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Supercritical Carbon Dioxide Extraction of Oils from Antarctic Krill

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Supercritical carbon dioxide extraction of the Antarctic krill yielded oils that were composed solely of nonpolar lipids, largely triglycerides, without phospholipids. The extracted oils were fluid and of red color due to astaxanthin, which tended to be decomposed at temperatures higher than 60 °C and a fixed pressure of 250 kg/cm^2. Analyses of the fatty acids showed a comparatively high proportion of eicosapentaenoic acid (11%). These results indicate that supercritical carbon dioxide is effective in obtaining nonpolar lipids from the krill by only one-step extraction and in excluding phospholipids that interfere with the utilization of krill oils.

Organic solvent extraction of oils from raw materials is a well-developed technology. However, after the extraction process, further purification steps are generally required to remove impurities and gum-forming compounds from the extracted oil, especially in foodstuffs intended for human use.

In recent years supercritical fluid extraction has received much attention, though its fundamental principles were known over 100 years ago (Hannay and Hogarth, 1879). The theory and practice of the supercritical fluid extraction process have been reviewed by Paul and Wise (1971), who predicted its application to foods, pharmaceuticals, fine chemicals, petrochemicals, mineral extraction, and fuel- and waste-processing technologies. Various kinds of supercritical fluids have been studied (Wilke, 1978), but most work done so far has used carbon dioxide as the extractant. Carbon dioxide has the advantages of nontoxicity, incombustibility, low critical temperature (31 °C) and pressure (75 kg/cm^2), and low price, all of which meet the recent energy and health concerns.

The application of supercritical carbon dioxide (SC-CO_2) extraction to foods has had limited success, as exemplified by decaffeination of green coffee beans in large-scale industrial plants (Zosel, 1974) and production of hop extract (Hubert and Vitzthum, 1978). Further, SC-CO_2 has been used to extract oils from soybean (Friedrich and List, 1982), coconut palm (Brannolte et al., 1983), butter (Kaufmann et al., 1983), etc. Applications of SC-CO_2 extraction to animal sources, in particular seafoods, are still more limited.

Lipids of aquatic organisms are generally rich in highly unsaturated fatty acids and phospholipids, which are readily deteriorated. The Antarctic krill, *Euphausia superba*, possesses an especially high proportion of phospholipids (Mori and Hikichi, 1976), which hampers the effective utilization of krill oils. We applied SC-CO_2 extraction to krill samples and proved that the extracted oils were composed solely of nonpolar lipids without contamination by phospholipids and their deteriorated lipids.

The present paper deals with the extraction and characterization of the extracted oils and residual lipids.

Materials and Methods Commercial preparations by Nippon Suisan Kaisha, Ltd., of frozen Antarctic krill and its meal were sampled. A freeze-dried sample was prepared by lyophilization of frozen krill. The samples were kept below −25 °C until used. Standard commercial preparations of lipid reagent were used without further purification.

Proximate composition of the samples was analyzed according to the AOAC procedures (No. 7.003, 7.009, 7.015, and 7.060; 1984).

The extraction with SC-CO_2 was carried out using a test plant instrument manufactured by Mitsubishi Kakoki, Co., Ltd. This equipment has a 75-mL extraction vessel with upper limits of pressure and temperature of 500 kg/cm^2 and 100 °C, respectively.

Ground freeze-dried krill (20 g) and krill meal (25 g) were put into the extraction vessel, gaseous CO_2 of which pressure was increased to a supercritical state by a pressure pump was introduced, and extraction was carried out at different pressures and temperatures. Gaseous CO_2 of commercial purity (Iwatani & Co., Ltd.) was used. Average flow rate was 0.6 kg/h. The extracted oils were measured gravimetrically.

The fractionation of the oils (150 mg) was carried out on a column (1 × 8.5 cm) using silica gel (Wakogel C-200, 100–200 mesh) and 50 mL of chloroform and then 50 mL of methanol. The eluted oils were measured gravimetrically after evaporation of the solvent.

The method of Bligh and Dyer (1959) was applied to the extraction of whole lipids from the krill samples and the residues after SC-CO_2 extraction. After evaporation of the solvent, the oils and lipids were measured gravimetrically.

The oils and lipids extracted with SC-CO_2 and by the method of Bligh and Dyer (1959) were analyzed by TLC on silica gel 60F_{254} (Merk, 0.25 mm thick) with petroleum ether–diethyl ether–acetic acid (90:10:1) or chloroform–methanol–water (65:25:4) as solvents and 50% aqueous sulfuric acid or Dragendorff reagent as indicator.

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Table I. Proximate Composition of Krill Samples (as Percentage of Total)

	frozen krill	freeze-dried krill	krill mean
moisture	77.7	7.83	1.75
protein[a]	14.0	52.0	60.7
fat	3.01	16.7	11.5
ash	2.74	12.2	13.4
total	97.5	88.7	87.4

[a] Total nitrogen was multiplied by 6.25.

Table II. Yields of Oils Extracted from Krill Samples with Supercritical Carbon Dioxide

		amt, g/100 g sample			
sample	extractn condn[a]	extr oil	resid lipid[b]	total lipid[b]	rec,[c] %
freeze-dried krill	250/40	11.2	7.6		95
	250/60	11.7	7.9	19.7	98
	250/80	11.5	8.1		99
krill meal	250/40	4.5	8.1		84
	250/60	4.1	9.0	16.2	81
	250/80	4.0	8.4		77
	400/40	4.0	9.0		80

[a] Pressure, kg/cm^2/temperature, °C. [b] Measured by the Bligh–Dyer method. [c] Recovery = [extr oil + resid lipid]/total lipid × 100.

After saponification and then esterification with boron trifluoride–methanol complex, the fatty acid composition of the oils was determined by GLC with a Shimadzu GC-5A gas chromatograph using a glass column (2 m × 3 mm) packed with 10% DEGS on Celite 545 (80–100 mesh) at a temperature of 200 °C, with nitrogen carrier gas at a flow rate of 25 mL/min. The temperature of the detector and injection port was 250 °C.

The composition of carotenoids in the SC-CO_2-extracted oils and in the residual lipids was analyzed by the method reported by Yamaguchi et al. (1983).

RESULTS AND DISCUSSION

Table I shows the proximate compositions of the krill samples. Total recoveries of components of the freeze-dried krill and the krill meal were rather low, because of the application of the Soxhlet method with diethyl ether for the extraction of lipid. The lipid of krill, which contains high proportions of polyunsaturated fatty acids and phospholipids (Mori and Hikichi, 1976), deteriorates rapidly and diethyl ether insoluble lipid increases when krill is dehydrated or treated at high temperatures. In this connection, the lipid content of the krill meal was found to be 16.2% when measured by the method of Bligh and Dyer (1959) with chloroform–methanol. Furthermore, the low recoveries in the freeze-dried krill and the krill meal may be also accounted for by chitin, contents of which should be higher than that in the frozen krill.

Figure 1 presents the SC-CO_2 extraction curves with time of oils from the freeze-dried krill and krill meal with SC-CO_2 at 250 kg/cm^2 and 80 °C. For freeze-dried krill, extraction of oil practically terminated in 3–4 h and after the use of 2–3 kg of CO_2 at a flow rate of about 0.6 kg/h. Extraction of the krill meal oil, however, ceased after 2–3 h and the use of 1–2 kg of CO_2. Reproducibility in the experiments was very high.

Table II shows the yields of oils extracted with SC-CO_2 under different conditions. In every sample the extracted oil was fluid and bright red from carotenoids. When the temperature was increased from 40 to 60 and 80 °C at a fixed pressure of 250 kg/cm^2 and when the pressure was increased from 250 to 400 kg/cm^2 at a fixed temperature of 40 °C, yields of extracted oils remained almost constant.

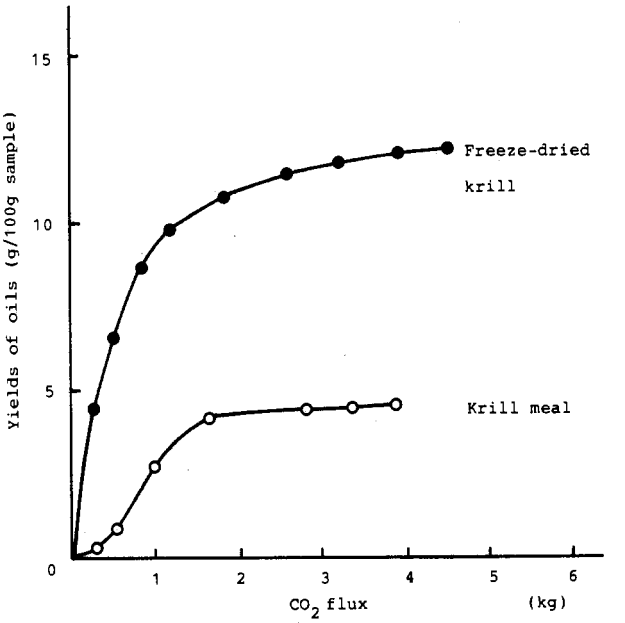

Figure 1. Extraction curves of krill oils with supercritical carbon dioxide at 250 kg/cm^2 and 80 °C.

In this connection, Brogle (1982) reported that the solvent power of SC-CO_2 for organic substances is highly dependent on its pressure and temperature; at low pressures, around 100 kg/cm^2, solvent power drops with rising temperatures, and at pressures higher than approximately 150 kg/cm^2, its solvent power increases. For the krill samples, however, the amounts of extracted oils were almost constant regardless of pressures and temperatures examined. That is, even under the lowest temperature/pressure combination (250 kg/cm^2 and 40 °C), nearly all oils extractable with SC-CO_2 were recovered. Further, yields of krill meal oil were one-third of those of freeze-dried krill oil. The lower yields from meal oil are probably attributable to the fact that the oil of the krill meal was in part deteriorated by oxidation or polymerization to such an extent that only limited extraction occurred with SC-CO_2. Judging from this result, we think that SC-CO_2 extraction is suitable method to obtain undenatured oils from meals of marine origin.

To determine the composition of the extracted oils, we partitioned them by chromatography on a silica gel column using a mobile phase of chloroform and methanol. Approximately 100% of the oils from both krill samples was recovered in the fraction eluted with chloroform, proving that the oils extracted with SC-CO_2 were composed exclusively of nonpolar lipids with practically no polar lipids. Figure 2 shows TLC patterns of the oils extracted with SC-CO_2 and the residual lipids. By co-TLC with standard reagents, the main component of the extracted oils was found to be triglycerides (spot 3) and the minor components were identified as hydrocarbon (1), cholesteryl ester (2), free fatty acids (4), diglycerides (5), cholesterol (7), monoglycerides (8), and carotenoids (6 and 9), as illustrated in chromatogram I. In addition, a small amount of a nonpolar lipid (spot 10) was found to be present in the oil from the krill meal, but it remained unidentified. The free fatty acid content of the oil from the krill meal was lower than that of the freeze-dried krill. Because of their rapid deterioration, some of free fatty acids in the meal should have been denatured during manufacture and storage of the meal.

As shown in chromatogram II, trace amounts of some nonpolar lipids (spots 3–9) were observed, but almost all the residual lipids (spot 11) were not affected when de-

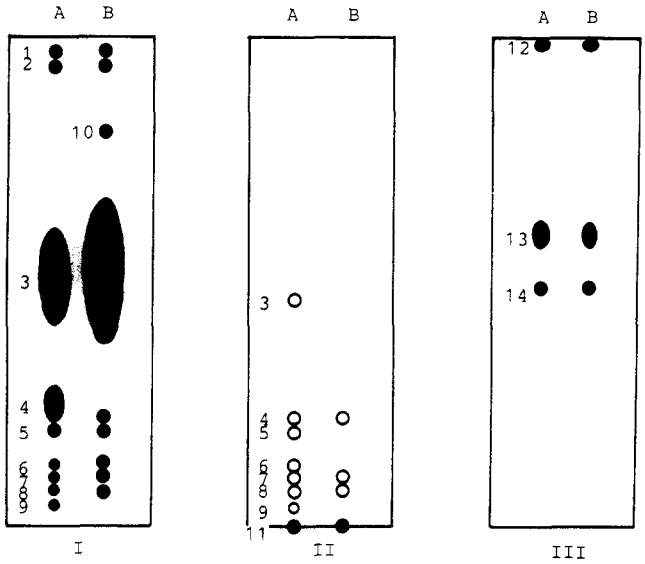

Figure 2. Thin-layer chromatograms of krill oils extracted with supercritical carbon dioxide (I) and the residual lipids (II and III): A, freeze-dried krill; B, krill meal. Stationary phase: silica gel $60F_{254}$ in chromatograms I–III. Mobile phase: petroleum ether–diethyl ether–acetic acid (90:10:1) in chromatograms I and II; chloroform–methanol–water (65:25:4) in chromatogram III. Identification: 50% H_2SO_4 in chromatograms I and II; Dragendorff reagent in chromatogram III.

Table III. Fatty Acid Composition of Oils Extracted from Krill Samples with Supercritical Carbon Dioxide[a]

fatty acid	freeze-dried krill	krill meal	fatty acid	freeze-dried krill	krill meal
12:0	0.25	0.28	18:4	1.98	3.21
14:0	17.42	19.08	20:1	1.82	1.86
15:0	0.28	0.21	20:3	0.15	0.09
16:0	22.05	18.78	20:4ω6 + 22:1	1.08	0.55
16:1	11.78	16.30			
17:1	1.09	1.70	20:4ω3	0.32	0.30
18:0	1.47	1.33	20:5	11.36	6.62
18:1	21.37	22.40	22:5	0.20	0.18
18:2	2.24	3.56	22:6	4.44	2.71
18:3	0.30	0.26	24:1	0.32	0.42
			unidentified	0.08	0.16

[a] Conditions of extraction: 250 kg/cm², 60 °C.

veloped with petroleum ether–diethyl ether–acetic acid (90:10:1), indicating that the residual lipids were composed almost exclusively of polar lipids together with denatured and polymerized lipids. Furthermore, as shown in chromatogram III, when the residual lipids were developed using chloroform–methanol–water (65:25:4) and visualized with Dragendorff reagent, at least three orange-red spots (12–14) of phospholipids were detected. These results confirmed that the SC-CO_2 extraction of krill samples yielded only nonpolar undenatured oils, as mentioned above.

Table III shows the fatty acid compositions of the oils extracted with SC-CO_2 at a pressure of 250 kg/cm² and a temperature of 60 °C. Essentially no differences were noticed in the fatty acid compositions of the oils extracted under other conditions. Whereas the main fatty acids were 14:0, 16:0, 16:1, 18:1, and 20:5 in oils of both freeze-dried krill and krill meal, significant differences were found between krill meal and freeze-dried krill oils in the 16:1, 20:5, and 22:6 acids.

Table IV shows the content and composition of carotenoids in the oils extracted with SC-CO_2 from the

Table IV. Content and Composition of Carotenoids in Oils Extracted from Freeze-Dried Krill with Supercritical Carbon Dioxide

carotenoid	temp of extractn,[a] °C 40	60	80
total content, mg/100 g oil	43.5–50.4	19.1–24.3	7.0–8.7
composition, %			
astaxanthin diester	48–58	78–83	*b*
astaxanthin monoester	33–44	13–15	*b*
astaxanthin	5–7	3–4	*b*
unidentified	0–2	1	*b*

[a] Pressure: 250 kg/cm². [b] Unable to be determined due to decomposition.

freeze-dried krill at a fixed pressure of 250 kg/cm² and stepwise increase of temperatures. In previous papers (Yamaguchi et al., 1983; Miki et al., 1983), we reported that the carotenoids of the Antarctic krill consist almost exclusively of astaxanthin and its esters and that their stabilities against heat and organic solvents are in the order of astaxanthin diester, astaxanthin monoester, and astaxanthin. It is evident that the astaxanthin tends to be decomposed according to their instabilities during extraction with SC-CO_2. Although Zosel (1978) pointed out that SC-CO_2 is suitable for the isolation of thermally labile substances because of its low critical temperature, the above finding indicates that some compounds like astaxanthin could be unstable under high pressures of SC-S-CO_2 at a temperature, for example 80 °C, that never induces the decomposition of astaxanthin under atmospheric pressure (Miki et al., 1983). Attention should be paid to this fact in the extraction of natural products with SC-CO_2.

In spite of such a disadvantage, we proved that SC-CO_2 is effective in obtaining nonpolar lipids from Antarctic krill by a simple, one-step extraction that excludes the phospholipids that have hampered the utilization of krill oils. In this connection, several workers (Stahl et al., 1980; Friedrich and List, 1982) have reported the removal of polar lipids from seed oils with SC-CO_2, but the reason why polar lipids can be removed with SC-CO_2 has not yet been elucidated.

As shown in Table III, the oils extracted from krill samples with SC-CO_2 contained fairly high proportions of eicosapentaenoic acid (EPA), which is known as a medically useful substance (Needleman et al., 1979). Recently, Krukonis (1984) reported that, by using SC-CO_2 for the fractionation of fish oils, EPA could be concentrated to 15% from 8%. Used in this manner, SC-CO_2 extraction will produce useful substances from aquatic organisms.

Abbreviations Used: SC-CO_2, supercritical carbon dioxide; EPA, eicosapentaenoic acid.

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Isolation of Estrogens in Bovine Plasma and Tissue Extracts Using Alumina and Ion-Exchange Microcolumns

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Estrogens (estradiol, estrone) in picogram quantities can be isolated quantitatively from bovine plasma and tissue extracts by a simple procedure. Bovine plasma (0.1–1.0 mL) was extracted with either acetone or ether while tissues (1 g) were extracted with acetone. Extracts were passed through two disposable plastic tubes vertically arranged in tandem. The top column (5-mL pipet tip) contained 1–1.5 g of dry basic alumina and removed interfering substances. The bottom column (transfer pipet) contained 0.3–1.0 g of wet anion-exchange resin in the phosphate form and trapped the estrogens through their phenolic hydroxyl group. The estrogens were then eluted with acetic acid in acetone following a thorough washing of the columns. Recoveries greater than 95% were obtained when extracts of bovine plasma and tissue extracts of liver, kidney, and heart were spiked with either tritiated 17β-estradiol or estrone. This technique offers the advantages of simplicity, rapidity, and accuracy over traditional methods employed routinely in the purification of estrogens.

INTRODUCTION

Partial purification of estrogens extracted from animal tissues and fluids is necessary prior to most methods of quantitation. The methods currently employed in routine analysis of estrogens such as paper chromatography (Shutt, 1969), Sephadex LH-20 (Sjovall and Nystrom, 1968; Murphy, 1970; Mikhail et al., 1970; Murphy and Diez D'Aux, 1975), and Celite column cleanup (Korenman et al., 1969; Abraham et al., 1970) are tedious with reported recoveries of only 65–85%. Aqueous solutions of estrogens had also been purified by ion exchange (Järvenpää et al., 1979) with reported recoveries of 50–90%. Covey and co-workers (1984) also used an anion-exchange resin for purification of diethylstilbestrol and dienestrol.

This paper describes a relatively simple and rapid technique that can quantitatively isolate the estrogens (estradiol, estrone) from acetone extracts of bovine blood plasma and tissues for subsequent chromatographic analysis. This study is a preliminary report on the development of screening methods to detect and measure residues of estrogens in the blood and edible tissues of food-producing animals given growth-promoting hormones such as 17β-estradiol and to ascertain their absence such that safe and wholesome food can be delivered to consumers.

Table I. Column Conditions for Isolation of [^{3}H]Estradiol Added to Bovine Plasma and Tissue Extracts Using the Alumina Ion-Exchange Columns

sample	extr appl to col, mL	basic alumina, g	resin suspensn, mL
A. Bovine Plasma (mL)			
acetone extractn			
0.1	2	1.0 (3)[a]	1.0 (2)[b]
0.5	4	1.0 (3)	2.0 (3)
1.0	8	1.5 (5)	3.0 (4)
1.0	8[c]	1.5 (5)	3.0 (4)
ether extractn			
1.0	4	1.0 (3)	1.0 (2)
B. Bovine Tissues (1 g/8 mL of Acetone)			
liver	2	1.0 (3)	2.0 (3)
muscle	2	1.0 (3)	2.0 (3)
heart	2	1.0 (3)	2.0 (3)
kidney	2	1.0 (3)	2.0 (3)

[a] Total volume 95% acetone (mL) to wash alumina column. [b] Total volume 10% HOAc in acetone (mL) to elute [^{3}H]estradiol. [c] Plasma/acetone mixture added directly to column with glass wool on top of alumina bed.

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