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Extraction of Phospholipids from Salmon Roe with Supercritical Carbon Dioxide and an Entrainer

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Abstract: Supercritical carbon dioxide (SC-CO_2) is a suitable substance to extract non-polar substances (triacylglycerols). However it has not proven effective in the extraction of polar substances. The efficient use of SC-CO_2 and ethanol mixture to extract and fractionate phospholipids from salmon fish roe was therefore investigated. Extraction was performed at low pressure and temperature (17.7 MPa and 33℃) to avoid oxidation of polyunsaturated fatty acids. Phospholipids were not found to be extracted with 0- and 5%-ethanol in SC-CO_2. However extractions with 10, 15 or 20%-ethanol in SC-CO_2 were effective in extracting phospholipids. The amount of extracted phospholipids increased with increased addition of ethanol. When the extraction was performed with SC-CO_2 and 20%-ethanol mixture, more than 80% of the phospholipids were recovered.

Key words: supercritical carbon dioxide extraction, salmon roe, triacylglycerol, phospholipid, entrainer, ethanol

1 Introduction

Supercritical carbon dioxide (SC-CO_2) fluid extraction is applied in the commercial production of flavoring cosmetics, pharmaceuticals and food products. Some examples are decaffeinating coffee (1), hop extraction (2), extraction of turmeric essential oils (3), and ginger flavoring (4). In the oleo-industry, a numerous of researchers have done oil-extraction from seeds and refined plant oils with SC-CO_2 (5-11). There are several advantages in using SC-CO_2 in industrial production. CO_2 has several desirable properties, such as, it is non-corrosive, non-toxic, non-flammable and non-explosive. Because CO_2 is stable chemically, it does not react with other materials in treatment. Easy separation and removal of CO_2 from the products eliminates any problem related to toxic residual solvents. An added bonus is, it is inexpensive and readily available. A low critical temperature and pressure (Tc=31.1℃, Pc=7.4 MPa) can be utilized to establish an energy saving process.

A great deal of research has been focused on the intake of polyunsaturated fatty acids (PUFA), especially n-3 PUFA, as they have been seen to showing them to play a beneficial role in the prevention of cardiovascular diseases (12), hypertriglyceridemia (13) and autoimmune diseases (14), etc.

Some works refer to the application of SC-CO_2 extraction of marine materials to obtain PUFA. Yamaguchi *et al.* (15) reported on the extraction of lipids from Antarctic krill. According to their report, only non-polar components such as cholesterol, carotenoid triacylglycerols and their derivatives were extracted. Phospholipids did not appear in the extracted fractions.

Cheung *et al.* (16) tried extracting lipids from brown seaweed. They reported that the extracting conditions affected the fatty acid profiles, that is, the concentration of total PUFAs increased reaching a higher value than

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that obtained by solvent extraction.

In our previous report (17) we extracted lipids from freeze-dried salmon roe to obtain an information on the effects of extracting conditions (pressure 9.8-31.4 MPa, temperature 40-80℃) and the behaviors of lipids in SC-CO_2. Triacylglycerols (TGs) were not extracted completely. Phospholipids (PLs) were not extracted with SC-CO_2 at all.

SC-CO_2 does not provide a means to dissolve PLs effectively, but recovery of PLs can be achieved by the addition of a polar entrainer to SC-CO_2. The presence of an entrainer enhances the solubility of SC-CO_2. The choice of a suitable entrainer must be based on thermodynamic considerations and with regard to food safety, that is, it should be "Generally recognized as safe" (GRAS)(18). Prosise (19) noted that ethanol was an excellent solvent for isolating PLs for food use. Some researchers have already studied its role as an entrainer to extract PLs in SC-CO_2. Temelli (20) extracted PLs from canola flakes and presscake with SC-CO_2 and ethanol. Dunford *et al.* (21) reported a positive effect of ethanol on the extraction of PLs from canola meal. Montanari *et al.* (22) observed the extraction of PLs from soybean flakes with SC-CO_2 and 10 wt% ethanol. They reported phosphatidylcholine (PC) enrichment at low pressures although the total yields increased with increasing pressure. Teberikler *et al.* (23) used SC-CO_2 to produce a 95% purified PC from soybean lecithin containing a low percentage of PC.

The authors report herein, the extraction of Docosahexaenoic acid (DHA)-rich PLs from freeze-dried salmon roe with SC-CO_2 and ethanol as the entrainer.

2 Experimental

2·1 Materials

Frozen salmon roe was obtained from Nippon Kaken (Tokyo, Japan) and stored at -20℃ before use. It was thawed and freeze-dried. In this report freeze-dried salmon roe powder is referred to as the FD-sample. The lipid extracted from the FD-sample by Folch's method (24) is defined as the total lipid (TL). It contains TGs, PLs and their derivatives (diacylglycerols, monoacylglycerols and lysophospholipids, etc.).

2·2 SC-CO_2 Extraction

The extraction vessel used in this work was of 10.0-mm interior diameter and 129 mm length (model EV-4, JASCO, Hachioji, Japan) with a volume of 10 mL. The equipment used for the work consisted of a high-pressure liquid chromatograph system (pump, JASCO PU-1586, column oven, JASCO 865-CO) and back pressure regulator (JASCO 880-81).

4.0 g of the FD-sample was applied in the vessel. Extraction trials were performed at 33℃ and 17.7 MPa. The extracted lipid was collected several times during extraction. CO_2 and ethanol were delivered by two separate pumps, mixed and passed through a preheating coil.

2·3 Analysis

In this work, the lipids extracted with SC-CO_2 are referred to as the extracted lipids (EL). After the SC-CO_2 extraction, the lipids retained in the spent FD-sample were extracted by Folch's method. This is referred to as the residual lipids (RL), in this work. The lipids content of EL, RL and TL were analyzed by means of silica gel thin layer chromatography (TLC, plate 5721, Merck, Darmstadt, Germany) with hexane-diethyl ether-acetic acid (80:20:1 v/v/v) or chloroform-methanol-water (65:25:4 v/v/v).

The extracting yield is referred to as the ratio of the weight of EL to the weight of TL.

TL and RL (150 mg) were run through the column (20 mm i.d. × 200 mm height) with silica gel 60 (mesh 70-230, Merck) to fractionate the TGs and PLs. The TGs and PLs were eluted with 300 mL of chloroform and 200 mL of methanol, respectively. The TGs and PLs fractionated from TL are referred to as the original TGs and the original PLs.

The fatty acid profiles were analyzed by gas chromatography of the methyl esters prepared by transmethylation with BF_3/methanol. An Agilent 6890A series gas chromatograph (Yokogawa Analytical Systems, Musashino, Japan) equipped with a flame ionization detector (FID) and DB-WAX capillary column (30 M × 0.25 mm i.d.) (J & W Scientific, Folsom, CA) was used. The column temperature was raised from 150 to 210℃ at 5℃/min. Both the injector and detector temperatures were 250℃. The carrier gas was helium with hydrogen and air supplied to the FID. The fatty acids were identified by comparison of the retention times with lipid standards (Sigma, Saint Louis, MA).

PLs analyses were performed by high pressured liquid chromatography (HPLC). A LC Model I HPLC system (Toso, Tokyo, Japan) equipped with DEVELOSIL

model 60-5 HPLC-column (259 mm × 4.6 mmi.d.) (Nomura-chemicals, Tokyo, Japan) was used. The mobile phase was acetonitrile/methanol/phosphoric acid (780:50:9, v/v/v). All solvents were HPLC grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The flow of mobile phase was 1.5 ml/min. The column oven temperature was 45℃. Detective absorbance was at 220 nm. Zephiramine (Wako Pure Chemical Industries, Ltd.,) was used as the inner standard to analyze quantitatively.

3 Results and Discussion

3·1 Effect of Extracting Temperature on the Lipid Yield at 17.7 MPa

The authors investigated the most suitable conditions to extract lipids from salmon roe with SC-CO_2. The results in our previous report (17) suggested that the extraction at higher pressure gained a higher extraction yield. Extractions performed at 50 MPa and 40, 60 and 80℃ gave high extraction yields; 44.18±0.21, 46.71±0.83, and 51.03±0.71% (average±SD), respectively. The extraction yields were significantly higher than those found at 31.4 MPa and 40-80℃, mentioned in the previous report. No significant amount of PLs was extracted at these conditions.

The authors further investigated improved the extraction conditions from the following standpoints. The authors wanted to avoid PUFAs such as eicosapentaenoic acid (EPA; C20:5, n-3) and docosahexaenoic acid (DHA; C22:6, n-3) being oxidized through being exposed to high temperatures during extraction. Extraction at higher pressure also increases the risks of accidents in the handling of equipment. The authors furthermore tried to establish an energy saving protocol. Extraction at higher pressures and higher temperatures consume a great deal of energy to produce and maintain them.

Since the authors had already shown that reducing the temperature lead to an increase in the extraction yield at 17.7 MPa (17), to extract lipids from salmon roe FD at lower temperatures than 40℃ suggested a higher extraction yield. The extraction yield at 33℃ was significantly higher than that at 40℃ ($p<0.05$, **Fig. 1**). This was comparable to that at 50 MPa and 60℃. No PLs were extracted under these conditions. The authors investigated the behavior of lipids in SC-CO_2 at 17.7 MPa and 33℃.

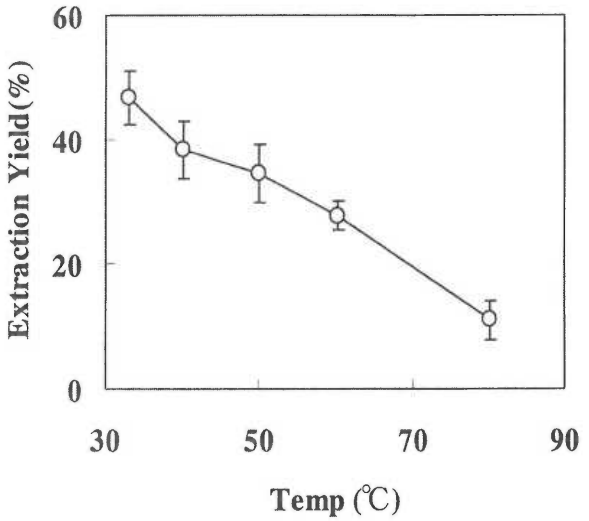

Fig. 1 Effect of Extraction Temperature on Extraction Yield.
SC-CO_2 flow was 3 mL/min, Extraction time was 10 hours.
The extraction pressure was 17.7 MPa.
Each result represents the mean ± S.D.

Many researchers have already reported since a pure carbon dioxide does not dissolve PLs effectively, extraction of PLs might be achieved by the addition of a polar entrainer to SC-CO_2. An entrainer is a substance of medium volatility added to a mixture of compressed gas and a low volatility substance (20). As the solubility in SC-CO_2 at the same extracting conditions (temperature and pressure) is drastically enhanced, extraction can be conducted at a lower pressure (25). The logical choice for a co-solvent in the food industry would be ethanol. The authors used ethanol as the entrainer to extract PLs in SC-CO_2 because: (i) It is suitable for food use; and (ii) the phase behavior of CO_2/ethanol mixes at high pressure is available (26, 27).

CO_2 and ethanol were mixed and passed through the preheating coil, and delivered to the vessel in the oven to extract the lipids. Extractions of PLs from canola, soybean and cottonseed with SC-CO_2/etanol mixture have been reported (21). In our study ethanol was used as the entrainer to extract PLs from salmon roe.

3·2 Effect of Ethanol on the Lipid Extraction

The extraction was performed at 17.7 MPa and 33℃ with 5, 10, 15 or 20%-ethanol in SC-CO_2. The ethanol

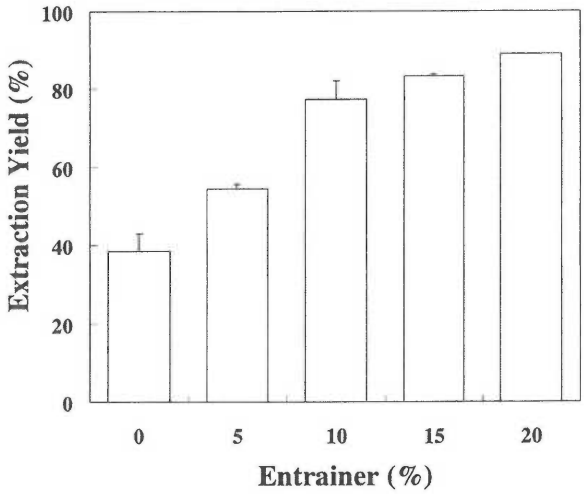

Fig. 2 Effect of Entrainer on Extraction Yield.
The extraction conditions were 17.7 MPa, 33℃.
Each result represents the mean ± S.D.

flows were 5, 10, 15 or 20% of CO_2 flow by volume (CO_2 flow = 3.0 ml/min, Ethanol flow = 0.15, 0.30, 0.45 or 0.60 ml/min). The effect of the entrainer on the extraction yield is shown in **Fig. 2**. The extraction yield increased with increase in the ethanol percentage. 39% of the total lipids were extracted without the entrainer after 4 hours. When the extraction was performed with SC-CO_2 and 5%-ethanol mixture, the extraction yield rose above 50%, while an addition of 10-, 15-, or 20%-ethanol in SC-CO_2 achieved an extraction yield of more than 75%.

The lipid compositions in all the fractions were observed using TLC. No PLs were observed in the EL fractions when extraction was performed with SC-CO_2 and 5%-ethanol mixture. When extraction was performed with SC-CO_2 and 10- or 15%-ethanol mixture, PLs were observed in the EL fractions. In all the extracted fractions both TGs and PLs were observed after a 4-hour extraction, and present in the RL fraction. When extraction was performed with SC-CO_2 and 20%-ethanol mixture within an initial 30 min extraction almost all the TGs were extracted. In the following fractions (1 h-4 h) and RL fraction, slight TGs and some amount of PLs were observed.

The PL concentrations in all the fractions were quantified by HPLC. The results are shown in **Table 1**. Extraction with SC-CO_2 and 20%-ethanol mixture produced fractions containing PLs without TGs. The rate of extracted PLs to the original PLs are indicated as the PL recovery rate. The rate of extracted PLs with SC-CO_2 and ethanol mixtures increased with increase in the ethanol amount in SC-CO_2 (**Fig. 3**). When extraction was performed with SC-CO_2 and 10%-ethanol mixture 30% of the PLs were recovered in the EL fractions. When the ethanol percentage was increased up to 20% more than 80% of the PLs were recovered in the EL fractions. The rate of extracted TGs to the original TGs are indicated as the TG recovery rate. When extraction was performed with SC-CO_2 and 5%-ethanol mixture, nearly 80% of the TGs were recovered during the four-hour extraction. On the other hand, 20% of the TGs were not extracted with SC-CO_2 and remained present in the RL fraction. When the lipids were extracted with SC-CO_2 and 10-, 15-, or 20%-ethanol mixtures more than 90% of the TGs were recovered to the EL fraction (**Fig. 4**).

3·3 Effect of Ethanol on Fatty Acid Profiles of Lipids

The fatty acid profiles of the TGs in all the fractions were analyzed. The concentrations of oleic acid (OA; C18:1, n-9) and DHA of the EL are shown in **Figs. 5** and **6**. The addition of the entrainer and extracting peri-

Table 1 Phospholipid Content (wt%) in the Extracted Lipid Fractions.

Ethanol (%)	Extraction time (h)				
	0.5	1	2	3	4
10	2.44±2.96	8.30±1.41	12.55±3.10	10.65±5.61	14.26±7.36
15	8.80±4.21	52.61±8.43	67.37±6.71	64.13±7.70	39.31±4.25
20	7.88±4.25	94.70±8.45	84.90±8.41	92.99±4.02	97.20±1.33

Phospholipid content was analyzed by HPLC.
Each result represents the mean ± S.D.

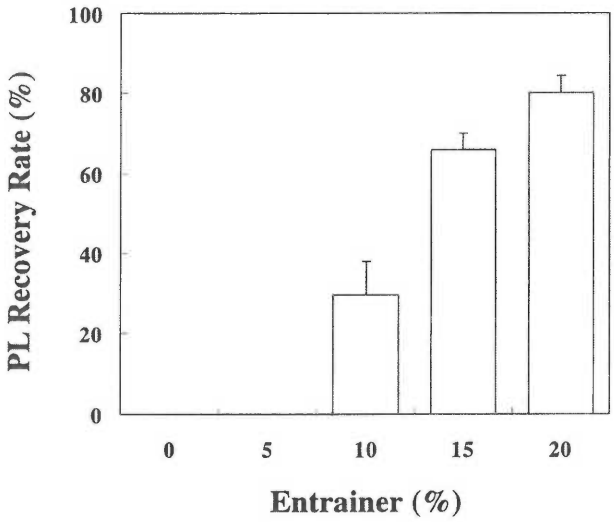

Fig. 3 Effect of Entrainer on Phospholipids Recovery Rate.
The extraction conditions were 17.7 MPa, 33℃.
Each result represents the mean ± S.D.

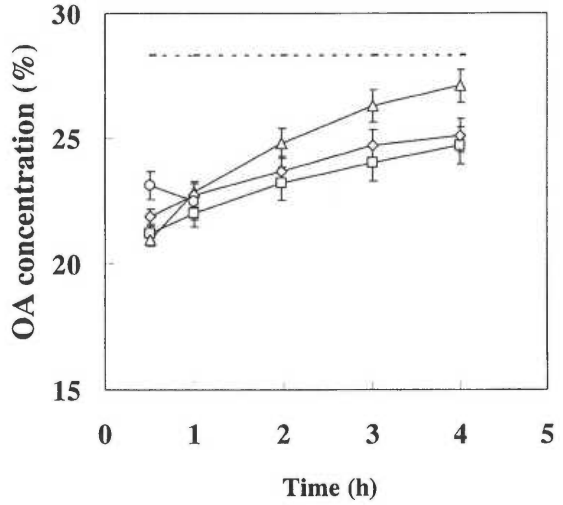

Fig. 5 Effect of Entrainer on Extracted Oleic Acid Concentration in TGs.
The extraction conditions were 17.7 MPa, 33℃.
Entrainer flows were 5 (□), 10 (△), 15 (◇) and 20 (○)%.
------- designates the oleic acid concentration in the original TG.
Each result represents the mean ± S.D.

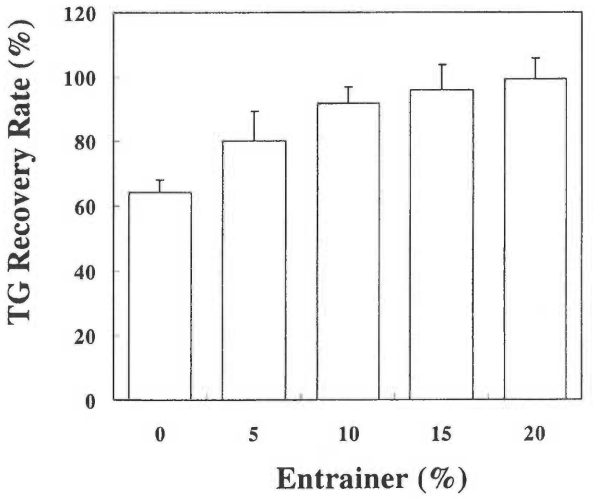

Fig. 4 Effect of Entrainer on Triacylglycerols Recovery Rate.
The extraction conditions were 17.7 MPa, 33℃.
Each result represents the mean ± S.D.

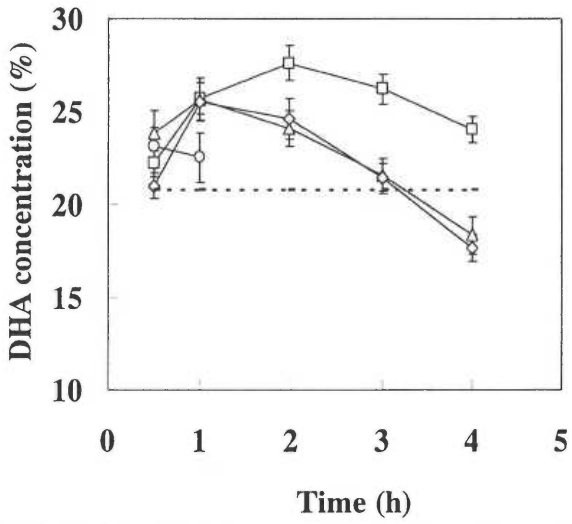

Fig. 6 Effect of Entrainer on Extracted Docosahexaenoic Acid Concentration in TGs.
The extraction conditions were 17.7 MPa, 33℃.
Entrainer flows were 5 (□), 10 (△), 15 (◇) and 20 (○)%.
------- designates the oleic acid concentration in the original TG.
Each result represents the mean ± S.D.

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