Lipase-Catalyzed Alcoholysis with Supercritical Carbon Dioxide Extraction 1: Influence of Flow Rate

Helga Gunnlaugsdottir* and Björn Sivik

Department of Food Technology, Lund University, 22100 Lund, Sweden

ABSTRACT: A combined process of lipase (E.C. 3.1.1.3) catalysis and extraction of product with supercritical carbon dioxide was studied. The effect of different flow rates of the extraction fluid on the selective removal of the ethyl esters (EE) synthesized in a lipase-catalyzed alcoholysis of cod liver oil with ethanol was investigated. The faster the flow rate, the faster the extraction rate and the higher the recovery of EE. For example, after a 270-min extraction, the total recovery of EE was 1520 mg for a flow rate of 0.3 liter carbon dioxide at atmospheric pressure and room temperature/min (NL/min) as compared to 250 mg when 0.015 NL/min was used. The concentration of EE in the carbon dioxide was found to decrease with increasing flow rate, which indicates that the rate of diffusion of EE limits their extraction at fast flow rates. A high flow rate was found to result in a more selective extraction of EE, i.e., less amounts of other lipid components present in the reaction mixture were coextracted with the EE. Further, by increasing the flow rate, the equilibrium of the reaction was shifted slightly toward ester synthesis. An increase in the flow rate from 0.015 to 0.075 NL/min resulted in an approximately 10% increase in total conversion (from 73 to 82%), whereas only a negligible increase was obtained when the flow rate was increased further to 0.15 NL/min.

JAOCS 74, 1483–1490 (1997).

KEY WORDS: Alcoholysis, enzyme, extraction, fish oil, flow rate, lipase, supercritical carbon dioxide.

In recent years, enzyme catalysis in water-poor nonaqueous media, such as organic solvents, has received some attention. Supercritical fluids (SCF) have been considered as an attractive alternative to ordinary organic solvents. Enzyme catalyses in SCF have been carried out by several research groups, and the status of this field has been reviewed (1–4). These fluids offer the same advantages for lipase (E.C. 3.1.1.3) catalysis as organic solvents. These advantages are solubilization of the hydrophobic lipid substrates, simple recovery of enzyme, as well as the possibility for the reversal of hydrolysis reactions in favor of synthesis. But SCF offer more. For in addition, a combined process of enzyme catalysis and product fractionation can be carried out. Further, the solvent easily can be recycled since the product can be recovered by pressure/temperature reduction. Supercritical carbon dioxide (SC-CO_2) is an especially attractive process solvent for enzymatic catalysis as it offers even more advantages over organic solvents, e.g., it is nonflammable, nontoxic, and inexpensive.

Fish oils are a potential source of long-chain n-3 polyunsaturated fatty acids 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 (5,6) and cardiovascular disease (7,8). As a result, the general public has been encouraged to increase the dietary intake of n-3 fatty acids. Consequently, there is a demand for enriched concentrates of these fatty acids. In this paper we describe lipase-catalyzed alcoholysis of cod liver oil in SC-CO_2. Cod liver oil is a fair source of EPA and DHA, as it contains approximately 10% of each fatty acid. The enzyme reaction under investigation results in a complex mixture of lipid components consisting of triglycerides (TG), diglycerides (DG), monoglycerides (MG), and ethyl esters (EE). We set out to extract the product (EE) from the reaction mixture, with the principal goal being efficient recovery of EE at high purity. The esters produced are of interest because they contain ethyl eicosapentaenoate and ethyl docosahexaenoate, which could be concentrated in a later step of the process. Further, by selectively extracting either the desired product or the by-product from the reaction mixture, one can shift the equilibrium of the enzyme reaction in the direction of further synthesis. The effect of shifting the thermodynamic equilibrium in this way was first investigated by Doddema and coworkers (9). They studied a continuous lipase-catalyzed transesterification of ethyl acetate and nonanol; the ethanol produced in this process (an inhibitor of the reaction) was removed. Adschiri *et al.* (10) shifted the equilibrium in a batch interesterification reaction between tricaprylin and methyl oleate by a selective removal of the by-product, methyl caprylate, from the reaction mixture with SC-CO_2. Likewise, Shishikura and coworkers (11) reported on improved lipase-catalyzed incorporation of long-chain fatty acids into medium-chain TG in a batch reaction. This they achieved by extraction of the by-product (medium-chain fatty acids) with SC-CO_2.

An integrated process of the type described here opens new possibilities for the application of biocatalysis in industrial processing. Clearly it offers potential for simplifications in downstream processing. This kind of process should

*To whom correspondence should be addressed at Department of Food Technology, Lund University, P.O. Box 124, 221 00 Lund, Sweden.
E-mail: Helga.Gunnlaugsdottir@livsteki.lth.se.

preferably be both fast and efficient. For this reason we have investigated the effect of flow rate of the extraction fluid on the selective removal of EE from the residual cod liver oil substrate and from the side products. The effect of changing the flow rate of the extraction fluid has not yet been reported by other researchers concerned with similar extractive reactions (enzyme catalysis with extraction of product/by-product in SC-CO_2). Although a detailed study of the effect of flow rate on the efficiency of supercritical fluid extraction (SFE) of analytes was carried out by Hawthorne and coworkers (12), their work exclusively dealt with extraction of components from various matrices and not from a reaction process.

Earlier results revealed that it is possible to extract the synthesized EE preferentially from the unconverted substrate and its side-products at low pressures (9 MPa/40°C) (13). These reaction conditions were therefore adopted for this work. Further, a separate study has established that the reaction system comprises three phases, i.e., a vapor and liquid phase in addition to the solid phase containing the immobilized enzyme (14).

The main objectives of this work were to study (i) the effect of flow rate on the extraction rate and recovery of EE; (ii) the effect of flow rate on the composition of extracts and liquid phases; (iii) whether the equilibrium of the enzyme reaction could be affected by the flow rate of the extraction fluid; and finally, (iv) to determine the amount of ethanol and CO_2 in the liquid phase and how changes in flow rate affect this.

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 units/g toward propyl laurate. The cod liver oil used was of commercial quality and was obtained from Lysi h.f. (Reykjavik, Iceland). CO_2 (purity 99.99%) was supplied by Air Liquide Gas AB (Malmö, Sweden). High-performance liquid chromatography (HPLC) grade hexane, 1-propanol, and water were from Fisher Scientific (Loughborough, United Kingdom), and acetic acid was p.a. grade from Prolabo (Paris, France).

Equipment and procedure. Figure 1 presents the experimental equipment (identified by numbers) used to conduct all experiments in SC-CO_2. Cod liver oil (4.0 g), ethanol (2.4 g; 99.5%) and enzyme (0.8 g) were placed in a 30-cm^3 reactor. The reactor was then connected to the equipment and pressurized by pumping CO_2 into the reactor. The substrates were continuously mixed using a magnetic stirrer. The sampling line for the vapor phase was located on the top of the reactor. The outgoing CO_2 was depressurized to atmospheric pressure by opening a heated micrometering valve [number 12]. This valve was first opened 60 min after the desired working pressure had been reached; thereafter the process became an extractive reaction. The outgoing CO_2 with the extracted reaction components and ethanol were first fed into a cold trap

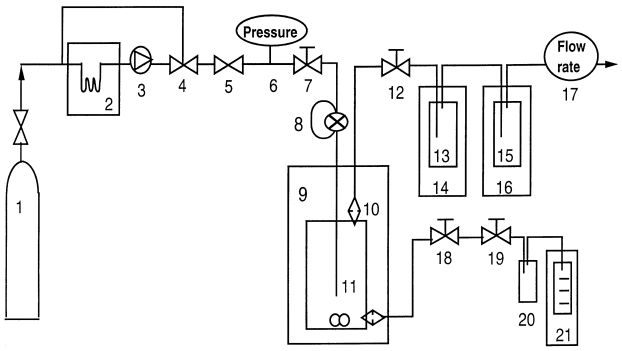

FIG. 1. Schematic diagram of the experimental apparatus: 1. gas tube; 2. cool bath (–10°C); 3. membrane pump; 4. relief valve; 5. check valve; 6. pressure meter; 7. shut-off valve; 8. injection loop; 9. water bath (40°C); 10. filter (3.2 mm o.d. × 0.8 mm thick, 0.25 mm); 11. reactor; 12. micrometering valve; 13. and 15. cold traps; 14. cooler (at 0°C); 16. cooler (at –60°C); 17. flow meter; 18. and 19. micrometering valves; 20. sampling vial; and 21. calibrated graduated tube.

[number 13] held at 0°C [number 14] and then further into a second cold trap [number 15] cooled with dry ice [number 16]. Both the flow rate and the amount of outgoing CO_2 were monitored by means of a mass flow meter [number 17]. The sampling line for the liquid phase was located on the bottom of the reactor. The sampling of this phase was carried out in the following manner: First, valve [number 19] was closed and valve [number 18] slowly opened and was kept open for approximately 10 min. Then valve [number 18] was closed and valve [number 19] opened slowly. This first sample of the liquid phase was discarded and a second sample was taken using exactly the same procedure. The second sample was collected in a small closed glass vial [number 20], and the volume of CO_2 present in the sample was determined by slowly expanding the CO_2 across the valve [number 19] and displacing water in a calibrated graduated tube [number 21].

The reaction was generally carried out for 330 min at 9 MPa and 40°C. The flow rate of CO_2 was varied between 0.015 NL/min to 0.3 NL/min. Three samples of both the vapor and liquid phases were taken at intervals of 110, 220, and 330 min after the experiment was started. While the samples were being taken, the magnetic stirrer was turned off. Ethanol (0.35 mL, 99.5%) was added to the reactor *via* a sample loop [number 8]. This was done three times: 83, 165, and 248 min after the experiment was started. When the flow rate of 0.3 NL/min was tested, the reaction was carried out for 660 min. For this flow rate, six samples were taken and ethanol was added six times to the reactor. After the reaction had been carried out for 330/660 min, the reactor was depressurized to atmospheric pressure, and 40 mL *n*-propanol was then added to the residue. This solution was then filtered through a Munktell No. 3 filter paper (Grycksbo pappersbruk, Stora Kopparberg, Sweden) into a previously weighed round-bottom flask. A rotary evaporator was used to remove the solvent, and the amount of residue was determined gravimetrically. The residue obtained was used in subsequent phase-behavior experiments (14).

During the experiment some ethanol (EtOH) was extracted and collected along with the reaction components in the cold traps [numbers 13 and 15]. This was removed using a stream of N_2 gas. Both the amounts of EtOH and of reaction components were determined gravimetrically. The amount of EtOH present in the liquid-phase samples was evaluated using the same procedure. All the data represent the means of two or four determinations.

Analysis of products. The overall content of TG, DG and MG, as well as free fatty acids (FFA) and fatty acid EE, of the lipid samples collected was determined by liquid chromatography. Separations were performed at ambient temperature by means of a column (250 × 3 mm) packed in the laboratory with diol-modified silica (5 µm, LiChrospher 100 DIOL) from Merck (Darmstadt, Germany). The mobile phase consisted of (i) hexane/acetic acid (99:0.5, vol/vol) and (ii) hexane/1-propanol/acetic acid/water (85:15:0.5:0.1, by vol). The linear gradient timetable was: at 0 min, 100:0; at 6 min, 50:50; at 18 min, 50:50; at 24 min, 100:0; at 45 min, 100:0, (%A:%B, respectively) at a solvent flow rate of 0.4 mL/min between 0 to 24 min, 0.8 mL/min between 24 to 35 min, and 0.4 mL/min between 35 to 45 min. A Waters (Milford, MA) model 600E HPLC instrument with a Rheodyne 7125 injector (Cotati, CA) (20 µL loop) was used. Detection was accomplished with an evaporative light-scattering detector (Sedex 55; Sedere, Alfortville, France) set at an air inlet pressure of 2.0 bar and a temperature of 45°C.

Calibration. A stock solution of a five-component composite standard, representing the lipid classes detected in the lipid samples, was made up in hexane. All standards were from Sigma (St. Louis, MO). The standards were as follows: palmitic acid EE; tripalmitin; palmitic acid; dipalmitin (mixed isomers); 1-monopalmitoyl-*rac*-glycerol. Dilutions of the stock solution were made, and a dose–response curve of the standards was constructed by injecting 20-µL aliquots of the composite standards (0.002–0.5 µg/µL). The correlation between dose and response of each standard was then fitted to an equation.

RESULTS AND DISCUSSION

Extraction of EE. The effect of flow rate on the extraction rate of EE from the reaction mixture is illustrated in Figure 2. The results show that the higher flow rate results in a faster extraction and a higher recovery of the EE. This behavior is likely due to the fact that the reaction mixture is exposed to more extraction fluid during a set time period. According to Hawthorne and coworkers (12), the extraction process of components in analytical SFE comprises two major steps. First, the analyte must be transported from the matrix into the SCF; this process has been called the desorption/kinetic step. Second, the analytes must be swept from the extraction unit; this process is termed the solubility/elution step. Normally, both steps occur more or less simultaneously. Our work deals not only with an extraction like the work of Hawthorne and coworkers (12). Nevertheless, we suggest that the same principles may be applied to determine the rate-limiting step in our extractive reaction process.

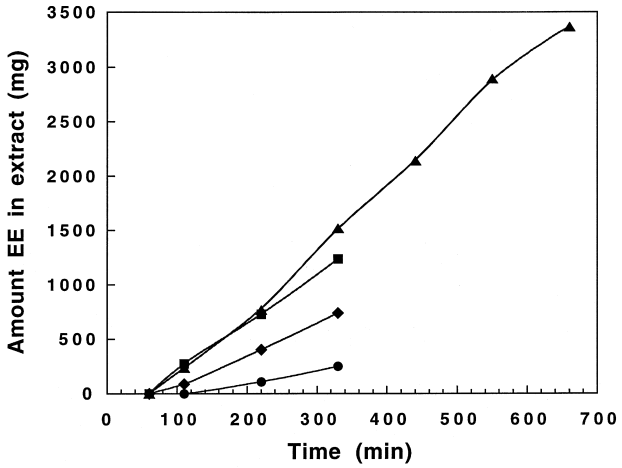

FIG. 2. Effect of flow rate on the extraction of ethyl esters (EE) from the reaction mixture. Symbols: ● 0.015 NL/min; ◆ 0.075 NL/min; ■ 0.15 NL/min; and ▲ 0.3 NL/min. NL/min = liter CO_2 at atmospheric pressure and room temperature/min.

Figure 2 reveals that the plots of the recovery of EE vs. time at different flow rates are almost straight lines. It has been suggested by Hawthorne and coworkers (12) that this feature indicates that the extraction of an analyte (in our case EE) is primarily controlled by the solubility/elution step. However, they have also reported that if the extraction is primarily limited by the solubility/elution step, the quantity of the analyte extracted must be proportional to the volume of CO_2 that has been used for extraction. This effect was not observed in our work (Table 1), which suggests that the extraction of EE from the reaction mixture is limited primarily by the solubility/elution step in the beginning of the extraction. Thereafter, the desorption/kinetic step starts to dominate the extraction rate. If this were the case it explains the lack of increase in the extraction rate of EE between 0.15 and 0.3 NL/min (Fig. 2). In our work, the so-called desorption/kinetic step includes both the internal mass transfer of substrates and products within the immobilization support and the external mass transfer in the bulk liquid phase.

Figure 3 shows the concentration of EE in the extract vs. time at various flow rates. The trend observed from the plots is that the concentration of EE in the CO_2 decreases with increasing flow rate. The higher concentration of EE at the 0.015 NL/min flow rate may be a consequence of the extra time for equilibration of EE in the extraction medium. How-

TABLE 1
Effect of CO_2 Flow Rate on Ethyl Esters (EE) Extracted from the Reaction Mixture at 9 MPa and 40°C

Flow rate (NL/min)[a]	0.015	0.075	0.15	0.3	0.3
Extraction time (min)	270	270	270	270	600
Total EE extracted (mg)	249	739	1238	1520	3368
Total CO_2 (L)	4.2	20.7	40.6	81.8	179.6

[a]Liter CO_2 at atmospheric pressure and room temperature/minute.

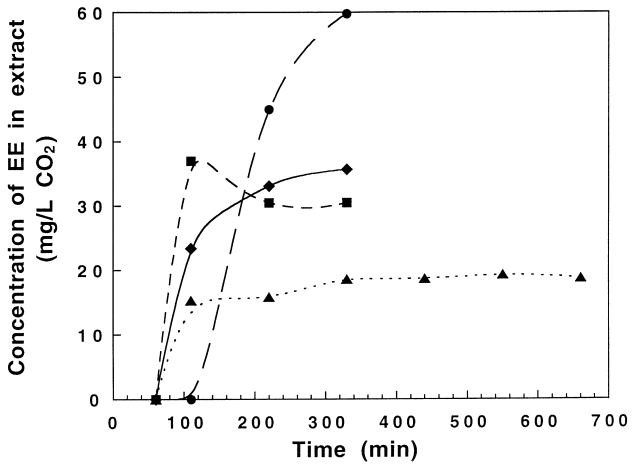

FIG. 3. Effect of flow rate on the concentration of EE in carbon dioxide. Symbols and abbreviation as in Figure 2.

ever, this high value might also be explained by experimental start-up effects. By this we mean that the extracted EE precipitated and built up in the tube section between the reactor and the first cold trap (Fig. 1), and thus the extract was collected periodically instead of continuously. This circumstance would also explain why no extract was obtained in the first sampling period at this flow rate (Figs. 2 and 3). The lower concentration of EE in the CO_2 at the faster flow rates (Fig. 3) suggests that it is the diffusion rate of EE within the liquid phase into the extraction fluid that controls the extraction of EE at these flow rates. Hence, equilibrium conditions were probably not reached. The apparent higher concentration of EE in the CO_2 observed in the beginning of the curve at 0.15 NL/min (Fig. 3) could be the result of two factors. First, when the micrometering valve is opened after a 60-min static period, there is suddenly a very effective flow through the reactor, which may have resulted in a very energetic mixing of the liquid phase and thus in an effective extraction of the EE from the liquid phase. Second, entrainment from the liquid phase could explain this high value. In our opinion, the first factor is the more likely explanation.

The equilibrium solubility of fish oil fatty acid EE from sand launce oil has been reported to be approximately 20 mg/L CO_2 at 9 MPa and 40°C (15). We found that the concentration of EE in the CO_2 was between 15–60 mg/L CO_2 depending on flow rate (Fig. 3). As discussed above, experimental start-up effects could explain the high value obtained at the slowest flow rate. Nevertheless, our values for the solubility of EE tended to be higher than the value reported by Staby and Mollerup (15). Probably, the EtOH dissolved in the SC-CO_2 results in the higher solubility of EE in the extraction medium. This supposition accords with the results presented by Nilsson and coworkers (16). They investigated partition coefficients (concentration of component *i* in the CO_2 phase/concentration of component *i* in the liquid phase) for fatty acid EE in SC-CO_2 with and without EtOH and found that the addition of EtOH increased the partition coefficients for all fatty acid EE studied.

The effect of changing the flow rate of the extraction fluid has not been previously reported by other researchers concerned with similar extractive reaction processes. The flow rate of the SCF medium has been used as a means of studying the effect of mass transfer in packed enzyme columns by measuring the reaction rate as a function of fluid velocity. Work of this nature has been carried out for continuous (17,18) and semicontinuous (19,20) lipase-catalyzed reactions in SC-CO_2. As that work is quite dissimilar to the extractive reaction studied here, direct comparison is not possible. Further, it is difficult to compare our results with literature values based solely on extraction of analytes with SC-CO_2 as in the work carried out by Hawthorne and coworkers (12).

Composition of extracts. The effect of flow rate on the lipid class composition of the three extracts collected during the experiment is shown in Figure 4. When the slowest flow rate (0.015 NL/min) was used, no extract was obtained during the first sampling period (Fig. 4A). However, the extracts collected using the other two flow rates tested contained coextracted lipid components such as TG, DG, MG, and FFA in addition to the EE. Further, the proportion of EE in the extract was lower at the 0.075 NL/min flow rate than at 0.15 NL/min (Fig. 4A). In the next extract collected (Fig. 4B) the proportion of EE also increased with increasing flow rate. At the fastest flow rate (0.15 NL/min), only EE were present in the extract. This trend is also seen in the third and last extract collected (Fig. 4C). The results show that, at all three flow rates investigated, the first extract collected contained proportionally the highest amount of coextracted lipid components such as TG, FFA, DG, and MG. The selectivity for EE then increased with time. The reason for this could be that the first sample was taken shortly after a 60-min static period where there was no flow through the reactor. During the static period, the lipid components probably had more time to partition and establish an equilibrium between the liquid and vapor phases, which was then reflected in a less-selective extraction of EE. When the other two samples of the extract were collected, CO_2 had been flowing through the reactor for some time and, as an equilibrium flow rate was probably not achieved, the continuous flow of CO_2 gave the lipid components only a short time to partition between the phases. Hence the most soluble components, i.e., the EE, were selectively extracted. This consideration also explains why the selectivity for EE increased with increasing flow rate.

Composition of the liquid phase. The effect of flow rate on the lipid class composition of the liquid phase is shown in Figure 5. In the first samples taken from the liquid phase, the proportional amount of EE was highest when the slowest flow rate (0.015 NL/min) was used (Fig. 5A). This trend reflects the faster extraction rate of EE at the 0.075 and 0.15 NL/min flow rates (Fig. 2). In the second sample taken of the liquid phase, the proportional amount of EE was found to be highest at the fastest flow rate (Fig. 5B). This occurs even though the extraction rate of EE increased with flow rate (Fig. 2). This result confirms that it is the rate of diffusion of EE within

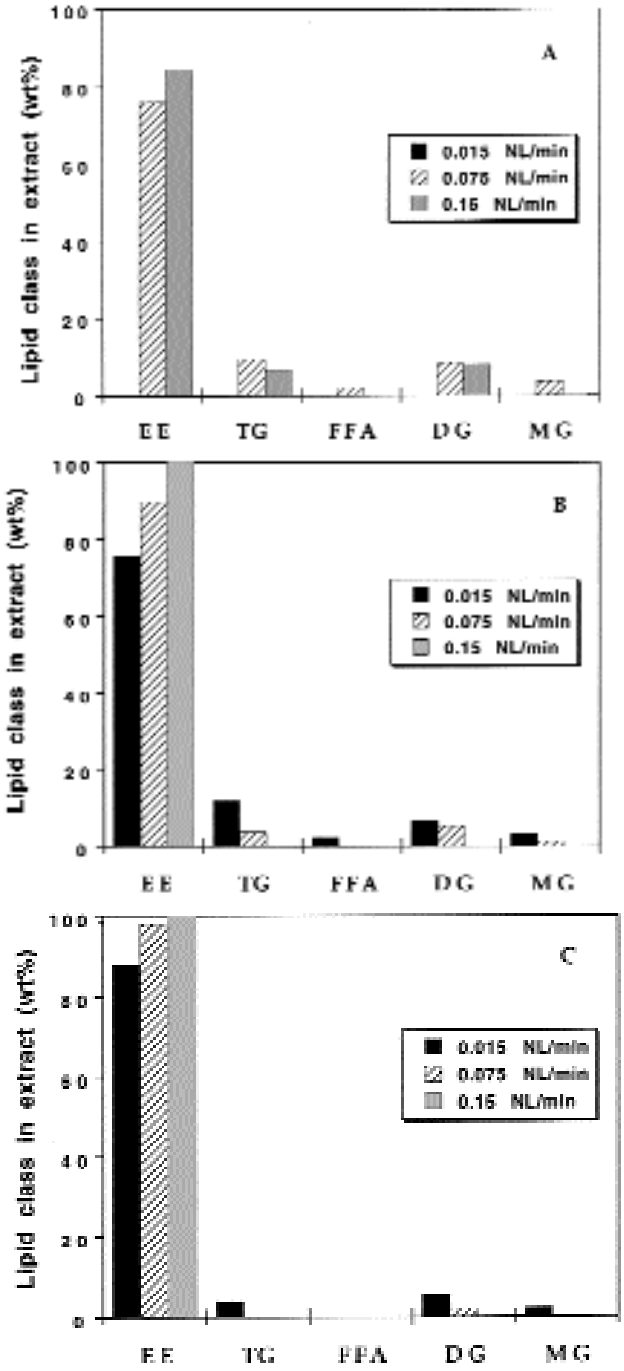

FIG. 4. Effect of flow rate on the lipid class composition in (A) the first samples of the extract taken 110 min after the experiment was started; (B) the second samples of the extract taken 220 min after the experiment was started; and (C) the third samples of the extract taken 330 min after the experiment was started. TG, triglycerides; DG, diglycerides; and MG, monoglycerides; FFA, free fatty acids. See Figure 2 for other abbreviations.

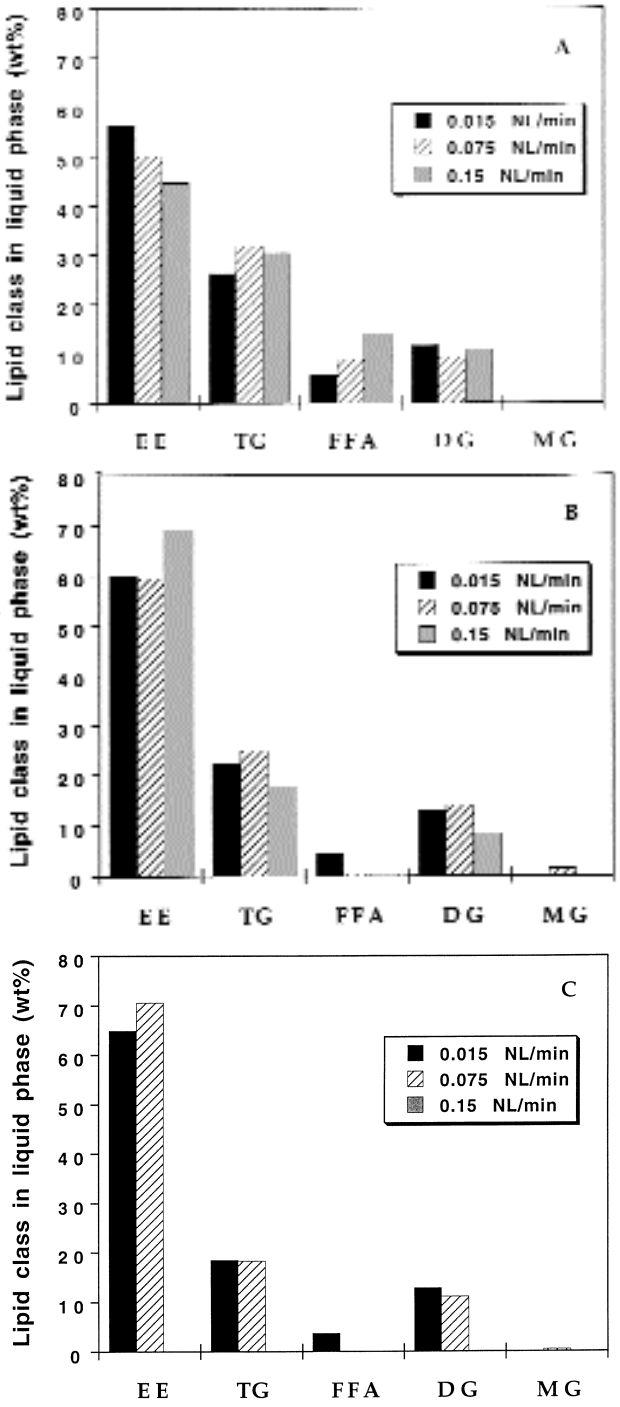

FIG. 5. Effect of flow rate on the lipid class composition in (A) the first samples of the liquid phase taken 110 min after the experiment was started; (B) the second samples of the liquid phase taken 220 min after the experiment was started; and (C) the third samples of the liquid phase taken 330 min after the experiment was started. See Figures 2 and 4 for abbreviations.

the liquid phase that limits the extraction rate of EE at this stage. The relatively high proportion of EE present in all samples of the liquid phase further supports this. A third sample of the liquid phase was only available at the 0.015 and 0.075 NL/min flow rates, as at the flow rate of 0.15 NL/min no bulk liquid phase was left to sample. The remaining residual lipids, obtained using *n*-propanol extraction, thus have to be located within or on the surface of the immobilization support. The lipid class compositions of the third samples were similar to those of the second samples (Fig. 5C and Fig. 5B). Figure 6

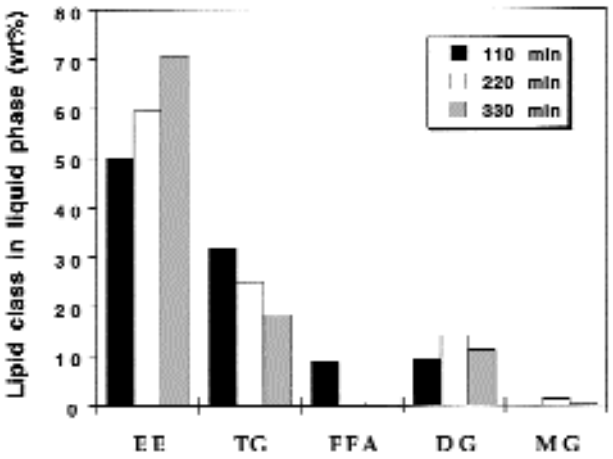

FIG. 6. Changes in the lipid class composition of the liquid phase with time at 0.075 NL/min flow rate. See Figures 2 and 4 for abbreviations.

illustrates the changes in the lipid class composition in the liquid phase with time at the flow rate of 0.075 NL/min. The results show that the proportional amount of EE in the liquid phase increased with time while the amount of TG and FFA decreased, the amount of DG first increased and then decreased, while the MG did not accumulate in the liquid phase to a great extent. Similar results were obtained at the other two flow rates tested. The observed effects reflect the production of EE with time and the reduction of the substrate TG as the enzyme reaction progressed. The initial increase and subsequent decrease in DG were due to the fact that DG are reaction intermediates. However, the MG reaction intermediates seemed to be consumed as soon as they were produced. The cod liver oil substrate consists almost solely of TG (98%); hence the FFA detected in the liquid phase were formed as a result of the lipase reaction. This result suggests that the reaction, to some extent, occurred by a two-step mechanism, i.e., a hydrolysis step followed by ester synthesis. The fact that the amount of FFA decreased with time further supports this conclusion. This kind of two-step mechanism has been described by other researchers investigating alcoholysis of TG at atmospheric pressures (21,22). Kanasawud and coworkers (21) suggested that the water (present in the immobilization support) may be a faster nucleophile than the alcohol, which lead to a kinetically controlled initial rise in FFA.

Conversion. In order to assess the effect of flow rate on the total conversion, the amount of substrate available to the enzyme was estimated from the original amount of substrate added to the reactor less the total amount of liquid phase removed as samples during the experiments. The total conversion is here defined as the sum of the amount of TG converted to EE in the residue and of that converted and present in the extract. The molecular mass of the cod liver oil substrate (880 g/mole) was calculated from the fatty acid composition.

The results are compiled in Table 2 and show that an approximately 10% increase in conversion was observed when the flow rate was increased from 0.015 to 0.075 NL/min. This

TABLE 2
Effect of CO_2 Flow Rate on the Total Conversion of the Cod Liver Oil Substrate to EE at 90 MPa and 40°C

Flow rate (NL/min)	0.015	0.075	0.15	0.3
Reaction time (min)	330	330	330	660
Total conversion[a] (%)	73.7	82.1	82.7	97.8

[a]Sum of the amount of triglyceride converted to EE in the residue and of that converted and present in the extract. See Table 1 for abbreviations.

fact indicates that the selective extraction of EE from the reaction mixture shifts the equilibrium of the reaction in the direction of further synthesis. These results are in agreement with the results presented by Adschiri *et al.* (10) and Shishikura *et al.* (11). However, there was only a marginal increase in the conversion when the flow rate was increased from 0.075 to 0.15 NL/min, possibly because the EE accumulated in the liquid phase (Fig. 5B) and within the tortuous structure of the immobilization support so that an equilibrium in the reaction was obtained at least in the microenvironment of the lipase. Table 2 also reveals that a total conversion (98%) of the substrate was obtained when an experiment was carried out (for 660 min) at a flow rate of 0.3 NL/min. The largest part (86%) of the EE produced was here collected in the extract fractions.

Coextraction of ethanol. Our previous work revealed that EtOH was extracted along with the EE (13). Hence, it was decided in this work to add EtOH periodically to the reactor *via* a loop (Fig. 1). The extraction of the EtOH from the reaction mixture is disadvantageous for two reasons. First, the EtOH is a substrate in the reaction, and a certain stoichiometric excess of EtOH probably promotes the reaction rate. Second, the coextracted EtOH would have to be separated from the product (EE). The ratio of the total amount of EtOH extracted to the total amount added to the reactor at the different flow rates under investigation is shown in Table 3. This ratio was found to be lowest at the slowest flow rate (0.015 NL/min), whereas more or less the same ratio (around 0.6) was obtained at the other three flow rates tested. These results indicate that increasing the flow rate above 0.075 NL/min did not result in an increased coextraction of EtOH. Further, it was observed that the largest amount of EtOH per liter CO_2 consumed was obtained in the first extracts collected, and after that the EtOH extracted per liter CO_2 consumed decreased (results not shown). This observation suggests that a large part of the EtOH placed in the reactor in the beginning of the experiment was, in fact, present in the vapor phase and was thus extracted with the outgoing CO_2 as soon as the extraction started. In order to reduce the amount of EtOH coextracted with the

TABLE 3
Effect of CO_2 Flow Rate on the Coextraction of Ethanol at 9 MPa and 40°C[a]

Flow rate (NL/min)	0.015	0.075	0.15	0.3
Extraction time (min)	270	270	270	600
EtOH extracted/EtOH added	0.44	0.66	0.62	0.64

[a]EtOH, ethanol; see Table 1 for other abbreviation.

TABLE 4
CO_2 and EtOH Content of the Different Samples Taken from the Liquid Phase at 9 MPa and 40°C

Sample	A	B	C	A	B	C	A	B
Time (min)[a]	110	220	330	110	220	330	110	220
Flow rate (NL/min)	0.015	0.015	0.015	0.075	0.075	0.075	0.15	0.15
g CO_2/g liquid phase	0.42	0.39	0.41	0.34	0.36	0.31	0.32	0.34
g EtOH/g liquid phase	0.13	0.065	0.063	0.088	0.05	0.037	0.044	0.020

[a]Time when the sample was taken. See Tables 1 and 3 for abbreviations.

product, it seems advantageous to reduce the amount of EtOH originally charged to the reactor and instead to add the EtOH frequently or continuously during the experiment.

Ethanol and CO_2 content of the liquid phase. Table 4 shows the proportional amount of EtOH in the liquid phase. From this, one can see that some EtOH was present in the liquid phase in all samples taken. This information is important as the EtOH is the nucleophilic substrate of the reaction and therefore has to be available to the enzyme for a reaction to occur. Figure 7 illustrates that there appears to be a linear correlation (r^2 = 0.7) between the CO_2 (g)/lipid (g) lipid and EtOH (g)/lipid (g) in the liquid phase. The lowest values of the plot represent the amount of CO_2 dissolved in pure cod liver oil. Here the proportion of CO_2 present in the liquid phase is approximately 27%. By contrast, when the EtOH content increases to, for example, 0.15 g EtOH/g lipid the proportion of CO_2 in the liquid phase becomes 34 to 38%. From this results it is obvious that the presence of EtOH in the liquid phase enables more CO_2 to become dissolved. Jennings and coworkers (23) have studied the vapor-liquid equilibria for CO_2 and ethanol mixtures, and have demonstrated that the mole fraction of CO_2 in liquid ethanol at 7.89 MPa and 40°C is 0.85. This favorable dissolution of CO_2 in liquid EtOH could explain the correlation observed between the CO_2 and EtOH contents of the liquid phase (Fig. 7).

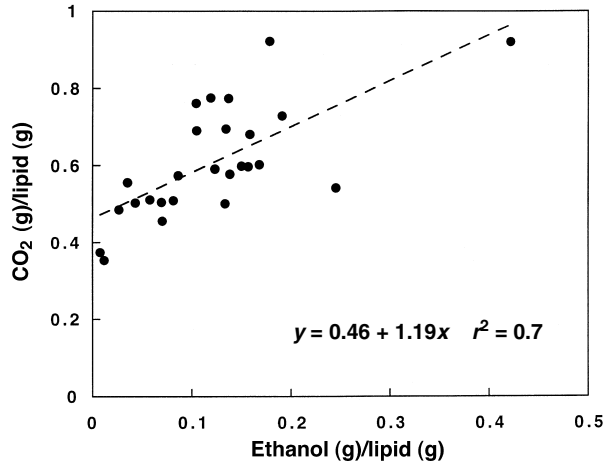

FIG. 7. The relationship between CO_2 (g)/lipid (g) and ethanol (g)/lipid (g) in the liquid phase.

The effect of flow rate on the CO_2 content of the samples taken from the liquid is also shown in Table 4. All three samples taken at the 0.015 NL/min flow rate contained proportionally the highest amounts of CO_2. The experimental apparatus used in this work (Fig. 1) could explain this observation. The CO_2 enters the reactor *via* a tube that initially is immersed in the liquid phase. When a low flow rate is used (e.g., 0.015 NL/min), the CO_2 slowly bubbles into the liquid phase where some of it dissolves or slowly diffuses through. A fast flow rate probably creates a funnel-shaped hole in the surface of the liquid phase, which reduces the contact between the CO_2 and the liquid phase, with the result that less CO_2 becomes dissolved and less diffuses through the liquid phase. Poor contact between the CO_2 and the liquid phase could explain the observed lower concentrations of EE in the CO_2 at the fast flow rates (Fig. 3).

The amounts of EtOH and CO_2 dissolved in the liquid phase at the different flow rates tested were of interest as they will reduce the viscosity of the liquid phase. This effect, in turn, might affect the external mass transfer of substrate and product. No apparent relationship can be seen between the amount of CO_2 and EtOH in the liquid phase and the efficiency (total conversion) of the process. Possibly the effect of reduction in viscosity in the liquid phase is more or less the same at all flow rates under investigation.

In conclusion, the results of this work have shown that a fast flow rate results in a faster extraction rate and higher recovery of EE. Further, the fast flow rate was found to result in a more selective extraction of EE. In addition, by increasing the flow rate, the equilibrium of the reaction was shifted toward synthesis to a certain degree. These are very encouraging results as they suggest that increasing the flow rate also increases the yield and the purity of the EE extracted. This enhancement was found to occur at the expense of a decreased concentration of EE in the CO_2 with increasing flow rate, which means that proportionally more extraction fluid has to be used to extract each gram of EE. However, the CO_2 easily can be recycled and reused in the process so the extra amount of extraction fluid needed does not have to increase the overall cost of the process. Coextraction of the ethanol substrate along with the product is a disadvantage. Presumably, this problem could be eliminated by adding the EtOH continuously to the reactor. Further, improved contact between the extraction medium and the liquid phase possibly

could increase both the extraction rate of EE and the reaction rate. Finally, it should be noted that the presented experimental apparatus is not optimized from an industrial point of view.

ACKNOWLEDGMENTS

Financial support from the Swedish National Board for Industrial and Technical Development (NUTEK) is gratefully acknowledged. The authors wish to thank Dr. Barry Ninham for linguistic advice.

REFERENCES

1. Aaltonen, O., and M. Rantakylä, Biocatalysis in Supercritical CO_2, *Chemtech 21*:240–248 (1991).
2. Perrut, M., Enzymatic Reactions in Supercritical Carbon Dioxide, in *High Pressure and Biotechnology,* edited by C. Balny, R. Hayashi, K. Heremans, and P. Masson, Colloque INSERM/John Libbey Eurotext Ltd., 1992, pp. 401–410.
3. Kamat, S.V., E.J. Beckman, and A.J. Russell, Enzyme Activity in Supercritical Fluids, *Crit. Rev. Biotechnol. 15*:41–71 (1995).
4. Ballesteros, A., U. Bornscheuer, A. Capewell, D. Combes, J.-S. Condoret, K. Koenig, F.N. Kolisis, A. Marty, U. Menge, T. Scheper, H. Stamatis, and A. Xenakis, Enzymes in Non-Conventional Phases, *Biocatal. Biotransform. 13*:1–42 (1995).
5. Kremer, J.M., A.V. Michalek, L. Lininger, C. Huyck, J. Bigauoette, M.A. Timchalk, R.I. Rynes, J. Zieminski, and L.E. Bartholomew, Effects of Manipulation of Dietary Fatty Acids on Clinical Manifestations of Rheumatoid Arthritis, *Lancet i*:184–187 (1985).
6. Kremer, J.M., D.A. Lawrence, W. Jubiz, R. Digiacomo, R. Rynes, L.E. Bartholomew, and M. Sherman, Dietary Fish Oil and Olive Oil Supplementation in Patients with Rheumatoid Arthritis, *Arthritis Rheum. 33*:810–820 (1990).
7. Leaf, W., and P.C. Weber, Cardiovascular Effects of n-3 Fatty Acids, *N. Engl. J. Med. 318*:549–557 (1988).
8. Kang, J.X., and A. Leaf, Antiarrhythmic Effects of Polyunsaturated Fatty Acids, *Circulation 94*:1774–1780 (1996).
9. Doddema, H.J., R.J.J. Janssens, J.P.J. de Jong, J.P. van der Lugt, and H.H.M. Oostrom, Enzymatic Reactions in Supercritical Carbon Dioxide and Integrated Product-Recovery, in *5th European Congress on Biotechnology,* edited by C. Christiansen, L. Munck, and J. Villadsen, 1990, Munksgaard, Copenhagen, pp. 239–242.
10. Adschiri, T., H. Akiya, L.C. Chin, K. Arai, and K. Fujimoto, Lipase-Catalyzed Interesterification of Triglyceride with Supercritical Carbon Dioxide Extraction, *J. Chem. Eng. Jpn. 25*:104–105 (1992).
11. Shishikura, A., K. Fujimoto, T. Suzuki, and K. Arai, Improved Lipase-Catalyzed Incorporation of Long-Chain Fatty Acids into Medium-Chain Triglycerides Assisted by Supercritical Carbon Dioxide Extraction, *J. Am. Oil Chem. Soc. 71*:961–967 (1994).
12. Hawthorne, S.B., A.B. Galy, V.O. Schmitt, and D.J. Miller, Effect of SFE Flow Rate on Extraction Rates: Classifying Sample Extraction Behavior, *Anal. Chem. 67*:2723–2732 (1995).
13. Gunnlaugsdottir, H., and B. Sivik, Lipase-Catalyzed Alcoholysis of Cod Liver Oil in Supercritical Carbon Dioxide, *J. Am. Oil Chem. Soc. 72*:399–405 (1995).
14. Gunnlaugsdottir, H., A.Å. Karlsson, and B. Sivik, Lipase-Catalyzed Alcoholysis with Supercritical Carbon Dioxide Extraction 2: Phase Behavior, *Ibid. 74*:1505–1508 (1997).
15. Staby, A., and J. Mollerup, Solubility of Fish Oil Fatty Acid Ethyl Esters in Sub- and Supercritical Carbon Dioxide, *Ibid. 70*:583–588 (1993).
16. Nilsson, W.B., G.T. Seaborn, and J.K. Hudson, Partition Coefficients for Fatty Acid Esters in Supercritical Fluid CO_2 With and Without Ethanol, *Ibid. 69*:305–308 (1992).
17. van Eijs, A.M.M., J.P.J. de Jong, H.J. Doddema, and D.R. Lindeboom, Enzymatic Transesterification in Supercritical Carbon Dioxide, in *International Symposium on Supercritical Fluids,* edited by M. Perrut, Société Française de Chimie, Nice, 1988, pp. 933–942.
18. Dumont, T., D. Barth, and M. Perrut, Continuous Synthesis of Ethyl Myristate by Enzymatic Reaction in Supercritical Carbon Dioxide, *J. Supercrit. Fluids 6*:85–89 (1993).
19. Aaltonen, O., and M. Rantakylä, Lipase-Catalyzed Reactions of Chiral Compounds in Supercritical Carbon Dioxide, in *Proceedings of the 2nd International Symposium on Supercritical Fluids,* Boston, 1991, pp. 146–149.
20. Miller, D.A., H.W. Blanch, and J.M. Prausnitz, Enzyme-Catalyzed Interesterification of Triglycerides in Supercritical Carbon Dioxide, *Ind. Eng. Chem. Res. 30*:939–946 (1991).
21. Kanasawud, P., S. Phutrakul, S. Bloomer, P. Adlercreutz, and B. Mattiasson, Triglyceride Interesterification by Lipases. 3. Alcoholysis of Pure Triglycerides, *Enzyme Microb. Technol. 14*:959–965 (1992).
22. Millqvist, A., P. Adlercreutz, and B. Mattiasson, Lipase-Catalyzed Alcoholysis of Triglycerides for the Preparation of 2-Monoglycerides, *Ibid. 16*:1042–1047 (1994).
23. Jennings, D.W., L. Rong-Jwyn, and A.S. Teja, Vapor-Liquid Equilibria in the Carbon Dioxide + Ethanol and Carbon Dioxide + 1-Butanol Systems, *J. Chem. Eng. Data 36*:303–307 (1991).

[Received March 3, 1997; accepted June 23, 1997]