Lipase-Catalyzed Synthesis of Triolein-Based Sunscreens in Supercritical CO_2

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ABSTRACT: Novozym® 435-catalyzed transesterification of ethyl 4-hydroxy-3-methoxy cinnamate (ethyl ferulate, EF) with triolein to form the ultraviolet (UV)-absorbing lipids monoferuloyl-monooleoyl-glycerol (FMO) and feruloyl-dioleoyl-glycerol (FDO) has been conducted using supercritical CO_2 (SC-CO_2) batch reactions. The alcoholysis of 0.1 M EF with 0.1 M 1-octanol in SC-CO_2 to form octyl ferulate was used as a model reaction to optimize pressure and temperature conditions. Conditions ranging from 45 to 80°C and 10.3 to 34.5 MPa (1,500 to 5,000 psi) were tested with a maximal conversion of 53% of the EF being achieved at 13.8 MPa (2,000 psi) and 80°C after 24 h. These optimized conditions applied to the transesterification of EF with triolein effected a combined FMO and FDO yield of 69%. Triolein exhibits higher solubilities in SC-CO_2 at higher pressures; therefore, the transesterification was performed at 80°C over a range of pressures from 13.8 to 34.5 MPa (2,000 to 5,000 psi). Results showed that a maximal yield of 74% of FMO and FDO was reached at 80°C and 24.1 MPa (3,500 psi) after 48 h. Compared to the FMO and FDO synthesis conducted neat or in toluene, the synthesis of the UV-absorbing lipids in SC-CO_2 affords higher yields within a shorter amount of time. Therefore, the transesterification of EF with triolein in a SC-CO_2 batch reaction is a viable route to UV-absorbing lipids that could be used as active ingredients in sunscreen formulations.

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KEY WORDS: Ethyl ferulate, lipase, Novozym® 435, sunscreen, supercritical CO_2, triolein.

We have previously reported a lipase-catalyzed route to feruloyl-substituted structured lipids (1). These lipids have been engineered to function as all-natural sunscreen ingredients (2), possessing the proper ultraviolet (UV) absorbance (290 to 375 nm) characteristics of a feruloyl moiety and the water insolubility of a lipid. Currently, the formulation of these UV-absorbing lipids as active ingredients in common sunscreen lotions is being tested [collaborations are currently underway with Flavor and Fragrances Specialties, Inc. (Mahwah, NJ) to formulate soy-based sunscreens into topically applied lotions]. The synthesis of these structured lipids is industrially attractive since the transesterification can be conducted neat, without the use of solvents, and at moderate temperatures. Purification of these UV-absorbing lipids consists of simply removing the enzyme-support from the reaction by filtration.

One concern, however, that could impede the industrial development of the described lipase-catalyzed synthesis is that 5 d are needed for reaction to reach completion. Also, to conduct the reaction under neat conditions, a 3:1 lipid-to-ferulate ratio is needed to provide enough solution volume to stir the suspended enzyme-support. This high lipid ratio reduces the concentration of the UV-absorbing lipids formed in the final reaction mixture. The lower UV-lipid concentration in the final reaction mixture would result in a lower weight percentage of UV-absorbing lipids as the active ingredient when formulated into a sunscreen lotion.

In previous studies, toluene was used as a reaction medium to enhance the mass transfer kinetics of the lipase-catalyzed transesterification of ethyl ferulate (EF) with triolein (1). The addition of toluene to the reaction allowed the use of a 1:1 EF-to-triolein mole ratio and halved the time needed for the UV-absorbing lipid synthesis to reach equilibrium. The use of the organic solvent, however, decreased the yield of the UV-absorbing lipids and introduced undesirable aspects to the sunscreen synthesis such as isolation of the product from the reaction medium, residual solvent in the product, and solvent waste disposal. Consequently, we have explored the use of supercritical CO_2 (SC-CO_2) as a benign alternative to using an organic solvent as the reaction medium (3,4).

SC-CO_2 and its critical point characteristics of 31°C and 7.6 MPa (1,100 psi) make it a suitable medium in which to perform enzyme-catalyzed reactions (5,6). More specifically, the commercially available lipase, Novozym®435, has been shown to remain fully active up to 100°C and at least partially active for 14 h at 140°C and ~15.2 MPa (2,200 psi) in SC-CO_2 (7). In addition, compared with organic solvents, SC-CO_2 has the advantage of being nontoxic, nonflammable, and it leaves no residual solvent in the reaction products.

The present study examined Novozym®435-catalyzed transesterifications of EF with triolein in SC-CO_2. Reaction conditions were optimized using the alcoholysis of EF with 1-octanol as a model reaction. Triolein was chosen as a representative triacylglycerol (TAG) for use in this study in place of vegetable oil to simplify the analysis of the reaction products. The lipase-catalyzed transesterification of EF, however, is not limited to triolein, and a patent application has been filed claiming the synthesis and potential sunscreen use of fer-

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ulyl-substituted TAG based on a variety of vegetable oils, including soybean, corn, and canola oils (8).

EXPERIMENTAL PROCEDURES

Materials. Novozym®435 (*Candida antarctica*) with a water content of 1 to 2% (by weight) was purchased from Novo Nordisk (Franklinton, NC). Ethyl 4-hydroxy-3-methoxy cinnamate (EF) was obtained from Sigma-Aldrich (St. Louis, MO) and kept desiccated under vacuum. 1-Octanol (Sigma-Aldrich) was dried over activated molecular sieves and stored under a nitrogen atmosphere. Triolein (Nu-Chek-Prep, Elysian, MN) was purchased in sealed ampoules and stored at –4°C. Acetone, acetonitrile, ethanol, *n*-butanol, and methanol (Sigma-Aldrich) were high-performance liquid chromatography (HPLC)-grade reagents and used as obtained. Supercritical fluid (SCF)/supercritical extraction (SCE) grade CO_2 (>99.9999% pure) was obtained from Air Products, Inc. (Allentown, PA).

Alcoholysis and transesterification of EF in SC-CO_2. The apparatus used for the SC-CO_2 batch reactions was a modified version reported by Ottoson *et al.* (9). The stirred SC-CO_2 batch reactions were performed using an Applied Separations (Allentown, PA) SPE-ED SCF Unit (Fig. 1). The 5-mL reaction cell was a modified, high-pressure gauge snubber (Chemiquip, West New York, NJ; Part # 30-31HF4-GS) seal on one end with a gland and plug (Autoclave Engineers, Erie, PA; Part # PA AGL 40-316 and AP40-316, respectively). The snubber head was modified with a stainless steel frit (Mott Metallurgical Corp., Farmington, CT; Part # 1000-.4375-.0625-10) and held in place by a modified gland (Autoclave Engineers; Part # HP50G). The assembled cell was vertically mounted above a magnetic stir plate inside the SPE-ED unit oven.

The reaction cell was loaded with 111 mg EF (0.50 mmol), an equal-mole amount of 1-octanol (79.2 μL) or triolein (484 μL), 111 mg Novozym® 435, and a Teflon stir bar. The reaction cell was sealed, plumbed into the SPE-ED unit, and allowed to equilibrate to the desired temperature for 30 min; then the CO_2 was adjusted to the desired pressure. The reactions were stirred for a designated amount of time. The reactor cell was then isolated by closing screw valve 1 (Fig. 1) and allowed to cool to ambient temperature (~25°C). Finally, the reactor cell was depressurized by venting the CO_2 through screw valves 2 and 3 (Fig. 1).

For the alcoholysis reactions of EF with 1-octanol, the contents of the reaction cell were extracted with 5.0 mL of ethanol with stirring for 30 min. A 10 μL aliquot of the extract was diluted to 2 mL in 50% aqueous ethanol and filtered (0.45 μm Gelman Acrodisc 13LC PVDF syringe filters; Sigma-Aldrich) before HPLC analysis. For the transesterification reactions of EF with triolein, the contents of the reaction cell were extracted with 5.0 mL of acetone with stirring for 30 min. A 100 μL aliquot of the extract was then diluted to 1 mL in acetone and filtered (0.45 μm Gelman Acrodisc 13LC PVDF syringe filters) before HPLC analysis.

HPLC analyses. Methods for the HPLC analyses of the alcoholysis and transesterification reactions of EF have previously been described (1). Octyl ferulate (OF) yields for the alcoholysis reactions were calculated based on the total peak area of EF, OF, and ferulic acid (FA) detected at 360 nm. The sum of the EF, OF, and FA peak areas remained constant over the time course of the reaction, allowing accurate OF yields to be calculated as the percentage ratio of the OF peak area to the total peak area of all three species.

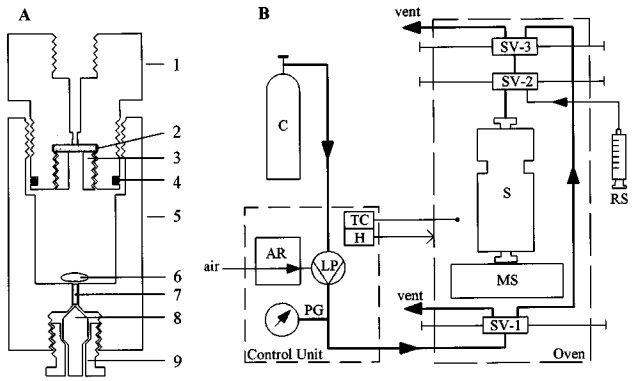

FIG 1. Apparatus for supercritical CO_2 (SC-CO_2) batch reactions. (A) Modified, high-pressure gauge snubber: (1) snubber head; (2) metal frit; (3) modified gland; (4) O-ring; (5) snubber body; (6) stirbar; (7) Teflon plug; (8) metal plug; (9) gland. (B) Modified Applied Separations SPE-ED SCF Unit: C = liquid CO_2 tank; LP = liquid pump; AR = air regulator; TC = thermal couple; H = heater; PG = pressure gauge; SV-1, 2, 3 = screw valves; S = snubber; MS = magnetic stirrer; RS = rinse syringe. See the Experimental Procedures section for manufacturers and part numbers.

RESULTS AND DISCUSSION

Alcoholysis of EF in SC-CO_2 batch reactions. The alcoholysis of EF with 1-octanol to form OF was catalyzed with Novozym®435 in SC-CO_2 in stirred batch reactions. A 1:1 molar ratio of EF to 1-octanol catalyzed by 1 weight equivalent of enzyme (based on EF) was previously reported to be the optimal conditions for obtaining the maximum conversion of EF in toluene (1). HPLC analysis of a control reaction in SC-CO_2 consisting of EF without enzyme showed quantitative recovery of EF from the reaction cell. Therefore, it was assumed that EF, OF, and FA were not lost during depressurization and subsequent extraction. The unwanted hydrolysis of EF, a consequence of the 1 to 2% (by weight) water content of the Novozym®435, resulted in less than 1.5% FA production. It was shown previously that additional water was deleterious to the alcoholysis of EF, resulting in increased hydrolysis (1). No effort was made to study the enzyme's performance at lower water concentrations.

The temperature effect on the alcoholysis of EF in SC-CO_2 batch reactions performed at 20.7 MPa (3,000 psi) is shown in Figure 2A. Each data point represents the average yield of three separate trials, while the error bars indicate the standard deviation from the mean of the three measurements. The relative standard deviation was typically ±3%. This indicated that the experimental procedure and analysis were precise; therefore, only one trial was performed for each data point for subsequent reactions.

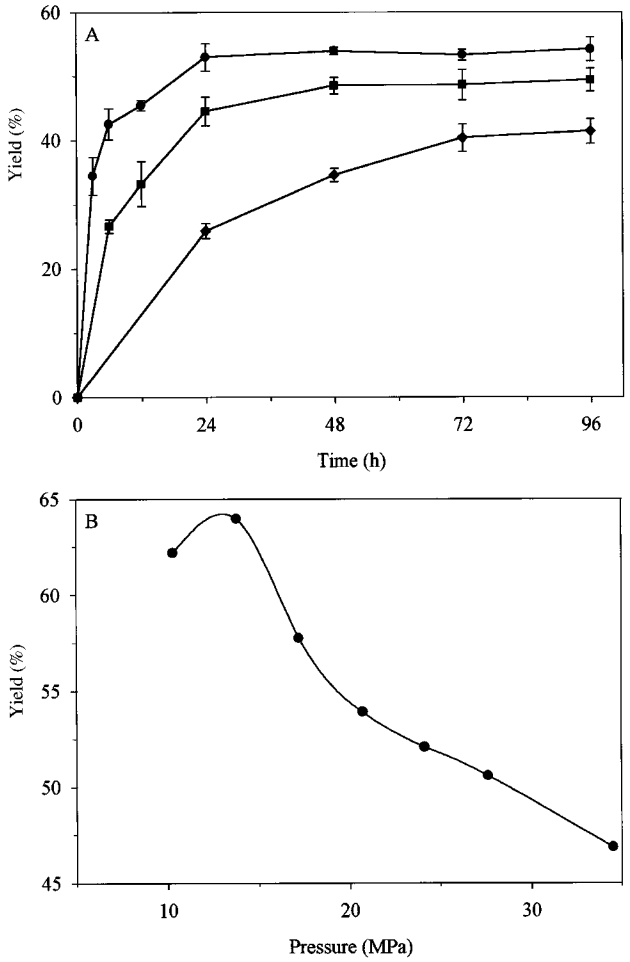

FIG. 2. (A) Temperature effect on the alcoholysis of 0.1 M ethyl ferulate with 0.1 M 1-octanol catalyzed by 110 mg of Novozym®435 (Novo Nordisk, Franklinton, NC) in SC-CO_2 at 20.7 MPa (3,000 psi) and 80 (●), 60 (■), and 45 (◆)°C. Error bars denote the standard deviation for a minimum of three trials. (B) Pressure effect on the alcoholysis of 0.1 M ethyl ferulate with 0.1 M 1-octanol catalyzed by 110 mg of Novozym®435 in SC-CO_2 at 80°C. For abbreviation see Figure 1.

When performed at 45°C the alcoholysis did not reach equilibrium in 96 h, at which time only a 41% yield of OF was achieved. Figure 2A shows, however, that the alcoholysis did reach equilibrium after 48 h at 60°C and 24 h at 80°C, resulting in 49 and 54% yields, respectively. It has been shown that the initial rates of lipase activity in SC-CO_2 may be dependent upon the formation of carbamate-lipase complexes, which were assumed to be less active than the dissociated enzyme (10). A lipase from *C. cylindracea* was shown to favor carbamate complexation at temperatures below 50°C while mostly dissociated CO_2 and lipase existed at temperatures above 60°C (10). It is reasonable to assume that Novozym®435 would also undergo similar carbamate complexation. Therefore, reactions performed at temperatures <60°C would have lower enzyme activity, which is consistent with the observed alcoholysis rates (Figure 2A).

Novozym®435 has been reported to remain active above 80°C (7); however, the effect of higher temperatures on the prolonged activity of the enzyme was not determined. Jackson and King (11) have reported that the activity of Novozym®435 is diminished at much lower temperatures, >50°C at 24.1 Mpa (3,500 psi), during the methanolysis of corn oil. Therefore, it was assumed that enzyme activity would be lower above 80°C, and alcoholysis experiments at temperatures exceeding this were not attempted.

As stated above, the alcoholysis of EF reaches equilibrium sooner at 80 than at 60°C (Fig. 2A), which is in agreement with previous studies which show that Novozym®435 exhibits higher reaction rates at higher temperatures (7). However, faster enzyme kinetics do not explain the higher OF yields obtained at 80 vs. 60°C. This effect may be a consequence of the increased solubility of one or more of the substrates at the lower CO_2 density.

The effect of pressure on the alcoholysis of EF is shown in Figure 2B. The alcoholysis of EF with 1-octanol was performed at 80°C for 48 h at pressures ranging from 10.3 to 34.5 MPa (1,500 to 5,000 psi). The alcoholysis reached a maximum yield of ~64% between 10.3 and 13.8 MPa (1,500 and 2,000 psi). Higher pressures were deleterious to the reaction. A similar pressure-dependent yield maximum at ~20.7 MPa (3,000 psi) has been reported for the formation of monoglyceride during the glycerolysis of soybean oil performed over the range of 5.5 to 62.0 MPa (800 to 9,000 psi) (12).

Transesterification of EF in SC-CO_2 batch reactions. The transesterification of EF with triolein catalyzed by Novozym® 435 in toluene or in neat reactions produces the UV-absorbing lipids monoferuloyl-monodeoyl-glycerol (FML) and feruloyl-dioleoyl-glycerol (FDO) (1). It is envisioned that the final reaction mixture containing FMO and FDO could simply be filtered from the enzyme-support and used without further purification in sunscreen formulations. To facilitate the formulation of the final reaction mixture into a typical sunscreen lotion containing 15% active ingredient, the highest possible weight percentage of FMO and FDO in the final reaction mixture is desired. A foible of the previously described FMO and FDO synthesis was that a 3:1 triolein-to-EF mole ratio was needed to perform the reaction neat, resulting in lower concentrations of FMO and FDO in the final reaction mixture. Also, the reaction took ~5 d to reach equilibrium when conducted neat. The reaction can be performed using a 1:1 triolein-to-EF mole ratio with toluene as a reaction medium. In the organic solvent, the reaction time was lowered to ~3 d; however, the overall yield of FMO and FDO was lower, and residual solvent in the final reaction mixture is undesirable. SC-CO_2 could be an ideal medium for the equal-molar transesterification of EF with triolein because it is environmentally benign and eliminates toxicity concerns previously raised by residual organic solvents in the final reaction mixture.

The transesterification of 1:1 molar mixtures of EF and triolein catalyzed by Novozym®435 were performed in SC-CO_2 in stirred batch reactions. HPLC analysis indicated that the products were identical to those obtained during the transesterifications performed either neat or in toluene (1). The four UV-absorbing species obtained during the transesterification were ferulyl glycerol and unreacted EF, both considered by-prod-

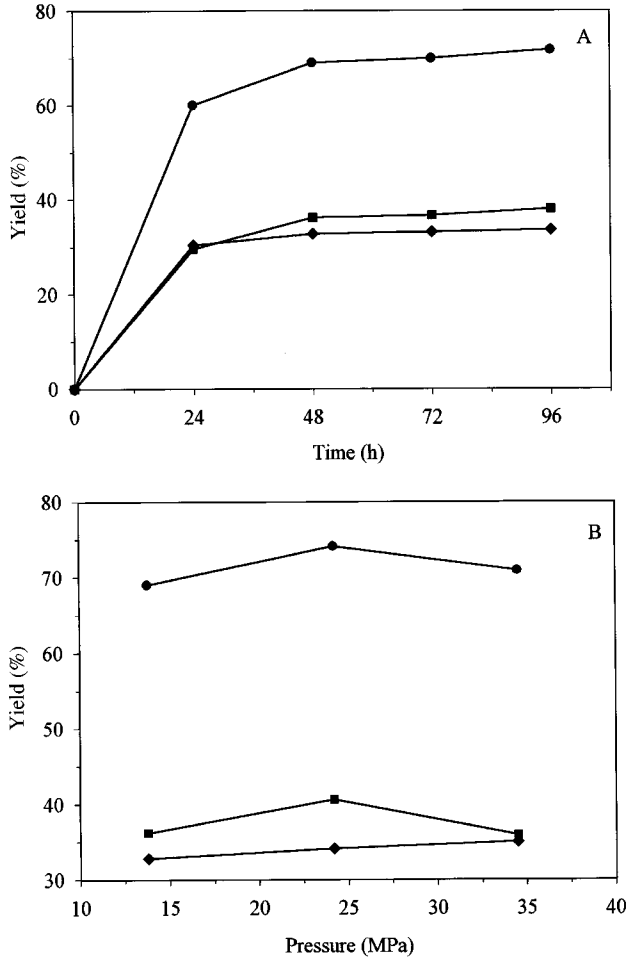

FIG. 3. Yield of monoferuloyl-monooleoyl-glycerol (FMO, ◆), feruloyl-dioleoyl-glycerol (FDO, ■), and FMO and FDO combined (●) obtained during the transesterification of 0.1 M ethyl ferulate with 0.1 M triolein catalyzed by 110 mg of Novozyme®435 in SC-CO_2 at (A) 80°C and 13.8 MPa (2,000 psi) and (B) 80°C for 48 h.

ucts, and FMO and FDO, the products of interest. Figure 3A shows that the synthesis of FMO and FDO at 13.8 MPa (2,000 psi) and 80°C reached equilibrium with a combined yield of 70% after 48 h. The FMO yield of 33% at equilibrium was slightly lower than that of the FDO, which was 37%. Autoclaved (250°C, 0.1 MPa) samples of Novozym® 435 did not facilitate the transesterification of EF with triolein; therefore, the transesterification is not spontaneous under these reaction conditions, and the catalytic activity can be attributed to the enzyme and not its acrylic support.

As discussed above, the alcoholysis of EF with 1-octanol attains a yield maximum at 13.8 MPa (2,000 psi) and 80°C; therefore, these reaction conditions were applied to the transesterification of EF with triolein. However, the solubility of triolein in SC-CO_2 (reported as the mole fraction of triolein in SC-CO_2, X_{TO}) increases with increasing pressure from 2.50×10^{-4} X_{TO} at 13.8 MPa (2,000 psi) to 5.25×10^{-3} X_{TO} at 24.8 MPa (3,600 psi) (13). Increasing the concentration of triolein in the SC-CO_2 mixture by increasing the pressure could affect the enzyme's kinetics as well as the overall yield of FMO and FDO. Figure 3B shows the effect of increasing pressure on the equilibrium yield of FMO and FDO during the transesterification of EF with triolein. The synthesis reached a maximal combined yield of FMO and FDO, 74%, at 24.1 MPa (3,500 psi) and 80°C. The FMO yield slightly increased from 33 to 35% with increasing pressure while the FDO yield reached a maximum of 41% at 24.1 MPa. Therefore, the optimal conditions for the transesterification of EF with triolein are 80°C and 24.1 MPa for 48 h.

Comparison of EF transesterifications in various solvents. The purpose of optimizing the transesterification of EF with triolein is to obtain a final reaction mixture containing the highest possible concentrations of FMO and FDO, which can be formulated as an "all-natural" active ingredient in sunscreen lotions. To be industrially attractive, the synthesis of FMO and FDO should be simple, maximize the FMO and FDO yields, be void of solvents, and reach equilibrium in a reasonable amount of time. Table 1 compares the transesterifications of EF with triolein in various solvents and their combined FMO and FDO yields. The least attractive of these synthetic options involves toluene. Although the addition of toluene allows the use of a 1:1 ratio of EF to triolein, the combined FMO and FDO yield after 48 h (60°C) was only 42% (1). The transesterification in toluene eventually reached a combined FMO and FDO equilibrium yield of 46% after 72 h, which is the lowest of the various transesterifications.

Using toluene as the reaction medium also introduces the issue of residual solvent left in the final product, negating the "all-natural" characteristics of the synthesis. The use of an organic solvent could be overcome by performing the transesterifications neat (60°C) during which a combined FMO and FDO yield of ~ 60% after 48 h was achieved (1). Eventually, the neat transesterification reached equilibrium after 5 d, resulting in a combined FMO and FDO yield of 77%. The time needed for the neat reaction to attain equilibrium may be too long to be industrially viable even though the combined FMO and FDO yield is 1.7 times higher than that obtained in the toluene reaction. Also, the mechanics of the neat reaction required a 3:1 triolein-to-EF ratio to allow the enzyme-support to be suspended

TABLE 1
Comparison of the Transesterification of EF with Triolein Performed in Various Solvents

	Neat[a]	Toluene[b]	SC-CO_2[c]
Triolein/EF ratio	3:1	1:1	1:1
Temperature (°C)	60	60	80
Time (h)	48	48	48
FMO + FDO yield (%)[d]	61	42	74

[a]Described in Reference 1, the transesterification attains a FMO + FDO equilibrium yield of 77% after 144 h. A 3:1 triolein-to-EF ratio is needed to facilitate stirring of the reaction.
[b]Previously described in Reference 1. This transesterification attains FMO + FDO equilibrium yield of 46% after 72 h.
[c]The transesterification was described in the Experimental Procedures section and was performed at 80°C and 24.1 MPa (3,500 psi).
[d]Percentage yields were calculated based on the total peak area of all ultraviolet (UV)-absorbing species recorded using a UV detector. Abbreviations: EF, ethyl ferulate; SC-CO_2, supercritical CO_2; FMO, monoferuloyl-monooleoyl-glycerol; FDO, feruloyl-dioleoyl-glycerol.

and stirred. This resulted in a 20% (w/w) concentration of FMO and FDO in the final reaction mixture, which would then have to be diluted by greater than twofold in order to be formulated into a lotion. Typical sunscreen formulations require 15% (w/w) active ingredient to produce a sun protection factor (SPF) of 10 to 15; therefore, the FMO and FDO weight percentage in the final reaction mixture is too low.

The optimal method for performing the transesterification of EF with triolein to obtain UV-absorbing lipids for sunscreen formulations is in SC-CO_2 batch reactions. A 1:1 ratio of triolein to EF can be reacted to produce a combined FMO and FDO yield of 74% after 48 h at 24.1 MPa (3,500 psi) and 80°C. This yield is comparable to the combined FMO and FDO equilibrium yield obtained in the reactions performed neat (77%), but it is achieved in less than half the time. Also the 74% conversion of EF corresponds to 46% (w/w) of FMO and FDO in the final reaction mixture, which is double the concentration that was obtained in the neat reaction. Finally, SC-CO_2 is nontoxic, noncarcinogenic, nonflammable, and leaves no solvent residue in the final reaction mixture (5).

The same enzyme-support load can be used to catalyze multiple triolein-EF transesterification batch reactions (1). Lipase from *C. cylindracea*, however, has been shown to lose ~40% residual activity in SC-CO_2 after 30 pressurization (~14.8 MPa)/depressurization cycles at 65°C (14). Therefore, an industrial scheme for the synthesis of FMO and FDO would preferably entail a semicontinuous flow, batch reactor that would not be depressurized. Reactants could be fed *via* a SC-CO_2 stream into a batch reactor and stirred for 48 h. Then the reactor would simply be flushed with SC-CO_2 to remove the final reaction mixture from the enzyme-support. The reactor would then be recharged with additional reactants.

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