 (0.59)
Palmitic acid ($C_{16:0}$)	15.83	98.66 (20.65)	95.48 (23.06)	118.15 (11.13)
Stearic acid ($C_{18:0}$)	19.75	23.07 (4.83)	29.83 (7.20)	40.42 (3.81)
Arachidic acid ($C_{20:0}$)	23.89	ND	ND	3.09 (0.29)
Heneicosanoic acid ($C_{21:0}$)	26.39	13.76 (2.88)	14.81 (3.58)	24.54 (2.31)
Behenic acid ($C_{22:0}$)	27.67	ND	ND	ND
Tricosanoic acid ($C_{23:0}$)	29.40	ND	ND	2.94 (0.28)
Lignoceric acid ($C_{24:0}$)	31.20	4.79 (1.00)	ND	9.82 (0.93)
Total SFAs		161.52 (33.80)	170.71 (41.23)	241.93 (22.79)
Myristoleic acid ($C_{14:1}$; *cis*-9)	13.29	ND	ND	1.76 (0.17)
Palmitoleic acid ($C_{16:1}$; *cis*-9)	16.34	18.40 (3.85)	8.76 (2.11)	45.23 (4.26)
Heptadecenoic acid ($C_{17:1}$; *cis*-10)	18.12	ND	ND	4.35 (0.41)
$C_{18:1}$; *trans*-9[1] , $C_{18:1}$; *cis*-9[2]	20.25	61.13 (12.79)	41.66 (10.06)	146.87 (13.83)
Eicosenoic acid ($C_{20:1}$; *cis*-11)	24.44	26.57 (5.56)	31.58 (7.63)	56.68 (5.34)
Erucic acid ($C_{22:1}$; *cis*-13)	28.02	2.80 (0.59)	12.21 (2.95)	5.94 (0.56)
Total MUFAs		108.90 (22.79)	94.21 (22.76)	260.83 (24.57)
$C_{18:2}$; *trans*-9,12[3] , $C_{18:2}$; *cis*-9,12[4]	21.28	5.33 (1.12)	ND	13.82 (1.30)
γ-Linolenic acid ($C_{18:3}$; *cis*-6,9,12)	21.83	ND	ND	1.85 (0.17)
Linolenic acid ($C_{18:3}$; *cis*-9,12,15)	22.55	ND	ND	7.12 (0.67)
Eicosadienoic acid ($C_{20:2}$; *cis*-11,14)	25.40	3.76 (0.79)	ND	7.80 (0.73)
Arachidonic acid ($C_{20:4}$; *cis*-5,8,11,14)	26.54	ND	ND	3.53 (0.33)
EPA ($C_{20:5}$; *cis*-5,8,11,14,17)[5]	27.64	71.81 (15.03)	58.49 (14.13)	218.96 (20.62)
Docosadienoic acid ($C_{22:2}$; *cis*-13,16)	28.95	ND	ND	ND
DHA ($C_{22:6}$; *cis*-4,7,10,13,16,19)[6]	31.77	126.53 (26.48)	90.62 (21.89)	305.88 (28.81)
Total PUFAs		207.44 (43.41)	149.11 (36.01)	558.96 (52.63)
Total		477.86 (100.00)	414.03 (100.00)	1061.72 (100.00)

[1]$C_{18:1}$; *trans*-9: elaidic acid, [2]$C_{18:1}$; *cis*-9: oleic acid, [3]$C_{18:2}$; *trans*-9,12: linolelaidic acid, [4]$C_{18:2}$; *cis*-9,12: linoleic acid, [5]EPA: eicosapentaenoic acid, [6]DHA: docosahexaenoic acid, [7]ND: not detected

Conclusion

SCO_2 extraction was performed at the temperature from 35 to 45°C and the constant pressure 25 MPa. The maximum extraction yields recorded at 40°C in squid viscera at 25 MPa. We obtained that protein denaturation was minimized using SCO_2 extraction from results of electrophoresis and amino acids analytical data. Also we suggested that SCO_2 extracted oil was more contained PUFAs such as EPA and DHA than SCO_2

untreated sample. Thus we can get the squid viscera that not denaturated protein without disintegrating the functional compounds using SCO_2 extraction. Therefore SFE technology is useful method to manufacturing useful materials using fish by-products and seems to be a useful processing technique for changing the composition of lipids in order to obtain high contents of the functional products. Furthermore from the viewpoint of protein-sourced materials, SCO_2 extraction would be able to substitute for the conventional solvent extraction. Therefore SFE technology provides more chance for the marine industry as functional seafood.

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References

1. AOAC. 2000. Official Methods of Analysis of AOAC INTERNATIONAL: AOAC Official Method 996.06. 17th ed., AOAC International, Gaithersburg, MD, USA.
2. Bandarra, M. M., Nunes, M. L. , Campos, R. M. and Batista, I. 1992. Variação Sazonal dos Lípidos de Sardinha sardina pilchardus, Relatorio Técnico e Cientifico.
3. Budowski, P. 1981. Review: Nutritional effects of ω-3 polyunsaturated fatty acids. *Irish J. Med. Sci.* **17**, 223-231.
4. Chun, B. S., Kang, K. Y., Ahn, D. H. and Wilkilnson, G. T. 2005. Extraction of liquids and cholesterol from squid oil with supercritical carbon dioxide. *Kor. J. Chem. Eng.* **22**, 399-405.
5. Chun, B. S., Kim, Y. W., Song, S. K., Kim, S. K. and Wilkinson, G. T. 1998. Extraction and fractionation of lipids from squid internal organs using supercritical carbon dioxide with entrainer. Proceedings of 5th ISASF Meeting. March 23-25. Nice, France.
6. Chyun, J. H. and Griminger, P. 1984. Improvement of nitrogen retention by arginine and glycine supplementation and its relation to collagen synthesis in traumatized mature and ageing rats. *J. Nutr.* **114**, 1705-1715.
7. Coenen, H. and Kriegel, E. 1984. Applications of supercritical gas extraction processes in the food industry. *Ger. Chem. Eng.* **7**, 335-344.
8. Dziezak, J. D. 1986. Innovative separation process finding its way into the food industry. *Food Technol.* **40**, 66-69.
9. Eisenbach, W., Gottsch, P. J., Niemann, K. and Zosel, K. 1983. Extraction with supercritical gases: the first twenty years. *Fluid Phase Equilib.* **10**, 315.
10. Fuke, S. and Konosu, S. 1991. Taste-active components in some foods: a review of Japanese research. *Physiol. Behav.* **49**, 863-868.
11. Grimmett, C. 1982. The use of liquid carbon dioxide for extracting natural products. *Chem. Ind.* **15**, 359-362.
12. Hong, I. K., Rho, S. W., Lee, K. S., Lee, W. H. and Yoo, K. P. 1990. Modeling of soybean oil bed extraction with supercritical carbon dioxide. *Kor. J. Chem. Eng.* **7**, 40-46.
13. Kang, K. Y., Ahn, D. H., Gordon T. Wilkinson and Chun, B. S. 2005. Extraction of lipids and cholesterol from squid oil with supercritical carbon dioxide. *Kor. J. Chem. Eng.* **22**, 399-405.
14. Kang, K. Y., Ahn, D. H., Jung, S. M., Kim D. H. and Chun, B. S. 2005. Separation of protein and fatty acids from tuna viscera using supercritical carbon dioxide. *Biotechnol. Bioproc. Eng.* **10**, 315-321.
15. Kang, K. Y., Ahn, D. H., Wilkinson, G. T. and Chun, B. S. 2005. Extraction of lipids and cholesterol from squid oil with supercritical carbon dioxide. *Kor. J. Chem. Eng.* **22**, 399-405.
16. Kang, S. S., Kim, B. J. and Chun, B. S. 1999. Recovery of high unsaturated fatty acid from squid processing wastes using supercritical carbon dioxide extraction method. *J. Kor. Fish. Soc.* **32,** 217-222.
17. Kim, H. S., Lee, S. Y., Kim, B. Y., Lee, E. K., Ryu J. H. and Lim, G. B. 2004. Effects of modifiers on the supercritical CO_2 extraction of glycyrrhizin from licorice and the morphology of licorice tissue after extraction. *Biotechnol. Bioproc. Eng.* **9**, 447-453.
18. Kim, J. S., Yang, S. K. and Heu, M. S. 2000. Food component characteristics of plain dried anchovies on the market. *J. Kor. Soc. Food Sci. Nutr.* **29**, 20-25.
19. Mansoori, G. A., Schultz, K. and Martinelli, E. E. 1988. Bioseparation using supercritical fluid extraction/retrograde condensation. *Bio-Technology* **6**, 393-396.
20. McHugh, M. A. and Krukonis, V. J. 1986. Supercritical fluid extraction principles and practice, Butterworth-Heinemann.
21. Raynie, D. E. 1997. Meeting the natural products challenge with supercritical fluids. In Supercritical Fluids Extraction and Pollution Prevention, M. A. Abraham and A. K. Sunol (Eds.). ACS SYMPOSIUM SERIES 670, ACS Washington DC, U.S.A., 68-75.
22. Roh, H. S., Park, J. Y., Park, S. Y. and Chun, B. S. 2006. Isolation of off-flavor and odors from tuna fish oil using supercritical carbon dioxide. *Biotechnol. Bioproc. Eng.* **11**, 496-502.
23. Sihvonen, M., Järvenpää, E., Hietaniemi, V. and Huopalahti, R. 1999. Advances in supercritical carbon dioxide technologies. *Trends Food Sci. Technol.* **10**, 217-222.
24. Stahl, E., Quirin, K. W. and Gerard, D. 1988. Dense gases for extraction and refining, Chap. IV. Springer-Verlag, New York.
25. Taylor, D. L. and Larick, D. K. 1995. Investigations into the effect of supercritical carbon dioxide extraction on the fatty acid and volatile profiles of cooked chicken. *J. Agric. Food Chem.* **43**, 2369-2374.
26. Timon, M. L., Ventanas, J., Martin, L., Tejeda, J. F. and

Garcia, C. 1998. Volatile compounds in supercritical carbon dioxide extracts of Iberian ham. *J. Agric. Food Chem.* **46**, 5143-5150.
27. Turner, C., King, J. W. and Mathiasson, L. 2001. Supercritical fluid extraction and chromatography for fat-soluble vitamin analysis. *J. Chromatogr. A* **936**, 215-237.
28. Villanueva, R., Riba, J., Ruíz-Capillas, C., González A. V. and Baeta, M. 2004. Amino acid composition of early stages of cephalopods and effect of amino acid dietary treatments on Octopus *vulgaris paralarvae*. *Aquaculture* **242**, 455–478.