

Supercritical-Fluid Extraction of Fungal Lipids Using Mixed Solvents: Experiment and Modeling^{†**}

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Miriam Cygnarowicz-Provost,* Dennis J. O'Brien, Robert J. Maxwell, and James W. Hampson

Polyunsaturated fatty acids, notably eicosapentaenoic acid (EPA), have been purported to have beneficial physiological activity, including the prevention of arthritis and cardiovascular disease. A possible source of these fatty acids are filamentous fungi (e.g., *Saprolegnia parasitica*). In this work, lipids are extracted directly from the fungal mycelia using supercritical CO₂ and CO₂ mixed with 10 wt % ethanol. Extractions are performed at temperatures from 40 to 60 °C and pressures from 205 to 680 bar. The recovery of lipid increases with increasing pressures and higher recoveries are obtained when a mixture of CO₂ with 10 wt % ethanol is used as the solvent (e.g., 89% recovered with 10% ethanol vs. 48% for 100% CO₂). The more polar CO₂ mixture is a better solvent since it is able to extract both the neutral and the polar lipid fractions. An unsteady extraction model which can give reliable representation of the entire extraction curve is presented. Mass transfer coefficients are computed using the experimental data, and these coefficients are correlated as a function of the interstitial velocity.

Keywords: polyunsaturated fatty acids, eicosapentaenoic acid, polar lipids, mass transfer

INTRODUCTION

The ω -3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are purported to have beneficial physiological activity, including reduced susceptibility to arthritis and cardiovascular disease. A typical source of these fatty acids is marine oils. Oily fish, such as anchovy, mackerel, sardine, and menhaden are primarily used for fish oil production. The composition and content of ω -3 fatty acids in fish oils is dependent upon the species of fish and the season and location of the catch. At present, most fish oil produced today is hydrogenated and incorporated into margarine or shortening, destroying the valuable polyunsaturated fatty acids. Therefore, it is unclear whether the supply of ω -3 fatty acids from marine sources will be adequate to satisfy the projected demand.¹ This fact motivates the search for alternative means of producing polyunsaturated fatty acids. Among the non-

conventional sources for EPA and DHA are algae² and fungi. In particular, the filamentous fungus, *Saprolegnia parasitica* has been demonstrated to contain a considerable amount of EPA and other polyunsaturated fatty acids in its lipid matter.³

In this work, supercritical fluids are used to extract lipids directly from the fungal mycelia. Several other researchers have studied the use of supercritical fluid extraction (SFE) for extracting and concentrating polyunsaturated fatty acids. Nilsson et al.⁴ and Rizvi et al.⁵ developed processes for the concentration of EPA and DHA from marine oils using supercritical CO₂. Ikushima et al.⁶ extracted the polyunsaturated fatty acids from freeze-dried mackerel powder and Yamaguchi et al.⁷ extracted the oils from antarctic krill. Choi et al.⁸ and Polak et al.⁹ used supercritical CO₂ (with and without ethanol as an entrainer) to extract the lipids from freeze-dried algae. Finally, Sako et al.¹⁰ and Sakaki et al.¹¹ have done extensive studies on the extraction of lipids from the *Mortierella* genus fungi. This fungus does not produce EPA, but does produce considerable amounts of the ω -3 fatty acid, γ -linolenic acid, which is present in the neutral lipid frac-

tion. They showed yields of up to 91% for extractions performed at 389 kg/cm² and 60 °C with 10% ethanol added as an entrainer. They also explored the use of alternative supercritical solvents, such as N₂O, CHF₃, and SF₆.

In contrast, the fungus extracted in this work contains a sizeable portion of EPA in the polar fraction of the lipids. The polar lipids are more difficult to extract with supercritical fluids, since they are often bound to the fungal tissue.⁸ In addition, they are prone to degradation and require special handling. The objective of this study was to explore the use of supercritical fluids to extract the polyunsaturated fatty acids from the fungus *S. parasitica*, using CO₂ and mixtures of CO₂ with 10 wt % ethanol. In addition, the mass transfer kinetics were studied and overall mass-transfer coefficients were computed using a non-steady extraction model.

EXPERIMENTAL

Fermentation and Harvesting of Filamentous Fungi. *S. parasitica* was grown in a 14-L New Brunswick fermenter in which the agitator and baffles were removed. Mixing was provided solely by aeration. Ten liters of growth medium¹² were used in which glucose was the carbon source. Air flow rate was 8 standard-L/min and the pH was maintained between 6.6 and 7.0 by the addition of KOH. The mycelial mass was harvested after 3.5 days by filtering through a Buchner funnel. Five batches grown under these conditions were used in these studies. The fungal cell walls were an impediment to efficient extraction and had to be broken before the experiments could proceed. In these studies, the fungi was lyophilized before extraction. The completely dried fungi easily crumbled to a fine powder, allowing the lipid to be readily extracted.

Supercritical-Fluid Extraction. The experimental apparatus used in these studies is shown in Figure 1. It consists of a high-pressure liquid pump (Haskel, Inc., Burbank, CA), a 20-mL extractor housed in an oven, a series of micrometering valves, a collection flask, and a flow totalizer. The air-driven Haskel pump includes an air-pressure regulator which maintains a constant outlet pressure. The extractor, valves, and fittings were supplied by Autoclave Engineers, Inc. (Erie, PA), and the flow totalizer was supplied by American Meter Co. (Philadelphia, PA).

To begin the experiments, liquid CO₂ from the supply tank is cooled in an ice bath and then compressed to the desired pressure. Once it enters the oven, the stream passes through approximately 2.5 m of 6.35-mm OD high-pressure tubing to allow it to reach thermal equilibrium. It then enters the extractor which has been packed with equal volumes of 5-mm glass beads and freeze-dried fungi (approximately 2 g of fungi were extracted in each experiment). The ends of the extractor were packed with glass wool to prevent entrainment. Upon leaving the

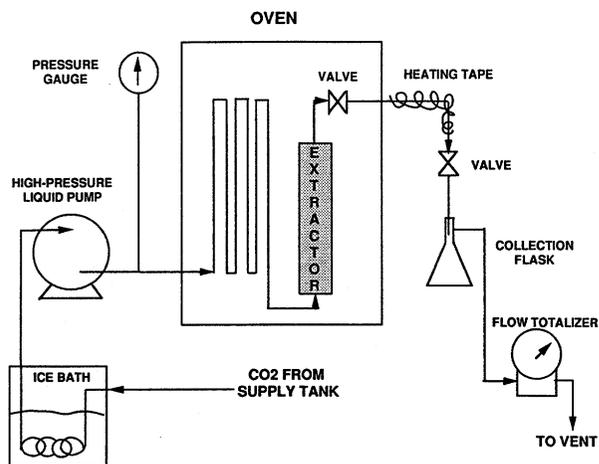


Figure 1. Experimental apparatus used to measure fungal lipid extraction curves in CO₂ and in CO₂ mixtures.

oven, the supercritical CO₂ stream, containing the dissolved lipid, passed through a series of metering valves which controlled the flow and pressure. The reduction in pressure caused the solubilized lipids to separate from the CO₂ and precipitate in the collection vessel. To prevent volatilization of the solute, the collection vessel was kept in an ice bath, and a trap was placed in the line immediately following it. The depressurized CO₂ gas flowed through the flow totalizer before being vented. In these experiments, the flow rate of gas was kept constant at values between 3 and 5 L/min. Extraction curves were determined by weighing the lipid that precipitated after passing through a known volume of CO₂. At the end of the experiment, the metering valve and tubing were flushed with methylene chloride to collect any lipid that precipitated. The system has no provision for adding cosolvents directly. Instead, cylinders of CO₂ mixed with 10 wt % ethanol were obtained from a supplier (Scott Specialty Gases, Plumsteadville, PA).

To determine the total lipid extracted, the collection flask and cold trap were washed with methylene chloride and transferred to a pre-weighed beaker and the solvent was evaporated. The lipid remainder (10–50 mg) was then weighed. To determine the percent recovered, the fungi remaining in the sample holder was extracted with a mixture of chloroform, methanol, and water, using the method of Folch.¹³ The solvent was evaporated under a stream of nitrogen, and the dry lipid weights determined. The percent recovery was thus equal to the weight of lipid extracted (including that precipitated in the metering valve and tubing), divided by the weight of lipid extracted plus the weight of lipid remaining. A complete mass balance could not be obtained since the starting material (i.e., the dried fungi) contained lipid amounts which were found to vary between 8–16 wt %. The solubility of the lipid at a given temperature and pressure was determined by computing the slope of the extraction curve during the initial (steady-state) period.

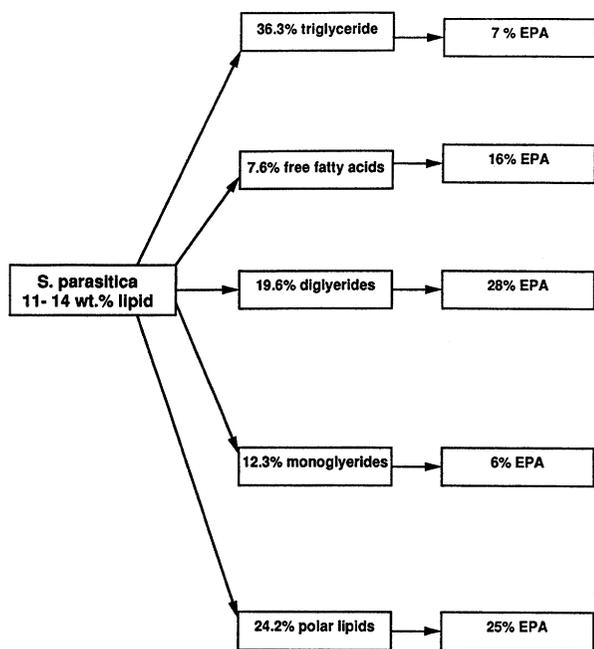


Figure 2. Composition of fungal lipids of *S. parasitica*.

Determination of Lipid Compositions.

The fatty acid composition of the lipid extracts was determined by capillary GLC of the fatty acid methyl esters (FAMES) as outlined by Wessinger et al.¹⁴ Analysis of the lipid classes was performed by thin-layer chromatography (TLC) using the method of Kurantz et al.¹⁵ The lipid extracts were dissolved in chloroform and applied to precleaned high performance thin-layer chromatography (HPTLC) plates. The lipids were separated using hexane-diethyl ether-formic acid (65:30:1, v/v/v). Fluorescence of the separated components was induced by dipping the dried plate into a mixture of sulfuric acid-absolute ethanol-hexane (1:35:64 v/v) and then heating the plates in a forced air oven at 100 °C for 45 min. Fluorescence densitometry of the chromatographed components was obtained with the TLC Scanner II (CAMAG, Muttenz, Switzerland). Densitograms were recorded and integrated (peak height mode) with a CAMAG SP 4290. Lipid classes were identified by HPTLC of standard lipids to locate the retention time of the polar and neutral components.

The composition of a representative sample of *S. parasitica* was determined using preparative thin layer chromatography on 500- μ m Silica Gel G plates. Standards were used to identify the lipid classes. The separated components were eluted from the silica, dried, and weighed.

RESULTS AND DISCUSSION

The organism *S. parasitica* produces an average of 11–14% lipid, on a dry weight basis. The composition of a single harvest of *S. parasitica*, grown under the conditions described in the previous section, is depicted in

TABLE I
Lipid Extracted under Various Conditions
(Based on 250 L Depressurized Gas)

Solvent	Temperature (°C)	Pressure (bar)	% Extracted
100% CO ₂	40	346	49
100% CO ₂	50	346	43
100% CO ₂	60	346	48
100% CO ₂	60	483	56
10% Ethanol	60	346	89

Figure 2. The lipid composition is 75.8% neutral and 24.2% polar. The neutral lipids are composed of 36.3% triglyceride, 19.6% diglyceride, 12.3% monoglyceride, and 7.6% free fatty acids. Note that although EPA is present in each fraction, a significant portion is contained in the polar lipids. Therefore, these lipids need to be extracted to obtain a reasonable yield of EPA.

An indication of the influence of the process variables is given in Table I, which shows the percent recovery of lipid after passing through 250 L of CO₂ (measured at atmospheric pressure). Note that an increase in pressure from 346 to 483 bar increases the recovery slightly (from 48 to 56% at 60 °C) and that temperature seems to have little effect. The largest increase in recovery is obtained when the solvent mixture is used, that is, 89% of the lipid is recovered at 60 °C and 346 bar. The increased recovery is partially due to the extraction of the polar lipids upon the addition of co-solvent. Analysis of thin-layer chromatography plates confirm the presence of polar lipids in the extracts obtained using the CO₂ mixture. No polar lipid peak was detected in the lipid extracted with pure CO₂ (Figures 3a–b). The extraction of polar lipids also results in higher fractions of EPA in the extract. Figure 4 compares the weight percent EPA determined from FAME analysis of the extracts obtained using 100% CO₂ and 10% ethanol at 310 bar and 60 °C. The fraction of EPA in the lipid extracted with 10% ethanol was higher than that for pure CO₂, and was essentially constant throughout the course of the extraction. When the solvent was pure CO₂, the concentration of EPA was initially low but increased near the end of the extraction. This was probably due to a preferential extraction of the lower-molecular weight glycerides. Once these were depleted, glycerides containing the higher-molecular weight, polyunsaturated fatty acids were extracted.

Modeling of Extraction Curves. Solubility of the lipids was determined at 60 °C for pressures ranging from 200 to 680 bar. The results were correlated to an empirical relationship, first presented by del Valle and Aguilera¹⁶ in which the solubility is represented as a simple function of temperature and solvent density as follows:

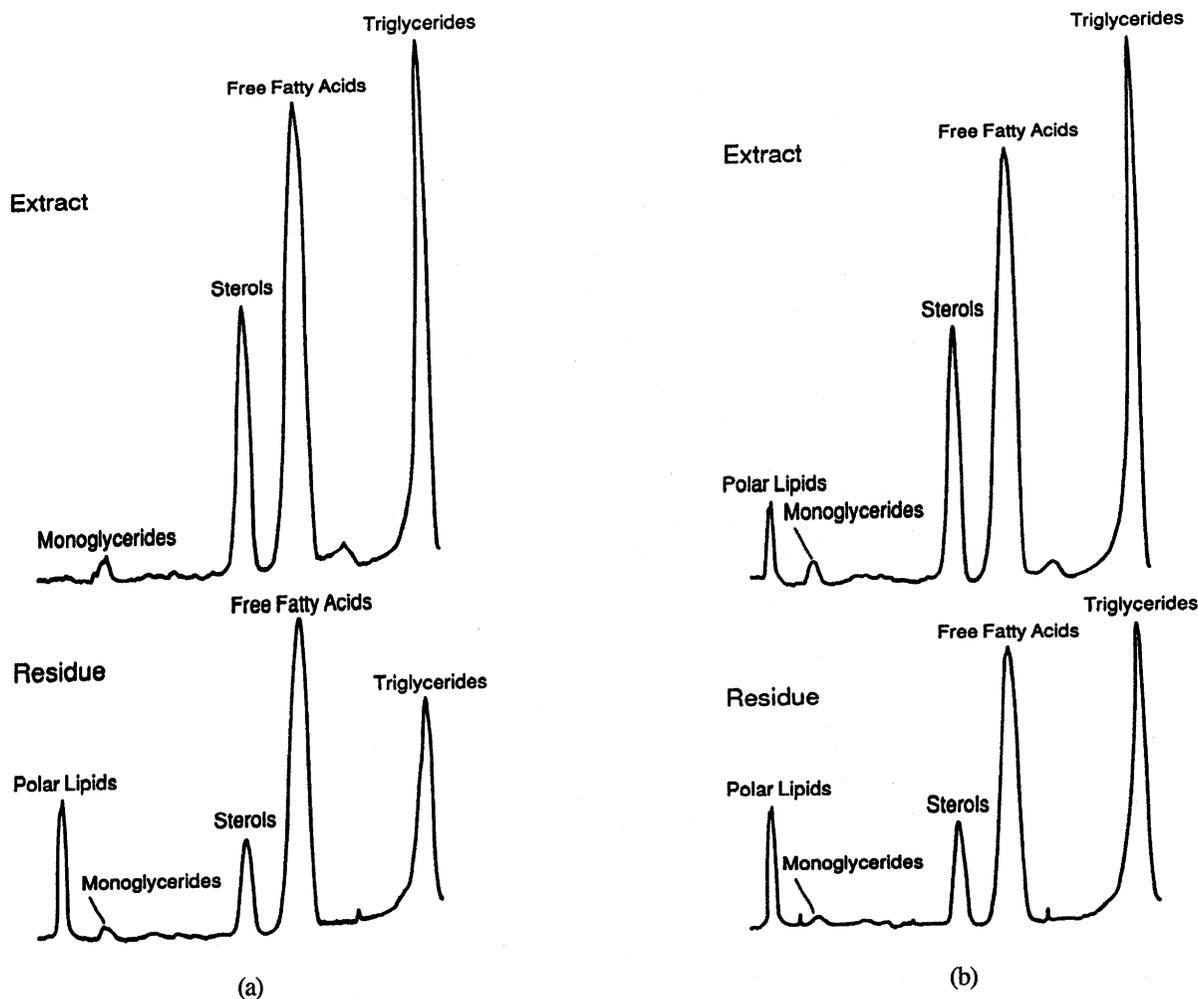


Figure 3. Thin-layer chromatograph of the lipid extract and the residue at 60 °C and 310 bar. a.) 100% CO₂ b.) CO₂ with 10 wt % ethanol.

$$\ln c = C_1 + \frac{C_2}{T} + C_3 \ln \rho \quad (1)$$

where c is concentration (g/L), T is temperature (K), and ρ is density (g/L) of the solvent. The density of pure CO₂ was determined using the Schmidt–Wagner equation-of-state¹⁷ and the density of the CO₂/ethanol mixture was estimated using the modified Peng–Robinson equation-of-state.¹⁸ In this work, only C_1 and C_3 were correlated since solubility data were only measured at one temperature. Experiments are underway to expand the database to include solubilities at temperatures from 40–80 °C. The experimental data and the model predictions are given in Figure 5. For comparison, the solubility of soybean oil (as predicted by del Valle and Aguilera) is also shown. Note that the solubility of the lipids in CO₂ is low (only 0.3 wt % at 680 bar) and that the addition of ethanol increases the apparent solubility by almost an order of magnitude.

The extraction model used in this work was first presented by Lee et al.¹⁹ to predict the extraction of canola oil using supercritical CO₂. The plug-flow model as-

sumes constant temperature, pressure and flow rate. The mass balances can be represented by a system of partial differential equations which describe the weight fraction of lipid in the CO₂ stream and the weight fraction of lipid in the fungi as a function of time and position in the extractor. The equations are

$$\varepsilon \rho \frac{\delta y}{\delta t} + \rho U \frac{\delta y}{\delta h} = R\{x, y\} \quad (2)$$

and

$$(1 - \varepsilon) \rho_s \frac{\delta x}{\delta t} = -R\{x, y\} \quad (3)$$

with initial and boundary conditions

$$\begin{aligned} x &= x_0 \text{ at } t = 0 \text{ for all } h \\ y &= 0 \text{ at } h = 0, t \leq 0. \end{aligned} \quad (4)$$

In these equations, y is the mass of lipid in the solvent stream per mass of lipid-free solvent, x is the mass of

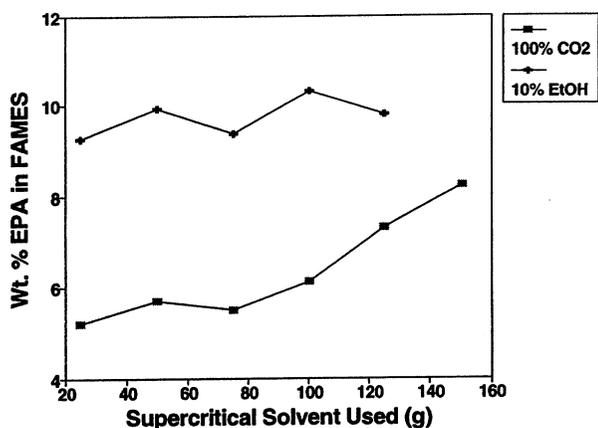


Figure 4. Weight percent EPA in fatty acid methyl esters (FAMES) of extracts as a function of CO₂ used for 100% CO₂ and CO₂ with 10% ethanol at 310 bar and 60 °C.

lipid in the fungi per mass of lipid-free fungi, ρ_s is the density of the dried fungi, U is the superficial velocity of the solvent, ϵ is the bed-volume void fraction, t is the time, h is the distance in the extractor from the inlet, and $R(x, y)$ represents the rate of mass transfer of the lipid from the fungi to the solvent phase. The boundary conditions assume that the solvent is pure at the extractor entrance and that all fungi particles have the same lipid content, and that it is uniformly distributed. Defining z as

$$z = \frac{\epsilon h}{U} \quad (5)$$

and substituting into eqs 2 and 3, the equations become

$$\frac{\delta y}{\delta t} + \frac{\delta y}{\delta z} = \frac{R(x, y)}{\epsilon \rho} \quad (6)$$

and

$$\frac{\delta x}{\delta t} = \frac{-R(x, y)}{(1 - \epsilon) \rho_s} \quad (7)$$

The above equations may be solved by the method of characteristics. The method is described in great detail by Acrivos,²⁰ Dranoff and Lapidus,²¹ and Lee et al.¹⁹ and will not be discussed here. In this work, the modified Euler method was used to integrate the equations. Good results were obtained when 500 time and spatial steps were used.

The mass transfer rate, $R(x, y)$, used by Lee et al. was simply

$$R(x, y) = A_p K (y^* - y) \quad (8)$$

where $A_p K$ is an overall mass transfer coefficient based on volume, y^* is the equilibrium concentration of lipid or oil in the solvent phase, and y is the actual concentration in the solvent phase. However, a constant mass transfer coefficient (i.e., one that is not a function of x) cannot de-

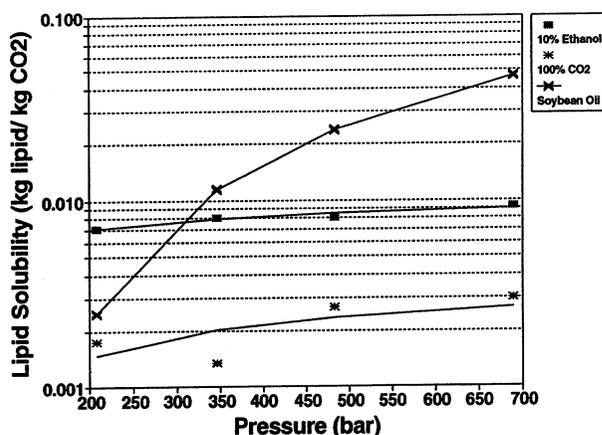


Figure 5. Experimental and predicted solubilities of lipid in CO₂ and CO₂/10% ethanol as a function of pressure. The solubility of soybean oil predicted by the equation of del Valle and Aguilera¹⁶ is plotted for comparison.

TABLE II
Physical Parameters and Constants Used in Fungal Lipid Extraction Model

	100% CO ₂	10% Ethanol
Void fraction, ϵ	0.3	0.3
ρ_s , kg/m ³	89.53	89.53
x_o	0.071*	0.098
x_{shift}	0.037	0.053

*Assumes only 75% of the lipids are extractable with pure CO₂.

scribe the entire mass transfer curve since the rate slows down considerably once the diffusion-controlled regime is reached. To describe the mass transfer coefficient throughout the entire range of extraction conditions, the following empirical correlation was developed by Cygnarowicz-Provost to represent the extraction of seed oils²²

$$A_p K = A_p K_o \exp \left[\ln(0.001) \frac{(x_o - x)}{(x_o - x_{\text{shift}})} \right] \quad (9)$$

In this expression, x_{shift} is the concentration in the fungi at which the extraction shifts from the mass-transfer controlled to the diffusion-controlled regime and $A_p K_o$ is the initial mass transfer coefficient. This relationship predicts that when $x = x_o$ (i.e., at the beginning of the extraction), $A_p K = A_p K_o$; and that when $x = x_{\text{shift}}$, $A_p K = 0.001 A_p K_o$. The latter relationship was determined through trial-and-error fits of the experimental data. The experimental measurements suggest that the shift to the diffusion-controlled regime occurs when the lipid concentration in the fungi drops to approximately 52% of its original value. This value is comparable to the results of King et al.,²³ who

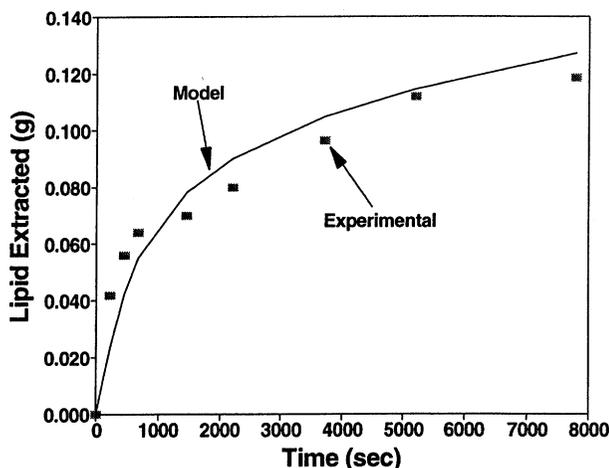


Figure 6. Experimental measurements and model predictions for the extraction of fungal lipids with 100% CO₂ at 60 °C and 483 bar.

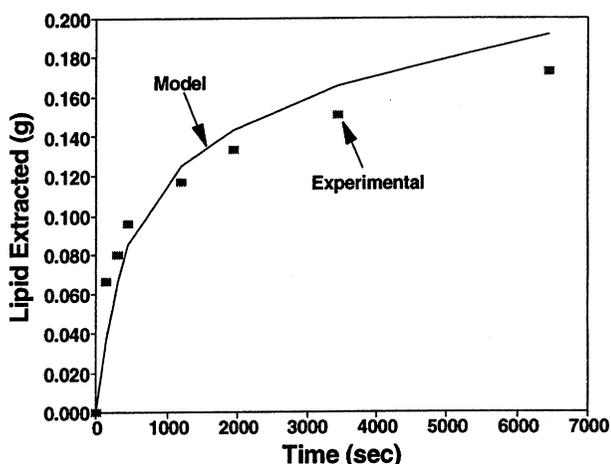


Figure 7. Experimental measurements and model predictions for the extraction of fungal lipids with CO₂/10% ethanol at 60 °C and 483 bar.

measured extraction curves for rapeseed oil in CO₂ and found the shift to occur when 65% of the oil had been extracted. Values of the physical parameters and constants used in this study are given in Table II.

The mass transfer coefficients were correlated through a least-squares fit of the experimental data. The successive quadratic programming algorithm²⁴ was used to minimize the sum of the squares of the residuals. Figure 6 shows a comparison of the experimental data and the model predictions for the extraction of lipids with 100% CO₂ at 60 °C and 483 bar. Figure 7 shows a plot of the extraction curve obtained with 10% ethanol at the same conditions. Note that the model fits the data well over the entire extraction curve. Analogous to the development of Lee et al.,¹⁹ the mass transfer coefficients computed from all of the experimental data were correlated as a function of the interstitial velocity (U/ϵ) as

$$A_p K_o = 793.32 \left(\frac{U}{\epsilon} \right)^{0.567} \quad (10)$$

The exponent obtained in this work is close to that computed by Lee et al. (i.e., 0.54). This extraction model can be incorporated into a model for the process flow sheet and optimization techniques can be used to assess the effect of the process variables on the design and economics. Such a strategy was used to investigate the extraction of β -carotene²⁵ and canola and soybean oil.²² Work is underway to build a process model for the isolation of fungal lipids and these results will be reported in future communications.

CONCLUSIONS

Supercritical-fluid solvents can be used to extract fungal lipids. Although the total recovery of lipid increases with increasing pressure, the highest recoveries were obtained when a mixture of CO₂ with 10 wt % ethanol was used as the solvent. Thin-layer chromatography of the extracts confirmed that the polar lipids were extracted when the solvent mixture was used. This led to higher solubility of the lipids and higher recoveries of EPA. The unsteady extraction model is able to give good representation of the extraction curves for both pure CO₂ and CO₂/ethanol mixtures.

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