Separation of Fatty Acid Esters from Cholesterol in Esterified Natural and Synthetic Mixtures by Supercritical Carbon Dioxide

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The solubility of cholesterol in supercritical carbon dioxide was determined by a continuous flow method. The solubility of cholesterol increased with increasing pressure and exhibited retrograde behavior. The Chrastil equation was used to describe the relationship between solubility and the density of carbon dioxide. A model mixture was made by adding cholesterol and fatty acid esters together. Squid visceral oil was esterified as the feed material. Both the model mixture and esterified squid visceral oil were extracted by supercritical carbon dioxide. The experimental results showed that cholesterol could be removed from a model mixture and from esterified squid visceral oil at low pressure (1500 psig) and high temperature (328.2°K). Under these conditions, cholesterol content in the extract was reduced from 2867 mg/100 g to 14.1 mg/100 g.

KEY WORDS: Cholesterol, fatty acid esters, supercritical extraction.

Supercritical fluids have solvent powers similar to liquids but with mass transfer characteristics like gases (1). The soluble extracts obtained can be recovered easily from the supercritical fluid by manipulating the operating conditions of pressure and temperature. Supercritical fluid extraction (SFE) has been used for isolating flavors, caffeine, and lipids since 1970 (2). Carbon dioxide has several advantages, such as its nontoxicity, nonflammability, no chemical residual problem, and low to moderate operating temperatures, etc., over other solvents and is the most widely used solvent in SFE. The solubility of a pure material in supercritical carbon dioxide (SC-CO_2) is the prerequisite data required to predict the separation efficiency from mixtures.

There are many studies reporting the solubilities of fatty acids and esters of fatty acids in SC-CO_2. Bamberger and co-workers (3) measured the solubilities of lauric acid, myristic acid, palmitic acid, and triglycerides in SC-CO_2. Brunetti (4) reported the solubilities of four fatty acids in SC-CO_2 at the pressure range of 2900.8–4531.1 psig (20–30 MPa) and the temperature range of 313.2–333.2°K. Chrastil (5) investigated the solubilities of certain fatty acids and triglycerides in SC-CO_2 within the pressure range of 1160.3–3626 psig (8–25 MPa) and the temperature range of 313.2–353.2°K and mathematically described the relationship between solubility and density of SC-CO_2. Several other investigators also have reported the results on extracting fatty acid esters from esterified fish oil (6–8). Reduction of cholesterol in dietary fat is considered beneficial to health. Wong and Johnston (9) and Chrastil (5) measured the solubility of cholesterol in SC-CO_2 using different methods. Chao and co-workers (10) investigated the feasibility of separating cholesterol and lipids from ground beef. However, studies applying solubility data to select process conditions are limited.

The objective of this work was to investigate whether the solubility data could be used to select appropriate process conditions for separating cholesterol from fatty acid esters. First, an empirical equation was used to correlate the solubility data of cholesterol with the density of carbon dioxide. Then the solubility of cholesterol at various conditions was estimated from the equation. Experiments extracting cholesterol from a model mixture and from esterified squid visceral oil were further conducted to validate the concept.

MATERIALS AND METHODS

Pure cholesterol (pure crystal, purity >99%) was purchased from Merck Co. (Darmstadt, Germany). Four kinds of ethyl esters of fatty acids were used in the model mixture. Ethyl palmitate and ethyl oleate (purity >99%) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Eicosapentaenoic acid (EPA) (purity 90.9%) and docosahexaenoic acid (DHA) (purity 90.3%) were bought from Nippon Oil & Fats Co., Ltd. (Tokyo, Japan). Squid visceral oil was obtained from Fung-I Co. (Kaohsiung, Taiwan). All materials were stored at −20°C until used. EPA, DHA, and squid visceral oil were esterified with absolute ethanol (11).

The flow diagram of the extraction system is shown in Figure 1. The equipment was purchased from Newport Scientific Inc. (Jessup, MD). The heart of the system is a 15,000 psi double-ended, diaphragm-type compressor. The pressure in the extractor is controlled by a back pressure regulator. Compressed CO_2 is pumped through high pressure tubing (stainless steel 304) into the extractor wrapped with heating tapes to maintain the desired temperature. The extraction vessel is 63 cm high with an inside diameter of 2.6 cm. The effective volume of the extractor packed with glass beads was 150 mL. A temperature controller was used to control the temperature in the extractor. The flow rate of CO_2 was adjusted to 6 L (ambient condition) per min by a variable speed controller, and the volumetric flow was recorded by a flow totalizer. In a typical experiment, the pressure and temperature in the extractor were adjusted to the set points. The system was stabilized for one hour after the feed materials were added into the extractor. The extract samples were eluted out continuously by CO_2 and collected periodically in a cold trap. The collected samples were weighed. The solubility of cholesterol was calculated as the initial

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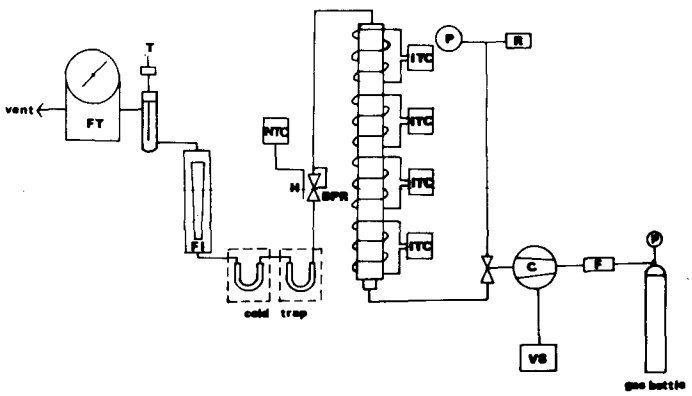


FIG. 1. The extraction system. BPR, back pressure regulator; C, diaphragm compressor; F, filter; Fl, flow indicator; FT, flow totalizer; H, heater; ITC, indicating temperature controller; NTC, nonindicating temperature controller; P, pressure gauge; R, rupture disc; T, thermocouple; VS, variable speed controller.

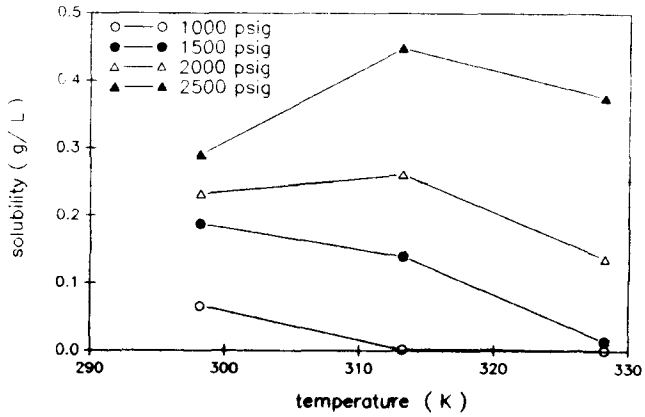


FIG. 2. The solubility of cholesterol in carbon dioxide at various conditions.

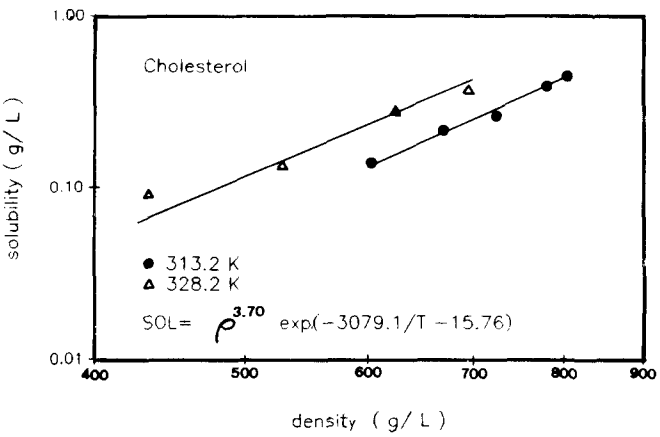


FIG. 3. The effect of the density of carbon dioxide on the solubility of cholesterol. Where SOL is the solubility mg/L, ϱ is density of carbon dioxide, and T is the absolute temperature in K.

slope of the weight of collected samples vs the eluted volume of CO_2. Three types of feed materials were used. They were 100 g of pure cholesterol, 50 g of model mixture containing 26.71% ethyl palmitate, 24.39% ethyl oleate, 23.54% EPA ethyl ester, 20.27% DHA ethyl ester, 2016 mg cholesterol/100 g, and trace amounts of fatty acid esters, and 100 g esterified squid visceral oil with the composition of 2.01% ethyl myristate, 10.96% ethyl palmitate, 1.63% ethyl palmitoleate, 3.29% ethyl stearate, 15.74% ethyl oleate, 0.66% ethyl linoleate, 0.37% ethyl linoleneate, 15.84% ethyl eicosenoate, 4.16% EPA ethyl ester, 12.01% DHA ethyl ester, and cholesterol (2,867 mg/100 g). All the percentages are in area percentages analyzed by gas chromatography.

The cholesterol content was measured by gas chromatography (Varian 3400, Varian Associates, Palo Alto, CA). The temperature of the FID detector was 250°C. A DB-1 fused capillary column 30 m long, with 0.25 mm internal diameter and 0.2 μm film thickness, was used. The column temperature was 250°C. The injector temperature was also 250°C. Hydrogen was used as the carrier gas at a flow rate of 1.5 mL/min. The split ratio was 100:1. An alcohol solution containing 0.4 g collected extract was used for analysis, and 0.001 g α-cholestane was used as the internal standard. Five to fifteen mL KOH solution (2N/95% C_2H_5OH) was added to undertake saponification at 40°C overnight. After the reaction was completed, 20 mL of water was added. The solution was extracted four times by 15 mL n-hexane. The combined extracts were washed twice using 10 mL 0.5N KOH aqueous solution and washed another three times using 10 mL of water. The extracts were then dehydrated by anhydrous sodium sulfate and concentrated to a volume of 1-2 mL. One μL of the concentrate was injected into the GC.

RESULTS AND DISCUSSION

Figure 2 shows the measured solubility of cholesterol in carbon dioxide at various conditions. The results show that the solubility of cholesterol in SC-CO_2 was less than 0.5 g/L under the experimental conditions. The solubility increased with increasing pressure. When the pressure was less than 1500 psig, the solubility decreased as the temperature increased. When the pressure was above 1500 psig, the solubility increased with increasing temperature from 298.2 to 313.2°K, and then decreased as the temperature continuously increased to 328.2°K. This is called the retrograde phenomenon (12). Our data compared closely with that reported by Wong and Johnston (9). The experimental data was fitted with an empirical equation similar to the Chrastil (5) equation:

$$SOL = \varrho^{3.70} \exp(-3079.1/T - 15.76) \quad [1]$$

where SOL is the solubility in g/L, T is the temperature in °K, and ϱ is the density of carbon dioxide.

The data of density of carbon dioxide was taken from the report of Angus *et al.* (13). The fit was good, as demonstrated in Figure 3. The power of density,

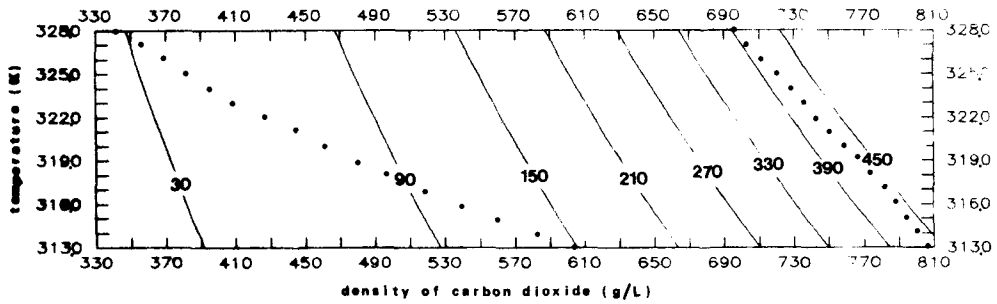


FIG. 4. Calculated solubility (mg/L) of cholesterol at different conditions. The numbers on the lines are the solubility in mg/L. The experimental conditions are located in the region between the two dotted lines.

TABLE 1

The Calculated Solubility of Cholesterol at Different Operating Conditions for Separating the Model Mixture

Run no.	Pressure (psig)	Temperature (K)	Density of CO_2 (g/L)	Calculated solubility of cholesterol (mg/L)
1	1500	313.2	602.4	146.9
2	1500	318.2	494.4	82.6
3	1500	323.2	408.1	47.2
4	1500	328.2	342.3	28.5
5	2000	313.2	723.4	289.2
6	2000	318.2	655.8	234.8
7	2000	323.2	588.5	182.7
8	2000	328.2	527.7	141.1

calculated as 3.7, was different from 12.095, which was obtained by Chrastil (5). The deviation could be due to a difference in experimental methods used. The continuous flow method was used both in this study and in Wong's report (9), whereas Chrastil used an equilibrium method. In the continuous flow method, the measurement obtained may not be at the equilibrium state. The experimental results could be lower than that which was obtained at the equilibrium state.

From the correlation of temperature and density, the solubility of cholesterol at different conditions can be estimated from Equation [1]. The calculated results are shown in Figure 4. It is a contour map. The numbers on the lines are the solubility in mg/L. The experimental conditions are located in the region between the two dotted lines. When the temperature was constant, the solubility increased as the density of carbon dioxide increased. At constant density of carbon dioxide, the solubility increased as the temperature increased. The solubility varied from 30 to 440 mg/L in this region. From Figure 4, the appropriate operating conditions can be selected according to the value of solubility data. Table 1 lists eight operating conditions with calculated solubilities estimated from Equation [1]. In the Table, the calculated solubility varied from 28.5 to 289.2 mg/L. High density of SC-CO_2 did not assure high solubility. For example, the solubility was 146.9 mg/L when the density of carbon dioxide was 602.4 g/L. However, the solubility was 182.7 mg/L when the density of carbon dioxide was 588.5 g/L. The solubility at 2000 psig was higher than that at 1500 psig at the same temperature. These eight solubility lines were used to extract cholesterol from the model mixture and to examine at which conditions cholesterol could be removed more easily.

The amount of carbon dioxide used to collect the extract from the model mixture at various conditions were different as listed in Table 2. The final amount of the extract was kept around 16–18 grams except in run No. 4. The last column in Table 2 is the average quantity of carbon dioxide needed to obtain a gram of the extract. Run No. 4 needed the most carbon dioxide. Run No. 5 needed the least amount of carbon dioxide. In the comparison of Table 1 and Table 2, the less the calculated solubility, the more carbon dioxide were needed to collect a gram of extract. To study the change of cholesterol content in the extract from the model mixture, the cholesterol contents in the fractions of the extract vs the weight percentages of the extract were plotted in Figure 5. The weight percentages were calculated based upon the final weights of the extracts listed in Table 2. Therefore, the change of cholesterol content during extraction can be understood.

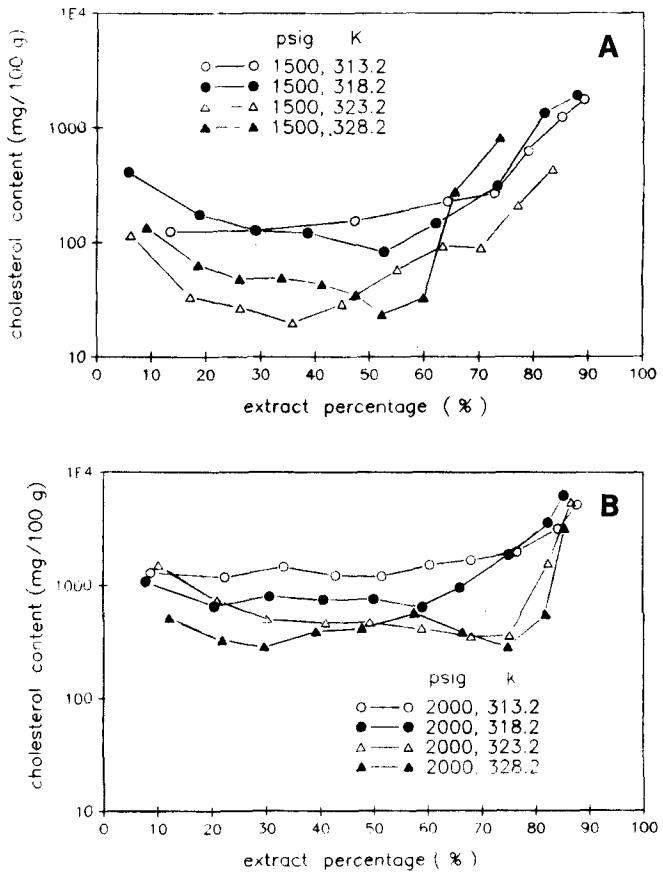
At 1500 psig, the cholesterol content decreased at first, and then increased during the extraction process as shown in Figure 5A. For example, at 323.2°K the

TABLE 2

The Amount of Cumulative Extracts from the Model Mixture and the Cumulative Volume of CO_2 Used at Different Conditions

Run[a] no.	Cumulative volume (NL)[b] of CO_2 used	Cumulative weight (g) of extract	Average CO_2 used per gram extract
1	460	18.13	25.37
2	890	17.81	49.97
3	2000	16.37	122.17
4	3600	6.80	529.40
5	260	17.47	14.88
6	330	17.07	19.33
7	300	17.33	17.31
8	550	17.31	31.77

[a]Run no. corresponds to those in Table 1.
[b]The volume in liters measured at ambient conditions (1 atm. and 25°C).



cholesterol content was 115.3 mg/100 g initially, decreased to 19.7 mg/100 g when 35% extract were collected, and then increased to 436.4 mg/100 g as the extraction proceeded. Chao and co-workers (10) reported the same pattern when extracting cholesterol from ground beef. In other words, the extract was richer in fatty acid esters initially since the content of fatty acid esters in the extract increased with the decrease in cholesterol content. As the extraction proceeded, the content of fatty acid esters decreased and cholesterol content increased. Therefore, leaving cholesterol as the residue in the extraction vessel would be an easy way to remove cholesterol from fatty acid esters. When the pressure was 2000 psig (Fig. 5B), the change in cholesterol content in the extracts exhibited a similar pattern as shown in Figure 5A. However, the change of cholesterol content was not so significant. At 313.2°K, the cholesterol content remained constant (1300.5 mg/100 g) until after 60% extract had been collected. Therefore, the extract was always high in cholesterol. When the temperature was 328.2°K, the cholesterol content was 519.5 mg/100 g initially, decreased to 286.9 mg/100 g, and then increased to 5482.2 mg/100 g during the extraction. Comparing Figures 5A and 5B, it can be seen that the cholesterol content of the extracts at 2000 psig was much higher than that at 1500 psig. A low initial cholesterol content was desirable for removing cholesterol from fatty acid esters. The model mixture contained a cholesterol content of 2016 mg/100 g. Therefore, the cholesterol content in the extract was reduced at least 100-fold at 1500 psig. The results indicate that cholesterol could be removed easier from fatty acid esters at low pressure and high temperature. The lower the solubility, the higher the separation efficiency was for cholesterol.

Table 3 lists six experimental conditions and the solubility of cholesterol estimated from Equation [1]. The calculated solubility increased with the density of carbon dioxide. These six operating conditions were used to extract the esterified squid visceral oil. The amounts of carbon dioxide used to collect the extracts are listed in Table 4. The average amount of CO_2 used to collect unit gram extract decreased as the density of carbon dioxide increased. The final weights of the extracts listed in Table 4 were the basis for calculating weight percentage of extracts. Figure 6 is plotted by the similar method to Figure 5.

The change of cholesterol content was the most remarkable at 1500 psig and 328.2°K, which yielded the lowest solubility of cholesterol. At these conditions, the cholesterol content decreased from 760.7 mg/100 g initially to 14.1 mg/100 g during extraction. The cholesterol content was reduced 200-fold as com-

TABLE 3

The Calculated Solubility of Cholesterol at Different Operating Conditions for Separating the Esterified Squid Visceral Oil

Run no.	Pressure (psig)	Temperature (K)	Density of CO_2 (g/L)	Calculated solubility of cholesterol (mg/L)
9	1500	328.2	342.3	28.5
10	1500	321.2	442.6	60.0
11	1500	316.2	537.6	105.9
12	1695	313.2	644.4	188.5
13	1973	313.2	715.8	278.1
14	2162	313.2	770.5	365.2

TABLE 4

The Amount of Cumulative Extracts from the Esterified Squid Visceral Oil and the Cumulative Volume of CO_2 Used at Different Conditions

Run[a] no.	Cumulative volume (NL)[b] of CO_2 used	Cumulative weight (g) of extract	Average CO_2 used per gram extract
9	2800	13.33	210.05
10	2600	32.67	75.57
11	2200	87.33	25.19
12	1600	90.40	17.70
13	1400	88.67	15.79
14	1400	93.33	15.00

[a]Run no. corresponds to those in Table 3.
[b]The volume in liters measured at ambient conditions (1 atm. and 25°C).

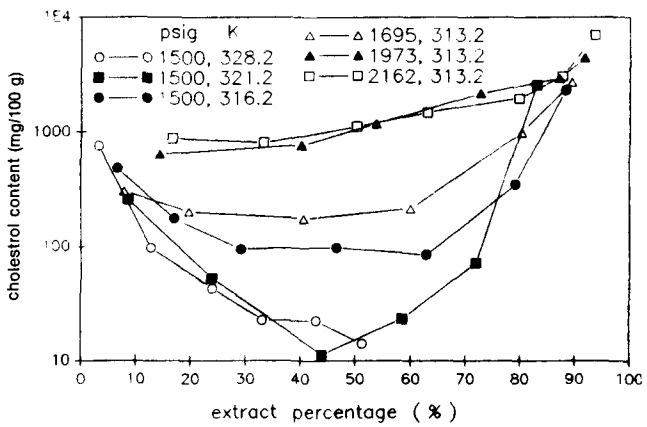


FIG. 6. The change of cholesterol content in extracts during extracton of esterified squid visceral oil at various conditions.

pared with the feed material. When the temperature was 313.2°K and the pressure was 2162 psig or 1973 psig, the cholesterol content kept increasing during the extraction. At 2162 psig, the lowest cholesterol content was 881 mg/100 kg. Low pressure and high temperature yielded low solubility but resulted in better separation. When the pressure was maintained at 1500 psig, the change in cholesterol content was more significant at high temperature. When at 313.2°K, the change in cholesterol content was more significant at low pressure. The experimental conditions with low solubility yielded high separation efficiency but needed more carbon dioxide. The choice of optimum conditions depends on the product specification and process cost analysis.

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