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Lipase-Catalyzed Randomization of Fats and Oils in Flowing Supercritical Carbon Dioxide

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ABSTRACT: Enzymes can frequently impart more selectivity to a reaction than chemical catalysts. In addition, the use of enzymes can reduce side reactions and simplify post-reaction separation problems. In combination with an environmentally benign and safe medium, such as supercritical carbon dioxide (SC-CO_2), enzymatic catalysis makes supercritical fluids extremely attractive to the food industry. In this study , randomization of fats and oils was accomplished with an immobilized lipase in flowing SC-CO_2. Triglycerides, adsorbed onto Celite, are solubilized in CO_2 and carried over 1–10 g immobilized lipase derived from *Candida antarctica*. The degree of randomization and rate of triglyceride throughput could be controlled by CO_2 pressure and flow rate and quantity of enzyme used. The dropping points and solid fat indices of the resulting randomized oils were compared to oils that were randomized by conventional methods with sodium methoxide. Reversed-phase high-performance chromatography with flame-ionization detection was used to quantitate changes in triglyceride composition of various substrates, such as palm olein and high-stearate soybean oil. The resultant randomized oil mixtures have properties, e.g., solid fat index, that make them potential candidates for incorporation into traditional margarine formulations.
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KEY WORDS: Dropping point, enzyme, lipase, palm olein, randomization, solid fat content, supercritical carbon dioxide.

Randomization, or interesterification, of fats and oils has been used for decades to improve the physical properties of triglycerides for use in the margarine and confectionery industries (1–4). The reaction was first described in 1924 by van Loon in a British patent (5) as being catalyzed by tin (II) chloride. In the 70 years since this patent, many other patents have been issued (6–9), and now the reaction is typically catalyzed by sodium methoxide or sodium metal (10). This requires that the oil be free of moisture or compounds that can react with and destroy such catalysts.

Randomization of oils has also been catalyzed by lipases (11–14). The use of biocatalysts is justified by the selectivity of lipases, which offers some control over the products; for example, 1,3-regiospecific lipases have been used to prepare cocoa butter substitutes (15,16) and designer triglycerides for nutritional studies and pharmaceuticals (17).

The use of biocatalysts with supercritical carbon dioxide (SC-CO_2) has been growing rapidly in recent years (18,19). Biocatalysts have the advantage of substrate specificity under mild reaction conditions, and SC-CO_2 has several advantages over organic solvents (20): The solvent properties of SC-CO_2 can be modified readily by adjusting pressure or temperature; the diffusivity of solutes in CO_2 is higher than in organic solvents; CO_2 can easily be removed from the reaction products, which minimizes the need for costly downstream cleanup; when CO_2 is used in lieu of organic solvents, it has the additional benefit of being environmentally benign. Several papers have appeared that describe interesterification (21), acidolysis (22), and methanolysis (23) reactions performed in SC-CO_2.

In this paper, we report on the randomization of triglycerides in flowing SC-CO_2. We also report on the physical properties of the resultant oils and fats with respect to their suitability as margarine hard stocks.

MATERIALS AND METHODS

High stearic acid soybeans, designated A-6, were supplied by W. Fehr of Iowa State University (Ames, IA) and those designated HS-1 were from the Jacob Hartz Seed Co. (Stuttgart, AR). Two bushels of A-6 from the 1989 crop year were divided into two equal portions prior to processing; HS-1 was from the 1993 crop year. Palm olein was obtained from PVO International (Granite City, IL). Celite 521 was from Sigma Chemical Co. (St. Louis, MO). Novozym 435 was purchased from Novo Nordisk (Boehringer Mannheim, Indianapolis, IN).

Randomizations were performed in a previously described apparatus (23). Approximately 16 g oil or fat was mixed with about 12 g Celite 521 to form a pourable powder. This was loaded into a stainless-steel cell (1.7 cm i.d. × 23 cm) and held in place with glass wool plugs. This cell was inserted into the flow stream right before a cell that contained the catalyst bed, which consisted of 1–10 g of supported Novozym 435 (usually 10 g in preparative runs where dropping point and solid fat content (SFC) were determined on the resultant products)

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in a second stainless-steel cell of the same dimensions as the cell containing the lipid substrate (Fig. 1). The Novozyme 435 was dried for approximately ½ h at 27.5 MPa and a CO_2 flow rate of 10 L/min by using a gas booster pump (Model ACT 62/152; Haskel Inc., Burbank, CA), before diverting the CO_2 into the cell with the substrate, and then through the enzyme bed.

All interesterifications were run at 65°C. CO_2 flow rates were set between 6 and 14 L/min with a micrometering valve (Series 30VRMM; Autoclave Engineers, Erie, PA) and measured as expanded gas by a dry test meter (American Meter Division, Philadelphia, PA). The micrometering valve was maintained at 90°C to give a steady flow. The product was collected at atmospheric pressure in a 100-mL round-bottomed flask. The reaction temperature was monitored by a Type-J thermocouple (Omega Engineering, Stanford, CT) placed at a tee (Series SW 250; Autoclave Engineers).

Supercritical fluid chromatography was performed as previously described (23). The reversed-phase high-performance liquid chromatography (HPLC) separations of triglycerides (24) were performed, in duplicate, by injecting 10-mg samples into a liquid chromatograph equipped with a flame-ionization detector (Tracor Model 945; Finnegan, Austin, TX) and two C-18 (0.49 × 50 cm, 5-µm particle size) Zorbax columns (DuPont, Inc., Wilmington, DE) placed in series. Triglyceride samples of 0.5 mg in 5–10 mL of methylene chloride were then resolved by using a 120-min gradient of 70:30 to 40:60 acetonitrile/methylene chloride (vol/vol) pumped at 0.8 mL/min. The columns were cleaned between analyses with a 100% methylene chloride rinse. The flame-ionization detector's block temperature was kept at 130°C. The detector used a gas flow of 140 mL hydrogen/min, whereas the cleaning flame used a hydrogen flow of 250 mL/min and oxygen at 175 mL/min. Triglycerides were identified by peak retention times with respect to synthetic standards. The flame-ionization detector signal was processed by a computer, and quantitation of the triglycerides was accomplished by comparison with a standard triglyceride mixture (#406; Nu-Check-Prep, Inc., Elysian, MN).

SFC was determined by following AOCS Official Method Cd16b-93 and by using a Bruker Minispec pc100 (Bruker Spectrospin, Ltd., Milton, Ontario, Canada) (25). Dropping points were determined by AOCS Official Method Cc 18-80 (26). These determinations were made with a Mettler FP90 Central Processor and a Mettler FP83 Dropping Point Cell (Mettler-Toledo, Inc., Hightstown, NJ). Measurements were made from 10 to 40°C at a heating rate of 2°C/min.

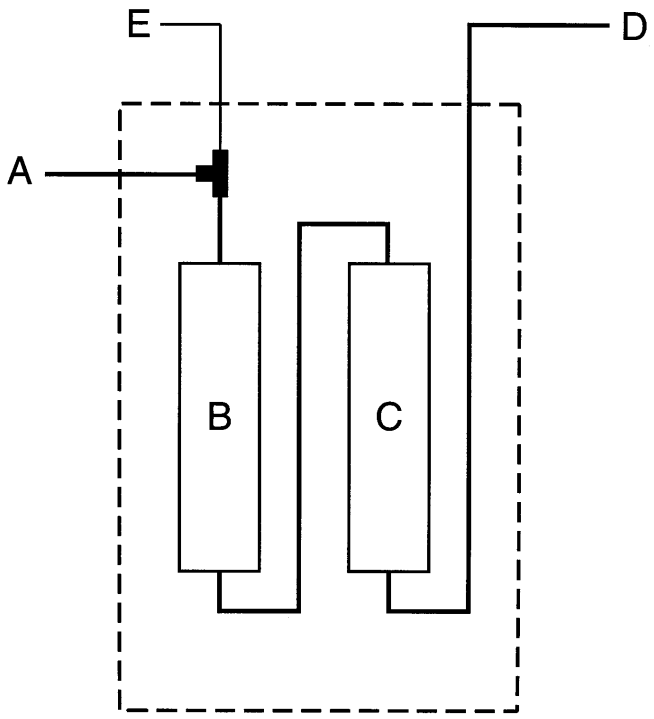

FIG. 1. Schematic of the system used for the randomization of fats and oils with flowing supercritical carbon dioxide. A: CO_2 inlet after the gas is brought to operating pressure; B: stainless-steel vessel that contains fat or oil adsorbed onto Celite (Sigma Chemical Co., St. Louis, MO); C: stainless-steel vessel with immobilized Novozym 435 (Novo Nordisk; Boehringer Mannheim, Indianapolis, IN); D: outlet leading to micrometering valve and receiver; E: in-line thermocouple for temperature control. The dashed line represents the oven.

RESULTS AND DISCUSSION

Table 1 shows the dropping points of several fats and oils before and after randomization. For all pure starting materials except tallow, the randomized triglyceride has a dropping point higher than the natural oil. This is in general agreement with randomizations performed without solvent and with chemical catalysts (27), indicating that randomizations performed in SC-CO_2 with lipase as catalyst are comparable to conventional methods.

Effect of pressure. The randomization reaction was carried out at four different pressures in the following order: 27.6, 34.5, 41.4, 17.2, and again at 27.6 MPa. The effect of pres-

TABLE 1
Comparison of Dropping Points of Various Fats and Oils Before and After Randomization in Dynamic SC-CO_2[a]

Sample[b]	Initial dropping point (°C)	Final dropping point (°C)
RBD soybean oil[c]	0.9	10.7
HS-1 soybean oil	18.3	27.6
A-6,3 soybean oil	20.5	30.4
Palm olein	21.7	37.7
Cocoa butter	32.0	41.6
Tallow	41.3	38.8
20% Tristearin 80% RBD soybean oil	60.4	37.1

[a]SC-CO_2, Supercritical carbon dioxide; experimental conditions: 27.5 MPa at 65°C, with 10 g of Novozyme 435 (Novo Nordisk; Boehringer Mannheim, Indianapolis, IN).
[b]Approximately 16 g oil or fat/12 g Celite (Sigma Chemical Co., St. Louis, MO).
[c]Refined, bleached, deodorized (RBD) soybean oil. A-6 (Iowa State University, Ames, IA); Palm olein (PVO International, Granite City, IL).

sure is twofold: Triglycerides are more soluble at higher pressure and enzyme activity tends to be adversely affected by higher pressure. Using the dropping point of the product as a measure of the extent of the reaction, we were able to show that randomization performed at 17.2 MPa resulted in a product that was similar to that obtained by chemical randomization (Table 1). At higher pressures, throughput was much higher, though the dropping point was not enhanced as much as for randomizations conducted at lower pressures. This indicates that the product is less random and that the randomization reaction is not complete. This partial reaction may be the consequence of higher lipid solubility or decreased enzyme activity. At 17.2 MPa, throughput was low owing to the low solubility of triglycerides at this pressure. The recorded lower dropping point of the product may be the result of this low solubility, i.e., randomization is an intermolecular reaction and not enough palm olein is available under these conditions to allow for randomization to occur.

Effect of CO_2 flow rate. The flow rate of CO_2dictates the rate at which the substrate passes through the catalyst bed and therefore serves as a means to control the reaction outcome. Ten grams of Novozym 435 was used to randomize palm olein (10–15 g) through which the CO_2 flow rate was approximately 6 L/min and 13 L/min. The dropping points of the products obtained were 37.9 and 36.4°C, respectively, which are higher than the dropping point of the starting palm olein (21.7°C). The subtle increase in dropping point at the lower CO_2 flow rate undoubtedly reflects the increased contact time between the oil dissolved in the fluid phase and the catalyst in the reactor. Thus, flow becomes another important variable to affect the physical properties of the resultant product. At higher flow rates, the restrictor became partly blocked, and flow control of the CO_2 was difficult.

Effect of enzyme quantity. Palm olein was randomized with 1–10 g of Novozym 435. All reactions were carried out at 65°C and 27.5 MPa at a CO_2 flow rate of 12.5 L/min. As would be expected, increasing the amount of catalyst resulted in a fat with a higher dropping point (Fig. 2). The dropping points obtained with 1.25, 2.5, 5.0, and 10.0 g catalyst were 26.3, 32.7, 35.8, and 37.1°C, respectively. The dropping point of the natural palm olein was 21.7°C, indicating that even the smallest amount of enzyme had a sizable effect on the dropping point of the triglyceride mixture. The throughput of randomized palm olein through the system decreased with an increase in the quantity of enzyme used (Fig. 2). When 1.25 g enzyme was used, palm olein was collected at 0.46 ± 0.01 wt% in CO_2. When 10.0 g of the enzyme was used, the throughput was 0.38 ± 0.07 wt%. Thus, it appears that the dropping point and product throughput are inversely related and that the maximum throughput and dropping point can be realized for this particular interesterification at 2.5 g of enzyme.

SFC of HS-1 and palm olein. Figure 3 shows the SFC of the high-stearate soybean oil, HS-1, and palm olein before and after randomization. After randomization, both of these oils show an increase in SFC at higher temperatures and a decrease at lower temperatures. The randomized HS-1 and palm olein begin to have a higher SFC than they do in their native state at approximately 15 and 20°C, respectively. Typical SFC values for soft-tub margarine oils, obtained by blending hydrogenated and liquid soybean oils, are as follows: 10°C, 18–24; 21.1°C, 6–11; 33.3°C; 1–2. Dropping points for such blends typically ranged from 30–33°C. A typical example of a hydrogenated blend product's SFC dependence on temperature is also shown in Figure 3. Again, above approximately 26°C, both enzymatically randomized oils have higher SFC than the hydrogenated blend. We have also included in Figure 3 a shaded area that designates the typical SFC-vs.-temperature range encountered for commercial soft-tub margarines. It is apparent that the randomized palm olein product has an SFC that exceeds that typically recorded for commercial products, and that randomized HS-

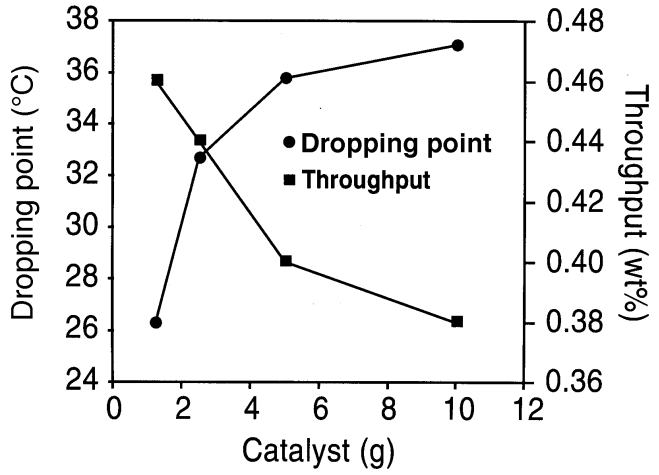

FIG. 2. Effect of amount of catalyst used for interesterification of palm olein on the dropping point and throughput of the resultant mixture.

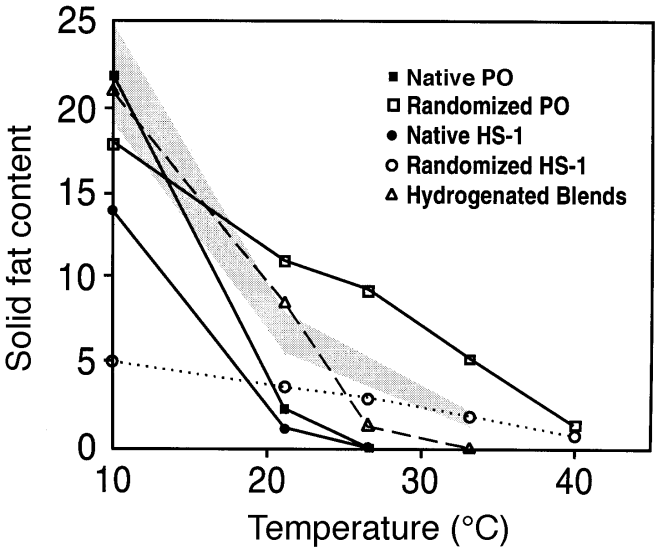

FIG. 3. Solid fat content of palm olein (PO) and HS-1 soybean oil (Jacob Hartz Seed Co., Stuttgart, AR) before and after randomization. Reaction conditions identical to those in Table 1.

1 product approaches the SFC values found for soft commercial margarines.

It should be obvious that palm olein in its natural state does not contain sufficient solids over the above temperature range (10–33°C) to qualify as a soft-tub margarine oil because its dropping point of 21.7°C is too low. However, after randomization, the SFC profile approximates that of a soft-tub margarine oil, and the dropping point increases to 37.7°C, a value comparable to hydrogenated oils. Hence, enzymatic randomizations of palm olein in SC-CO_2 appear to show promise in soft margarine formulations.

The HPLC profiles (Fig. 4) for these oils show the change in triglyceride composition that results from randomization of the HS-1 and palm olein moieties. Inspection of these profiles reveals that the relative proportions of LOP and SSO triglycerides have been changed via randomization in the palm olein sample; likewise, LLO, LnSS, LLS, and SOS have all increased in the randomized HS-1 sample relative to the amounts of other triglyceride species found in the native HS-1 sample where L = linoleic, O = oleic, P = palmitic, S = stearic, Ln = linolenic acids.

Cumulative data taken from these chromatograms are presented in Table 2 in terms of the combined effect of triglyceride carbon number and saturate-to-unsaturate content. Both oils show loss of disaturates with concommitant increases in tri- and diunsaturates as well as small increases in trisaturates. This result is quite different from those obtained with 1,3-specific lipases (28). The loss of disaturate functionality *via* randomization should aid in any crystallization process (29).

In summary, unlike batch reactions with an inorganic catalyst, randomizations catalyzed by a lipase in flowing SC-CO_2 offer some control over the outcome of the reaction. Adjustments in pressure, CO_2 flow rate, or enzyme quantity each affects the rate of triglyceride throughput and the degree of randomization achieved. It is also interesting to consider the possibility of combining the above reactions, conducted in the presence of SC-CO_2, with upstream fractionation of the constituent oil *via* supercritical fluid extraction or fractionation (30). A host of oleochemical products can thus be custom-designed as a consequence of the flexibility offered by a supercritical fluid processing step.

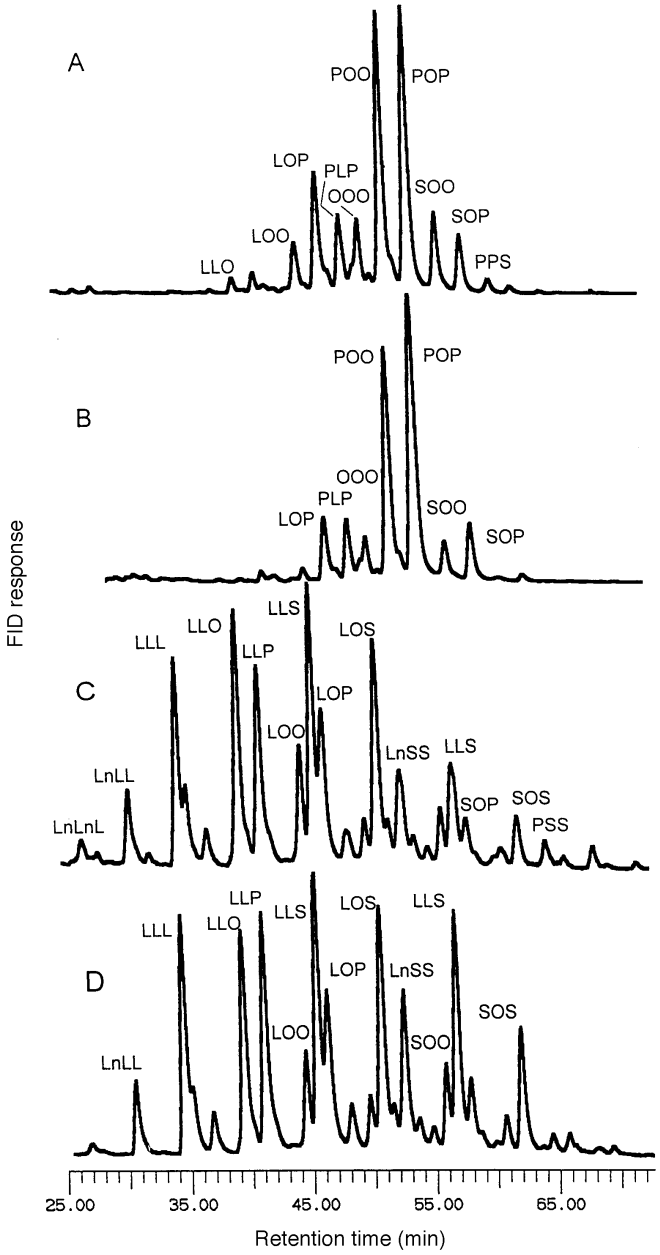

FIG. 4. Reversed-phase high-performance liquid chromatography with flame–ionization detector (FID) chromatograms of palm olein and HS-1 soybean oil triglycerides before and after randomization. A: randomized palm olein; B: palm olein; C: randomized HS-1 soybean oil; D: HS-1 soybean oil. Abbreviations for constituent triglyceride fatty acids: L, linoleic; Ln, linolenic; P, palmitic; O, oleic; S, stearic acids. Conditions are given in the Materials and Methods section and in Reference 24. See Figure 3 for company source.

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TABLE 2
Composition of Palm Olein and High-Stearate Soybean Oil Before and After Randomization in SC-CO_2[a]

Oil	Triunsaturates	Diunsaturates	Disaturates	Trisaturates
Palm olein	10.2	40.8	46.1	2.6
Random palm olein	13.0	44.5	39.2	2.8
HS-1 soybean oil	28.7	44.4	23.5	2.4
Random HS-1	29.1	45.3	18.9	3.5

[a]All reactions run at 27.5 MPa, 65°C; 16 g of fat or oil/12 g of Celite; 10 g of Novozyme 435. See Table 1 for company sources.

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