# Lipase-Catalyzed Alcoholysis of Cod Liver Oil in Supercritical Carbon Dioxide

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**ABSTRACT:** Enzymatic alcoholysis of cod liver oil, with an immobilized lipase, was carried out in supercritical carbon dioxide. The enzyme was catalytically active under the experimental conditions used. The reaction medium was investigated to preferentially extract ethyl esters, synthesized during the course of the experiment, from the unconverted cod liver oil substrate and side-products. The effect of pressure changes on the amount of tri-, di-, and monoglycerides and ethyl esters, present in both the extract and the remaining lipid residue, was determined. Furthermore, the fatty acid compositions of the lipid classes were analyzed, and the relative amounts of both eicosapentaenoic acid and docosahexaenoic acid to palmitic acid were determined. The results show that it is possible to preferentially extract the synthesized ethyl esters at low pressures. The extract collected at 9 MPa contained 64 g ethyl esters/100 g extract, while the total amount of all other lipid classes detected was 19 g/100 g extract. As the pressure was increased, the relative amount of the other lipid classes detected in the extract, especially triglycerides, was enhanced. The relative amounts of both eicosapentaenoic acid and docosahexaenoic acid to palmitic acid increased for some lipid classes in the extract. This increase was most pronounced for the monoglyceride lipid class. The integration of biocatalysis and product fractionation, applied in this study, suggests that the potential for biocatalysis in industrial processes is considerably wider than had been thought.

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KEY WORDS: Enzyme, fish oil, lipase, supercritical carbon dioxide.

Supercritical fluids (SCF), particularly carbon dioxide, are currently receiving widespread attention as possible media for enzymatic reactions. The main advantage of SCF over other liquid solvents is that the high diffusivity, low viscosity, and low surface tension of SCF can speed up mass transfer-limited enzyme reactions (1). In addition, SCF offer the possibility of modifying product solubilities through modification of pressure and/or temperature. Thus, enzymatically synthesized products may be fractionated from unconverted substrates and side-products by exploiting differences in solubility in supercritical carbon dioxide ($SCCO_2$) at different temperatures and pressures. Enzymatic catalysis in $SCCO_2$ is of particular interest to the food and pharmaceutical industry because $SCCO_2$ is a nontoxic, nonflammable, inexpensive solvent that does not present a waste-disposal problem. Further, its critical temperature (31.1°C) is sufficiently low for processing of heat-liable materials and close to the typical optimum temperatures for enzyme reactions.

After initial findings that some enzymes were active and stable in SCF (2,3), several other studies on enzyme catalysis in SCF have been carried out (4–17). The majority of systems explored to date have used model reactions, carried out to investigate the stability of various enzymes in $SCCO_2$ (2,4–7) and the kinetics of enzyme catalysis in the supercritical media (8–16). Some of these investigations have been devoted to a comparison of reaction kinetics in hexane and $SCCO_2$ (9,10,12,15,17). Only a few of these model reactions have immediate practical and commercial purposes.

We have studied the enzymatic alcoholysis of fish oil in $SCCO_2$ with a commercially available immobilized lipase (E.C. 3.1.1.3). Our interest in investigating this enzyme reaction is derived from the fact that fish oils are a potential source of long-chain n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Clinical studies suggest that these n-3 fatty acids may have beneficial effects in the treatment of arthritis (18,19) and cardiovascular disease (20). As a result, the general public has been encouraged to increase the dietary intake of n-3 fatty acids. Consequently, there is a demand (especially within the pharmaceutical industry) for enriched concentrates of these fatty acids. Commercial fish oils are a fair source of EPA and DHA, containing approximately 10% of each fatty acid (21). Due to the random association of fatty-acid chains on the glycerol backbone, the triglycerides (TG) (containing EPA and DHA) present in fish oil cannot be concentrated to any significant degree (21,22). Hence, the fatty acids have to be split from the glycerol backbone, by either chemical or enzymatic catalysis (21,23). Enzymatic catalysis is generally considered to be the more favorable method, due to its high selectivity and the mild reaction conditions required (23,24).

In our experiments, the substrates are cod liver oil (CLO), ethanol, and an immobilized lipase. At the beginning of the

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experiment, the oil is present in the form of TG. During the reaction, the lipase splits the individual fatty acids from the glycerol backbone to form ethyl esters (EE), and this reaction results in a complex mixture of lipid components that consists of TG, diglycerides (DG), monoglycerides (MG), and EE. We set out to extract the EE produced from the reaction mixture. Preferential extraction should be feasible because esters are known to be much more soluble than free fatty acids (FFA) in $SCCO_2$ (25). The extraction of the EE produced from the reaction mixture may also shift the enzyme reaction in the direction of further synthesis.

A number of researchers have studied the solubility and phase equilibria of EPA and/or DHA EE in $SCCO_2$ (26–28), as well as those of other fish oil fatty acid EE (25,29,30). Further, the separation of EPA and DHA EE from a mixture of fatty acid EE has been investigated (27,31–33). What is new in the present study is the simultaneous combination of enzyme catalysis of fish oil in $SCCO_2$ and fractionation of the product, EE, from the complex reaction mixture that results from the enzyme synthesis. The application of $SCCO_2$ as a solvent for enzyme catalysis thus offers a unique opportunity for integration of biocatalysis and product fractionation that has hardly been exploited previously. The only related study known to us is that of Marty and co-workers (34), who reported on a continuous lipase-catalyzed reaction in $SCCO_2$ coupled to a post-reactional separation process.

The primary objective of this study was to investigate the potential for the integration of biocatalysis and product fractionation in $SCCO_2$ for a complex natural substrate such as fish oil. To achieve that goal, we decided to determine whether it was possible to carry out enzymatic alcoholysis of CLO in $SCCO_2$ and to study the possibility of preferentially extracting the synthesized EE from the unconverted TG and other side-products. In addition, we wanted to determine the effect of pressure changes on both product, EE, quality and yield. A secondary objective was to investigate the possibility of using this method to produce PUFA-enriched EE.

## MATERIALS AND METHODS

*Materials.* Immobilized lipase (Novozym 435) from *Candida antarctica*, supported on a macroporous acrylic resin, was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). The enzyme had a quoted activity of 5500 PLU/g (see Ref. 35 for further information). The fish oil used was extracted in our laboratory from cod livers originating from the Baltic sea. Methyl heptadecanoate and methyl tricosanoate were from Larodan Fine Chemicals AB (Malmö, Sweden). Sodium methoxide (30% solution in methanol) was from Merck (Darmstadt, Germany), and 14% boron trifluoride–methanol from Sigma (St. Louis, MO). Carbon dioxide (purity 99.99%) was supplied by Air Liquide Gas AB (Malmö, Sweden).

*Equipment and procedure.* Figure 1 presents the experimental equipment used to conduct all experiments in $SCCO_2$. CLO (3.0 g), ethanol (1.8 g; 99.5%), and enzyme (0.6 g) were placed in a 30-$cm^3$ reactor. The reactor was then connected to

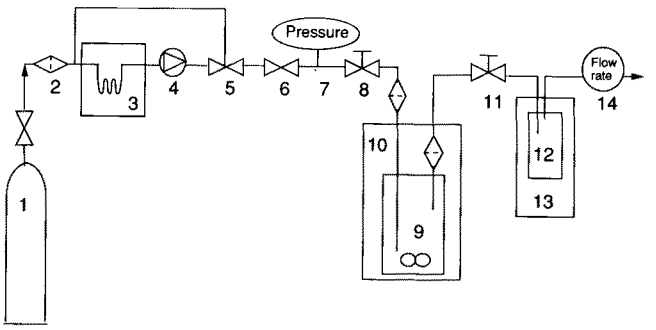


**FIG. 1.** Schematic diagram of the experimental apparatus: 1. gas tube, 2. filter, 3. cool bath (–10°C), 4. high-performance liquid chromatography pump, 5. relief valve, 6. check valve, 7. pressure meter, 8. shut-off valve, 9. reactor, 10. water bath (40°C), 11. micrometering valve, 12. cold trap, 13. cooler (0°C), 14. flow meter.

the equipment (Fig. 1) and pressurized by pumping $CO_2$ into the reactor. The substrates were continuously mixed by a magnetic stirrer. The outgoing $CO_2$ was depressurized to atmospheric pressure by opening a heated micrometering valve (11). It was first opened 60 min after the desired working pressure had been reached; thereafter, the reactor was semicontinuous. The outgoing $CO_2$ with the extracted reaction components was fed into a cold trap (12) in which the extract was collected. Both the flow rate (0.01–0.02 L $CO_2$/min) and the amount of outgoing $CO_2$ were monitored on a mass flowmeter (14). When the amount of exhausted $CO_2$ had reached 4.5 L, the shut-off valve (8) was closed, and the cold trap (a glass tube) (12) containing the extract was removed. The reactor was depressurized to atmospheric pressure, the residue was dissolved in 30 mL cylohexane and filtered through a Munktell No. 3 filter paper into a previously weighed round-bottom flask. A rotary evaporator was used to remove the solvent, and the amount of residue was determined gravimetrically. During the experiment, some ethanol was extracted and collected along with the reaction components in the cold trap (12). This was removed with pressurized $N_2$, and both the amounts of ethanol and reaction components were determined gravimetrically. All the data presented here represent the means of two or three determinations. Experiments with a high-pressure viewing cell have established that the reaction medium (in absence of enzyme) comprises two phases.

*Thin-layer chromatography (TLC).* Stock solutions (concentration: 75 mg lipid/mL chloroform) were prepared from both the extract and the remaining lipid residue obtained from the enzymatic reaction experiment. A 250-μL Unimetrics syringe (Labassco, Göteborg, Sweden) was used to apply the solution (200 μL) as bands on TLC plates (Kiselgel 60, Art 5721, 0.25 mm; Merck, Darmstadt, Germany). The solvent system used to separate TG, DG, MG, FFA, and EE consisted of hexane/diethyl ether/formic acid (80:20:2). The component lipid classes were detected by spraying with 0.2% 2′-7′-dichlorofluorescein in ethanol and identified by comparison with standard mixtures. Bands were scraped off into screw-

capped (Teflon-lined) centrifuge tubes. To these were added methyl heptadecanoate (17:0) and methyl tricosanoate (23:0) in the form of a 1.0-mL solution in tetrahydrofuran. The methyl heptadecanoate served as an internal standard for shorter-chain fatty acids (C14–18), and the methyl tricosanoate for longer-chain fatty acids (C ≥ 20).

*Preparation of methyl esters from the TG, DG, and MG lipid classes.* Fatty acid methyl esters were formed by adding 2.0 mL of 0.5 M sodium methoxide in methanol to the scrapings (and internal standard) from the TLC plates. The reaction tube was flushed with nitrogen ($N_2$), and the mixture was then thoroughly vortexed and heated for 10 min at 50°C. After incubation, 1 mL hexane and 2 mL saturated NaCl solution were added. The mixture was vortexed and centrifuged, and the upper layer was recovered for gas-chromatographic (GC) analysis.

*Preparation of methyl esters from FFA.* One mL of 7% boron trifluoride reagent was added to the FFA band (and internal standard) from the TLC plates. The tube was flushed with $N_2$, vortexed thoroughly and heated for one hour at 100°C. The reacted mixture was then cooled, hexane (1 mL) and water (1 mL) were added, the mixture was vortexed, and the top layer was recovered. The aqueous layer was then again extracted with hexane. The combined methyl ester extracts were concentrated slightly before injection into the GC.

Because the EE band required no derivatization, EE were extracted directly from the TLC scrapings, and the concentrated extract was used for GC analysis.

*GC.* Fatty acid esters were analyzed on a Varian 3700 gas chromatograph equipped with a flame-ionization detector, a digital integrator, and a DB-WAX column (30 m × 0.25 mm i.d., phase thickness 0.25 μm; J&W Scientific Inc., Folsom, CA). The split ratio was 1:100, helium (carrier gas) flow rate, 1 mL/min, and injection port temperature, 240°C. The GC was temperature-programmed from 140 to 220°C at 3°C/min, with a final 8-min hold at 220°C.

*Quantitation of fatty acids and lipid classes.* Area data for the fatty acid analyses were corrected for flame-ionization response (36–38), and absolute values for each fatty acid detected were obtained by the use of an internal standard. The method described by Christie *et al.* (39) was used to obtain a close approximation to the actual weight of the lipid classes present in the sample.

## RESULTS AND DISCUSSION

The enzyme reaction was studied in a batch reactor at 40°C, and the pressure was varied from 9 to 24 MPa. Our results show that Novozym 435 catalyzed the alcoholysis of CLO under these conditions (25% conversion at 9 MPa). A literature search revealed that so far this enzyme has not been used to catalyze enzymatic reactions in SCF. The stability and activity of other enzymes in $SCCO_2$ have, however, been studied by a number of authors. Our primary objective was to determine whether one could preferentially extract the EE synthesized during the course of the reaction in $SCCO_2$. Preliminary studies showed that the extract contained other lipid classes, such as TG, DG, MG, and FFA, in addition to the EE. The next question was whether changes in pressure could be used to manipulate the selectivity of the $CO_2$. Figure 2 clearly shows that the selectivity of the $CO_2$ for EE decreases as pressure increases. Thus, $CO_2$ is most selective at the lowest pressure (9 MPa), where the collected extract contains the largest proportion of EE. It is least selective at the highest pressure (24 MPa), where the extract contains virtually equal amounts of EE and TG.

Fundamental data on the solubility of various pure lipid components in $SCCO_2$ are available (40). The conclusion from those studies is that the solubilities of lipid components tend to increase with pressure. Liang and Yeh (27) measured the solubilities of some selected pure EE and studied the effect of changes in pressure and temperature on the separation efficiency of a model mixture of EE. They reported that high solubilities at high pressures and low temperatures resulted in low separation efficiency. Staby and Mollerup (25) measured the K-values (the concentration of a component in the gas phase, divided by its concentration in the liquid phase) of fish oil fatty acid EE, prepared from sand launce oil. They compared the K-values of selected fish oil fatty acid EE (EPA, DHA, myristic, palmitic, and oleic acid) and observed that the relative difference in the K-values became greater with reduced pressure, whereas the absolute size of the K-value became smaller. They also suggested that the optimal extraction conditions for the separation of EE by chainlength would be 9 MPa at 313.2 K (40°C). The results of these investigations are thus all in agreement with the observed loss in selectivity at higher pressures in this study. Another explanation for this observation is that the enzyme activity itself might be sensitive to changes in pressure.

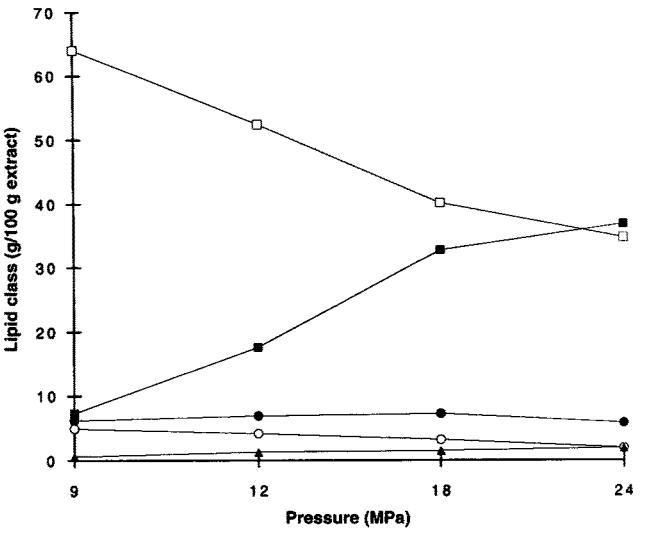


**FIG. 2.** Amounts of lipid classes present in the total material extracted after 4.5 L $CO_2$ consumption, collected at different working pressures. Symbols: ■, Triglyceride; □, ethyl esters; ●, diglyceride; ○, ,monoglyceride; ▲, free fatty acid.

The yields of the lipid classes in the extract based on 100 g of CLO substrate are illustrated in Figure 3a. Comparison of Figures 2 and 3a confirms again that, although the extract contains the largest proportion of EE at the lowest pressure (9 MPa), the yield of EE is also lowest at this pressure. The relative amount of EE is slightly lower in the extract collected at 12 MPa than in that collected at 9 MPa (Fig. 2). However, the yield of EE is much higher at this pressure (Fig. 3a). These results suggest that, to achieve extracts of acceptable purity (with regard to EE) in high yields, the working pressure should be around 12 MPa. At higher flow rates of $CO_2$ than were used here (0.01–0.02 L $CO_2$/min), it might be possible to increase the yield of EE at 9 MPa. This is currently under investigation in our laboratory. The yields of the lipid classes in the remaining lipid residue were also determined (Fig. 3b).

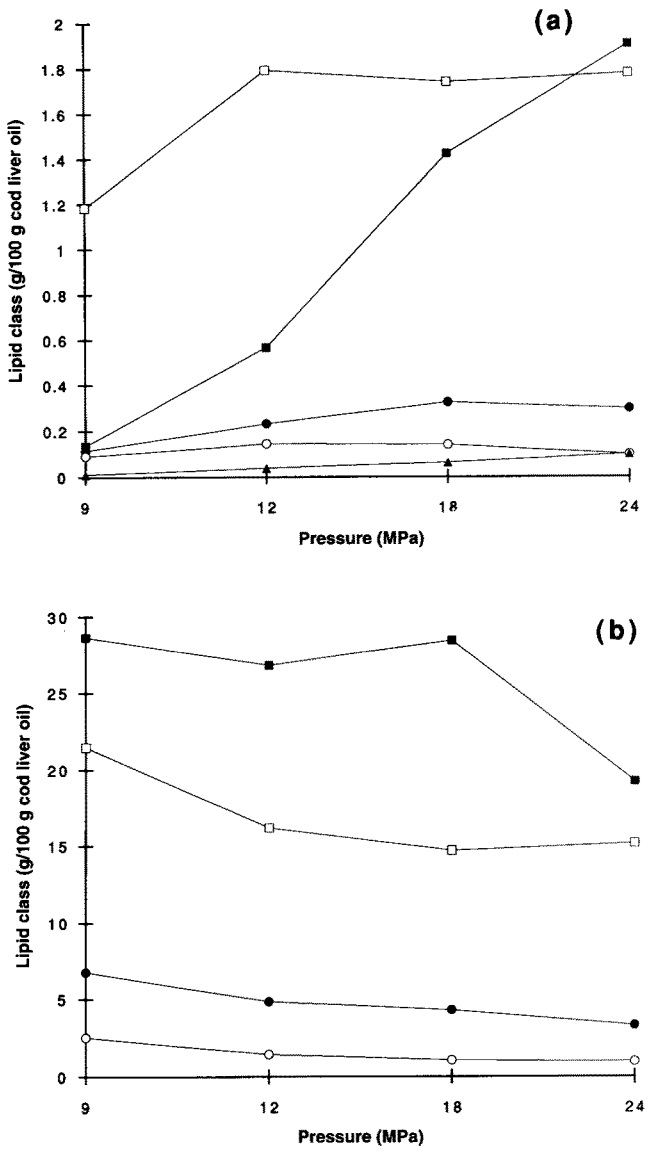


**FIG. 3.** Yield of lipid classes (a) in the total material extracted after 4.5 L $CO_2$ consumption and (b) in the remaining lipid residue at different working pressures. Symbols as in Figure 2.

The results show that EE could be detected in the residue at all working pressures. The yield of all lipid classes detected in the remaining residue decreased with increasing pressure. This is consistent with the fact that the yield of each lipid class in the extract increases with increasing pressure (Fig. 3a). Figure 3 shows that overall mass balance in the experiments is violated. This lipid loss defect increased with increasing pressure (Table 1). The main source of lipid loss is probably extracted lipid components that precipitate and build up in the tube section between the reactor (9) and the cold trap (12). Again, some of the extracted lipid components might not fall out of solution when the $CO_2$-rich phase is expanded (11). They will thus be carried along with the exhausted $CO_2$ gas rather than being collected in the cold trap (Fig. 1). In addition, some lipid components may be entrapped in the pores of the enzyme support.

The secondary objective of this study was to investigate whether this technology could be used to produce PUFA-enriched EE. The percentage of EPA and DHA of the total fatty acids detected in the CLO substrate were 13 and 17%, respectively. However, in the EE lipid class, present in the extract, the amount of EPA was 19–20% and that of DHA 5–8%, depending on pressure. The technology presented here can be used to carry out enzymatic catalysts and product, EE, fractionation simultaneously. However, this technology per se is not an effective way to produce PUFA-enriched EE, and further purification of the valuable EPA and DHA EE from the produced mixture of fish oil EE may be carried out either by previously documented methods (31,41), or by preparative SCF chromatography in connection with the enzyme process. It is feasible that such a subsequent purification step can be carried out on-line.

To determine if the selectivity of $CO_2$ for either DHA or EPA present in the various lipid classes is affected by changes in pressure, the amount of those fatty acids relative to the amount of palmitic acid was plotted against increasing pressure (Figs. 4 and 5). In the CLO substrate, the relative amount of DHA to palmitic acid was 1.66, and for EPA to palmitic acid it was 1.34. Only the relative amount of DHA present in the MG lipid class of the extract increased significantly with pressure. A slight increase was also observed in the TG, DG, and EE lipid classes of the extract. In all other instances, the

**TABLE 1**
**The Total Amount of EE Produced, the Loss of Lipid Components and the Loss of Ethanol During the Experiment[a]**

| Pressure (MPa) | Conversion (%) | Corrected conversion (%) | Lipid loss[b] (%) | Ethanol loss[c] (%) |
|---|---|---|---|---|
| 9 | 25 | 37 | 25 | 21 |
| 12 | 20 | 34 | 35 | 21 |
| 18 | 19 | 30 | 36 | 20 |
| 24 | 19 | 30 | 41 | 24 |

[a]Data shows the average of two or three determinations.
[b]The initial amount of fish oil less the sum of the residue and the extracted lipid, as a percentage of the initial fish oil weight.
[c]The amount of ethanol in the extract as a percentage of the initial ethanol weight.

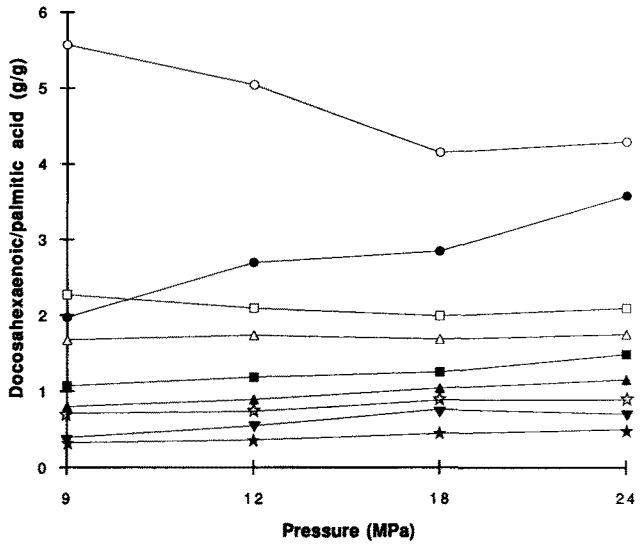


**FIG. 4.** The relative amount of docosahexaenoic acid to palmitic acid in the lipid classes present in the extract and the remaining lipid residue as a function of system pressure. Symbols: ■, triglyceride in the extract; □, triglyceride in the residue; ▲, diglyceride in the extract; △, diglyceride in the residue; ●, monoglyceride in the extract; ○, monoglyceride in the residue; ★, ethyl esters in the extract; ☆, ethyl esters in the residue; ▼, free fatty acids in the extract.

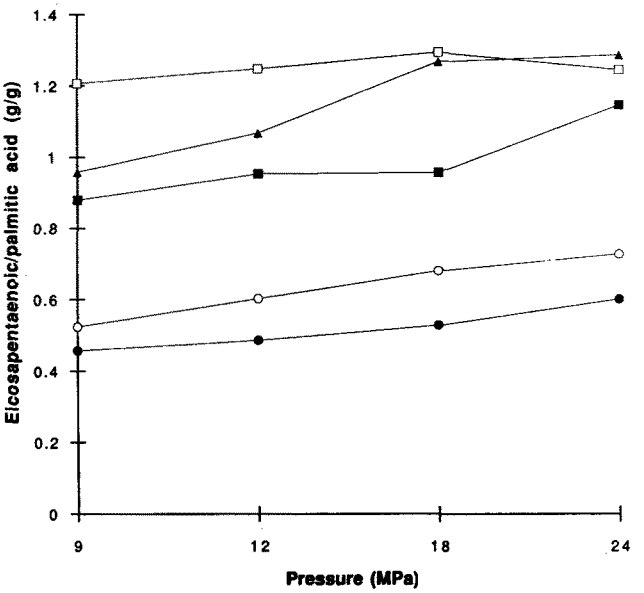


**FIG. 5.** The relative amount of eicosapentaenoic acid to palmitic acid in the lipid classes present in the extract as a function of system pressure. Symbols as in Figure 2.

amount remained virtually unchanged (Fig. 4). Similarly, the relative amount of EPA to palmitic acid increased with pressure in the TG, DG, MG, and FFA lipid classes present in the collected extract (Fig. 5). No such increase was noted in the relative amount of EPA/palmitic acid for the lipid classes present in the remaining lipid residue (results not shown here).

The solubilities of pure ethyl palmitate, ethyl EPA and ethyl DHA in $CO_2$ (binary mixtures) have been reported by Liang and Yeh (27) to increase with increasing pressure. However, in general, the solubilities of the pure fatty acid EE decreased with the increase of carbon number in the molecule. Thus, at the same temperature and pressure, ethyl palmitate was more soluble than ethyl EPA and ethyl DHA, respectively (27). These findings contradict our results. By contrast, we observed an increase with pressure in the relative amounts of both EPA and DHA present in some lipid components. If our results followed the same pattern as that observed for the pure EE by Liang and Yeh (27), the relative amounts for both fatty acids under investigation should have remained unchanged with pressure.

The apparent contradiction can be resolved as follows. Staby (29) measured the K-values of various fatty acid EE present in a natural mixture of fish oil fatty acid EE. He compared the measured K-values of ethyl oleate, eicosapentaenoate, and docosahexanenoate with the binary data of Bharath *et al.* (26) as a function of pressure (9–15 MPa) at 313.2 K (40°C). The experimental K-values reported by Staby (29) for both ethyl EPA and ethyl DHA were higher than the binary value at pressures between 10–15 MPa. For the DHA EE, the difference between the two values increased with increasing pressure. Hence, the solubilities of both EPA and DHA EE were enhanced when present in the fish oil fatty acid EE mixture. However, the experimental K-value for ethyl oleate was slightly lower than the binary value at all pressures under investigation. Staby (29) suggested that the enhanced solubility of the EPA and DHA EE might be due to an entrainer effect from the lighter components and that the increased solubility of the heavy components in the SCF phase led to a decrease in the solubility of the lighter EE when compared with binary data. Because we are also studying a complex multicomponent mixture, the enhanced solubility of the DHA EE with pressure, reported by Staby (29), may explain the slight increase with pressure in the relative amount of DHA EE in the EE lipid class of the extract (Fig. 4).

The observed increase with pressure in the relative amounts of both EPA and DHA in some of the other lipid classes present in the extract (Figs. 4 and 5), especially in the MG lipid class, might be explained by the fatty acid specificity of the lipase enzyme used in this study. There are reports in the literature of fatty acid specificity of some lipases. As an example, *Candida rugosa* (previously *C. cylindracea*) has been reported to have a low activity toward some fatty acids, especially the PUFA such as EPA and DHA (42). *Mucor miehei* has also been reported to discriminate strongly against DHA (43,44). The high ratio of DHA observed, roughly 52% of all fatty acids detected in the MG lipid class in the remaining residue at 9 MPa, suggests that the enzyme used in this study (from *C. antarctica)* liberates the shorter and less unsaturated fatty acids before DHA. The result is that DHA accumulates to the largest extent in the MG lipid class but also to some degree in the TG and DG lipid classes. Such accumulation of DHA in the MG lipid class would result in

an apparent enhanced solubility of this lipid class with pressure in the SCF phase. The effect is similar to that reported by Staby (29) for the DHA EE in the fish oil EE mixture. The same argument can be invoked to explain the observed relative increase in extracted EPA with pressure in some lipid components present in the extract. It is also possible that the fatty acid selectivity of the lipase is pressure-dependent.

It should be kept in mind that our system consists of a complex multicomponent mixture and that various factors may effect the solubility of the lipid components present in this mixture. Only a few investigators have studied the solubility of a mixture of lipid components in $SCCO_2$ in detail (45–47). Nilsson and co-workers (46) determined the partition coefficient of mono-, di-, and trioleylglycerol in a quaternary mixture (MO–DO–TO–$CO_2$). They observed that, compared to the solubility of pure MO and DO, their solubility was enhanced when present in the quaternary mixture, whereas the solubility of TO remained unchanged. Later, Nilsson and Hudson (47) measured the solubility of a mixture of TG containing tripalmitoylglycerol (PPP), trioleoylglycerol (OOO) and various isomers of palmitoyl–dioleoylglycerol (POO) and oleoyl–dipalmitoylglycerol (PPO). They found that PPP was much more soluble in $SCCO_2$ when present in the triacylglycerol mixture then it was as a pure component. The studies carried out by Nilsson and co-workers enable us to understand why it is difficult to predict the phase behavior of a complex multicomponent mixture.

Conversion of the CLO substrate to EE at various pressures is given in Table 1. To calculate the conversion, the molecular mass of the CLO substrate (879 g/mol) was calculated from the fatty acid composition. The total amount of EE produced during the experiment was obtained by adding the amount of EE in the remaining lipid residue to the amount determined in the extract. We assume that the entire CLO substrate used in the experiments (usually 3.0 g) can be converted to EE. The results suggest that the conversion decreases with increasing pressure (Table 1). However, Table 1 also reveals that the loss of lipids increased with pressure. Thus, some of the EE, synthesized during the experiment at higher pressures, might not be accounted for. The sources of lipid loss were discussed above. To account for the lipid loss, a corrected conversion was calculated by assuming that the lipid components lost in the tubes and valves and entrained with the exhausted $CO_2$ gas provide the main source of lipid loss. The lipid class composition of the lost lipid was then taken to be the same as that of the extract (this assumption has been confirmed in later experiments). However, even the corrected value suggests that the conversion decreases with increasing pressure (Table 1). Probably, the increasing pressure changes the way the reactants partition between the supercritical phase and the liquid phase. The lipid class composition of the extract (Fig. 2) suggests that, as the pressure increases, a larger proportion of the TG becomes dissolved in the SCF phase. Hence, these are then presumably no longer available as reactants. This expectation is reinforced by considering some experiments of Mittelbach (48). He studied lipase-catalyzed alcoholysis of sunflower oil. The experiments were carried out at atmospheric pressure with petroleum ether as solvent, and the proportion of reactants and lipase were virtually the same as in our experiments. However, using a *Candida* (SP-382) lipase, Mittelbach (48) reported a 79% conversion of the sunflower oil substrate to EE, a much higher conversion than that observed in this study (Table 1). The differences are almost certainly due to the fact that different solvents and pressure were used. An additional complicating factor that inhibits direct comparison of our results with other experiments is that some of the ethanol (see discussion below) and TG charged into the reactor in our experiments will not be available as reactants due to their dissolution in the SCF phase. This may slow down the rate of the enzyme reaction, which, in turn, would explain the low conversion observed in this study. Another factor might be the water content of the enzyme, because $SCCO_2$ has been reported to strip away the enzyme's essential monolayer of water (8,15).

Unfortunately, the ethanol substrate dissolved to some degree in the SCF phase, so that it was extracted along with the lipid components. The amount of ethanol determined in the collected extract was virtually the same at all working pressures (Table 1). In the experiments, a quantity of approximately 3.8 times the stoichiometric amount of ethanol was used. An estimate of the loss of ethanol during the experiment was obtained from a total system blank study carried out at 9 and 24 MPa. The results showed that, even in the most extreme case (24 MPa), a quantity equal to 1.3 times the stoichiometric amount of ethanol remained in the reactor at the end of the five and one-half hour experiment. It is apparent that some of the ethanol charged into the reactor dissolves in the SCF phase and is, therefore, no longer available as a reactant. To optimize yield, it might be necessary to use an even larger excess of ethanol. A better process would be adding ethanol continuously to the reaction mixture, because an abundant excess of ethanol has been reported to inhibit esterification of oleic acid and ethanol catalyzed by an immobilized lipase from *M. miehei* (9).

Notwithstanding the complexity in interpretation, the results of this study do show that the synthesized EE can be preferentially extracted at low pressures from the unconverted CLO substrate and its side-products. Further studies are required to optimize the various process parameters involved to achieve higher yields of the synthesized EE. The prospect exists that it might be possible to use such an integration of biocatalysis and product fractionation to synthesize EE from other types of oils (such as vegetable oils) that could be used as diesel fuel substitute.

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