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Enzymatic Interesterification of Extra Virgin Olive Oil with a Fully Hydrogenated Fat: Characterization of the Reaction and Its Products

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Abstract The lipase-catalyzed interesterification of extra virgin olive oil (EVOO) and fully hydrogenated palm oil (FHPO) was studied in a batch reactor operating at 75 °C. The compositions of the semi-solid fat products depend on the reaction conditions and the initial ratio of EVOO to FHPO. The dependence of the quasi-equilibrium product TAG profile on the reaction time was determined for initial weight ratios of EVOO to FHPO from 80:20 to 20:80. Lipozyme TL IM, Lipozyme RM IM and Novozym 435 were employed as biocatalysts. The interesterification reaction was optimized with respect to the type and loading of biocatalyst. Equilibrium was approached in the shortest time with Novozym 435 (80% conversion in 4 h). The chemical, physical, and functional properties of the products were characterized. Appropriate choices of the reaction conditions and the initial ratio of EVOO to FHPO lead to TAG with melting profiles and solid fat contents similar to those of commercial products. Differences were observed in the solid fat contents, melting profiles, and oxidative stabilities of the various interesterified products and also between the indicated properties of each category of product and the corresponding physical blend of the precursor reagents.

Keywords *Candida antarctica* · HPLC-mass spectroscopy · Lipase · Interesterification · Margarines · Extra virgin olive oil · Semisolid fat · Zero *trans* fats

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Introduction

The physical characteristics of oils and fats are determined by their chemical compositions [chain length and degree of unsaturation of fatty acid (FA) residues, as well as the distribution of FA residues along the glycerol backbone]. Enzymatic interesterification (IE) is an emerging method for modifying the physico-chemical properties of fats and oils. EI permits one to incorporate greater amounts of hard fats (e.g., palm stearin and palm kernel olein) in fat based spreads than is possible in simple physical blends of the precursor TAG [1]. Thus, softer products with lower melting points and with no phase separation problems can be produced by EI of a hard fat with a vegetable oil. Consequently, interesterified shortenings are softer than the corresponding (non-interesterified) physical mixtures and undergo minimal changes during storage.

Enzymatic processes based on the use of either *sn*-1,3 specific or nonspecific lipases are advantageous over chemical interesterification (CI) because of their enhanced selectivity (generation of fewer undesired byproducts compared to CI), mild reaction conditions (temperatures lower than 100 °C), ease of product recovery [2], reduced losses of oil/fat, fewer process steps, lower requirements of investment capital, and, decreased degradation of tocopherols. The most important disadvantage of EI is the

high cost of the biocatalyst. Nevertheless, this cost can be reduced if the biocatalyst can be reused.

Dietary ingestion of olive oil has been reported to have physiological benefits such as lowering serum cholesterol levels, suppressing certain types of cancer, enhancing liver function, and reducing the effects of aging and heart disease. Preparation of semi-solid fats from enzyme modified olive oil is an attractive alternative for the food industry, given the high oxidative stability of the product in frying situations, and the health enhancing properties of this material.

The goal of this research was to conduct a detailed study of the influence of reaction conditions on the compositions and functional characteristics of the products. Hence, we utilized an extra virgin olive oil and a solid fat (fully hydrogenated palm oil, FHPO). Semi-solid fats were prepared by enzymatic IE using seven different weight ratios of EVOO to FHPO (80:20, 70:30, 60:40, 50:50, 40:60, 30:70 and 20:80). Chemical, physical, and functional properties of the resulting products were characterized to identify potential applications of these materials.

Materials and Methods

Materials

The extra virgin olive oil was from Carbonell (Spain). The fully hydrogenated palm oil (P60-D) was from Vandemoortele (Belgium). For EVOO and FHPO, the free fatty acid (FFA) contents were 0.4 and 1.5%, respectively. An *sn*-1,3 non-specific immobilized lipase from *Candida antarctica B* (Novozym 435), and two *sn*-1,3 specific immobilized lipases from *Thermomyces lanuginosus* (TL IM) and *Rhizomucor miehei* (RM IM) were kindly provided by Novozymes (Bagsvaerd, Denmark). All solvents were HPLC grade (Scharlau, Barcelona, Spain). Standards of 1-oleyl-rac-glycerol, 1,3-diolein, tristearin (SSS), trilaurin (LaLaLa), tripalmitin (PPP) and triolein (OOO) [all at least 99% pure] were from Sigma Chemical (St Louis, MO). Standard mixtures of fatty acids and fatty acid methyl esters were purchased from Supelco (Bellefonte, PA). The hexadecane used as an internal standard for the GC analyses was obtained from Aldrich.

Reaction Protocol

The IE reaction between FHPO and EVOO was carried out at 75 °C (above the melting point of FHPO, 63 °C) in 50-mL stoppered flasks with orbital shaking at 300 rpm. The weight ratio of EVOO to FHPO was varied from 80:20 to 20:80. Different amounts of biocatalyst were studied (from 2.5 to 20% of the total weight of substrates). Aliquots (400 μL) of the reaction mixture were periodically withdrawn and analyzed. Reactions were allowed to proceed for 24 h.

Gas Chromatographic Analyses for Fatty Acid Composition

Samples (ca. 20 mg/mL) of the precursor reactants (the feed mixtures of EVOO and FHPO) and the products of all reaction trials (semi-solid fats) were prepared in chloroform solution. Hexadecane was employed as an internal standard. Samples (400 μL) were methylated as described by Otero et al. [3], and subjected to gas chromatographic analyses. One microliter of sample was injected into an Agilent (Palo Alto, CA) gas chromatograph (model 6850) fitted with an Omegawax column (30 m × 0.25 mm × 0.25 μm film thickness) purchased from Supelco (Bellefonte, PA). Injector and detector temperatures were 250 and 230 °C, respectively. The temperature program was as follows: starting at 50 °C for 2 min and then heating to 220 °C at 30 °C/min, holding at 220 °C for 20 min, followed by heating from 220 to 260 °C at 5 °C/min. Finally, the temperature was raised to 265 °C and held at this temperature for 10 min. Identification of the various fatty acids was based on a PUFA No. 3 standard (no. 4-7085) and FAMEMIX No. 37 from Supelco.

sn-2 Positional Analyses of Fatty Acid Residues

A known weight of oil or fat and an appropriate amount (30–50 mg) of porcine pancreatic lipase were placed in a stoppered flask. Then, 0.65 mL of Tris buffer (1 M, sodium salt), 0.35 mL of borax (0.19 M, sodium borate), 0.1 mL of calcium chloride (22% w/w) and 0.25 mL of bile salts (0.1% w/w) were added. The resulting mixtures were heated for 1 min to 40 °C without agitation, and then shaken at 250 rpm at 40 °C for 8 min. Then, addition of 1 mL of acetic acid (0.1 M) quenched the hydrolysis reactions. The product solutions were then extracted with three 1-mL portions of chloroform and filtered through a 0.45-μm nylon syringe filter to remove the enzyme.

The extracted reaction products were then concentrated to 250 μL in chloroform and analyzed by thin-layer chromatography. The chromatogram was developed with hexane/ethyl ether/acetic acid (50:50:1 v/v/v). The bands were visualized after being sprayed with 2% sulfuric acid in ethanol and heating. The bands corresponding to 2-monoacylglycerols were scraped from the silica plate and extracted with 3 mL ethyl ether, then filtered and concentrated to 300 μL in chloroform solution. The resulting solutions were methylated by addition of 1 mL of 0.1 N methanolic NaOH. These mixtures were heated at 60 °C for 30 min. The methyl esters of the fatty acid residues

were extracted as described by Otero et al. [3] and analyzed by gas chromatography.

sn-1,3 Positional Analyses of Fatty Acid Residues

A modified version of the method of Dourtoglou et al. [4] was employed. In a capped test tube 200 mL of the sample (oil or fat) and 200 mL of 1-butanol were placed, 250 mg of 1,3-specific lipase (Lipozyme RM IM or Lipozyme TL IM) were added, and the entire mixture was agitated. No differences were found in the results obtained using the two different lipases. The reaction was allowed to proceed for 2 min. Then the mixture was agitated gently using a vortex mixer. This procedure was repeated five times.

The reaction products were rinsed with 20 mL of pentane and 5 mL of a saturated aqueous solution of NaCl. After agitation and separation into two phases, the aqueous phase was discarded. The organic phase was extracted twice with 5 mL of the saturated solution of NaCl and then with 5 mL of distilled water. The organic phase was dried over sodium sulphate. The reaction products were determined using TLC analyses, whereas butyl esters were analyzed by gas chromatography as described above.

Normal Phase HPLC (NP-HPLC) Analyses

Aliquots of the reaction were taken at different times and dissolved in chloroform (12.5–15 mg/mL). 10-μl aliquots of these solutions were injected into an HPLC system consisting of a Merck–Hitachi HPLC equipped with a Sedex 55 Evaporative Light Scattering Detector (ELSD from SEDERE, France). The acylglycerol species were separated using a Kromasil silica column (260 mm × 4.6 mm × 5 μm) from Análisis Vínicos (Ciudad Real, Spain). The ELSD drift tube temperature was 70 °C, and the air pressure was 2 bar. The mobile phases were a hexane/2-propanol/ethyl acetate/formic acid mixture (80:10:10:0.1, by volume) and a hexane/formic acid solution (100:0.02, by volume). The solvent gradient was similar to that previously described by Liu et al. [5].

TAG Families in the Reactor Effluent

Reversed phase HPLC (RP-HPLC) was used to separate and quantify the TAG families present in samples of the feedstock mixtures (EVOO and FHPO) and the reaction products. The column was a Kromasil C18 column (260 mm × 4.6 mm × 5 μm) from Análisis Vínicos maintained at 25 °C. The ELSD drift tube temperature was 70 °C, and the air pressure was 2 bar. The flow rate of the eluant was 1 mL/min for a acetonitrile/dichloromethane solvent system (56:44, by volume) for 45 min. The conversion values are based on two of the most abundant TAG species present in FHPO (PSP plus SPS). The acylglycerol species present in the reaction mixture were identified by HPLC-MS analyses. The conversion values were calculated as the ratio of the increment in the area percentages of the above mentioned species to the corresponding initial area percentage:

$$\text{Conversion}\ (\%) = 100\times \Delta[\%\ \text{area (PSP)} + \%\ \text{area(SPS)}]_t / \times [\%\ \text{area (PSP)} + \%\ \text{area(SPS)}]_{t_0}$$

Chemical Characterization of TAG Species by HPLC-MS

The TAG present in the feedstocks (EVOO and FHPO) for the interesterification reaction, as well as the semi-solid fats produced by the reaction, were analyzed using a reversed phase HPLC system coupled to a mass spectrometer through an atmospheric pressure chemical ionization source (RP-HPLC/APCI-MS) as described elsewhere [3].

Determination of Free Fatty Acids

The FFA contents of the feedstocks and the fat products, calculated as oleic acid, were determined as described by Ronne et al. [6].

Differential Scanning Calorimetry Analyses of the Reaction Products

A Pyris 1 Perkin-Elmer (Shelton, CT) differential scanning calorimeter (DSC) was used to determine the thermal profiles of the reaction products. The DSC was calibrated using indium (m.p. = 156.6 °C) and mercury (m.p. = –38.87 °C). Samples containing 5–10 mg of the melted fat sample were placed in aluminum pans and sealed. These samples were rapidly heated from 25 to 80 °C at 20 °C/min, then held at 80 °C for 5 min. The samples were then immediately cooled from 80°C to –20°C and heated again to 80 °C at a constant rate of 5 °C/min. The final temperature of 80 °C was again maintained for 5 min. Duplicate analyses were used to generate the thermal profiles of all samples.

Solid Fat Content of the Reaction Products

The solid fat contents (SFC) of product samples were evaluated using a Bruker Minispec Solid Fat Analyzer. NMR tubes (10 mm in diameter) were filled with 1 mL of fat mixture and capped. Tempering pretreatment of all samples was carried out using IUPAC Method 2.150. SFC values were determined at intervals of 5 degrees between 0 and 60 °C. Duplicate analyses were conducted for each sample.

Oxidative Stability of the Reaction Products

The values of the induction period of three representative reaction products (those prepared with weight ratios of EVOO to FHPO of 80:20, 60:40, and 30:70) were determined with a 743 Metrohm Rancimat apparatus (Herisau, Switzerland) operating at 98 °C. For the oxidative stability analyses, *ca*. 2 g of the oil or semi-solid fat were weighed into a glass vessel. The conductimetric cells were filled with 50 mL of de-ionized water and air was passed through the heated oils at a flow rate of 20 L/h.

Results and Discussion

Time Course of the IE Reaction in a Batch Reactor

The progress of the lipase-catalyzed IE reactions was monitored until equilibrium was approached. The compositions of the two precursor reagents (EVOO and FHPO) are summarized in Tables 1, 2 in terms of both the overall and positional distributions of the component fatty acid residues and the constituent TAG, respectively. EVOO has oleic acid as its primary fatty acid residue and OOO and OPO as primary TAG species. Like the majority of vegetable oils, it contains an elevated proportion of unsaturated fatty acid residues at the *sn*-2 position. The FHPO contains mainly saturated fatty acid residues. The major TAG species contain palmitic and stearic fatty acid residues.

For all the experimental trials involving a variety of weight ratios of EVOO to FHPO, rapid depletion of the major TAG components of both precursor reagents (OOO for EVOO and PSP + SPS for the FHPO) was observed. As a consequence, rapid growth of the primary product TAG species was also noted. Figure 1 depicts the time course of the reaction for the trial involving 30% (w/w) FHPO with Novozym 435 as biocatalyst. Quasi equilibrium conditions were reached after 4 h of reaction.

As anticipated, the ready availability (high concentrations) of residues of oleic, stearic and palmitic acids governed the types and relative amounts of the newly formed TAG species. The IE reaction produces increases in the concentrations of five TAG species (OPO, POP, and the three positional isomers of SOP). Production of the three positional isomers of SOP in this trial is probably more a consequence of the lack of positional specificity of Novozym 435 than of acyl migration (e.g., migration after hydrolysis of SPS and/or PSP and subsequent re-esterification with O at the *sn*-2 position).

Effects of the Type and Loading of Biocatalyst

Several trials were conducted to elucidate the effects of enzyme type and degree of loading on the conversion achieved in 1 h of reaction. Three different immobilized lipases (Novozym 435, Lipozyme TL IM and Lipozyme RM IM) were employed in these trials (Fig. 2a, b). The conversions obtained in 1 h ranked in the order: Novozym 435 > Lipozyme TL IM > Lipozyme RM IM. In all these trials, the initial rate increased as the charge of enzyme increased from 2.5 to 20% (w/w) biocatalyst (Fig. 2a). In a batch reactor subjected to orbital agitation, Novozym 435 seems to be the most appropriate biocatalyst to use. We selected an enzyme loading of 10% (w/w) to perform subsequent experiments because at this loading one can achieve high conversions in reasonable times without encountering difficulties in obtaining uniform suspensions of the enzyme. Moreover, when one increases the amount of biocatalyst, the extents of the undesirable hydrolysis reactions increases also (data not shown).

The IE reactions catalyzed by each of the three biocatalysts were conducted until quasi-equilibrium conditions

Table 1 Molar composition and distribution of fatty acid residues of the starting materials

Fatty acid residue	Molar composition (in percent)					
	Overall		At *sn*-2 position		At *sn*-1(3) positions	
	EVOO	FHPO	EVOO	FHPO	EVOO	FHPO
C12:0 Lauric (La)		0.5				0.7
C14:0 Myristic (M)		1.5		N.D		2.2
C16:0 Palmitic (P)	10.8	57.3	14.9	56.0	8.7	58.0
C16:1 Palmitoleic (Pa)	1.4		N.D		2.1	
C18:0 Stearic (S)	2.8	36.0	8.6	44.0	N.D	32.0
C18:1 Oleic (O)	75.1	2.3	68.0	N.D	78.7	3.5
C18:2 Linoleic (L)	8.5	1.9	8.4		8.5	2.8
C18:3 Linolenic (Li)	0.7				1.0	
C20:0 Arachidic (A)	0.4	0.5	N.D		0.6	0.8
C20:1 Eicosenoic (E)	0.3				0.4	

FHPO fully hydrogenated palm oil, *EVOO* extra virgin olive oil, *N.D* Not detected

Table 2 Triacylglycerol compositions of the starting materials and the reaction products

Acylglycerols species	EVOO	FHPO	Physical blends			Interesterified products		
			Weight ratio of EVOO to FHPO					
			80:20	60:40	30:70	80:20	60:40	30:70
Diacylglycerols		2.13	0.43	0.85	1.49	3.96	3.98	5.12
LLL	0.61		0.49	0.37	0.18			
LLO, LOL	(LLO) 3.97		(LLO) 3.18	(LLO) 2.38	(LLO) 1.19	(LLO) 2.46	(LLO) 0.79	(LLO) 0.83
LLP, LPL	(LLP) 2.13		(LLP) 1.70	(LLP) 1.28	(LLP) 0.64	(LLP) 0.87	(LPL) 0.79	
OLO	11.62		9.30	6.97	3.49	9.27	4.62	1.95
OPaO, PaOO	(OPaO) 2.69		(OPaO) 2.15	(OPaO) 1.62	(OPaO) 0.81	(OPaO) 1.76	(OPaO) 0.96	
LOP + Isomers	6.81		5.45	4.09	2.04	6.10	4.85	2.73
PPaO + Isomers						1.48	1.12	0.61
PLP	2.76		2.21	1.66	0.83		1.44	1.52
OOO	32.53		26.02	19.52	9.76	22.48	13.27	5.88
OOO + OSO + OLO + OPO	1.27		1.01	0.76	0.38	1.51	1.84	0.78
OPO	21.82		17.46	13.09	6.55	20.25	16.77	8.72
PMP		3.14	0.63	1.26	2.20			
PSL + Isomers							1.38	1.61
POP	5.20		4.16	3.12	1.56	7.74	10.81	11.91
OSO, PPP	(OSO) 5.51		(OSO) 4.41	(OSO) 3.30	(OSO) 1.65	(OSO) 10.36	(OSO + PPP) 12.17	(PPP) 14.94
PPP		28.84	5.77	11.54	20.19			
OSP + Isomers	1.68		1.34	1.01	0.50	6.37	10.10	12.11
PSP		36.45	7.29	14.58	25.52	2.53	7.15	16.16
OAO	0.97		0.78	0.58	0.29			
SOS	0.42		0.34	0.25	0.13	1.74	2.75	3.16
SPS		23.18	4.64	9.27	16.23	1.12	4.01	9.66
SSS		6.26	1.25	2.50	4.38		1.18	2.30

Reaction conditions: 10 g reaction mixture, 10% Novozym 435 based on the total weight of substrates, 75 °C, orbital shaking at 300 rpm, 4 h reaction time. Acylglycerol species compositions in area percentage. Compositions of the physical blends have been calculated using a mass balance based on the compositions of the precursors. Diacylglycerols species: LP, OO, OP, PP, OS, SP, SS

EVOO extra virgin olive oil, *FHPO* fully hydrogenated palm oil. *P* Palmitic, *S* Stearic, *L* Linoleic, *Pa* Palmitoleic, *M* Myristic

were approached. The times required to approach equilibrium were 4, 8 and >8 h for Novozym 435, Lipozyme TL IM and Lipozyme RM IM, respectively (Fig. 2b). Hence from a kinetic point of view, Novozym 435 is the best enzyme to employ. This lipase is non-specific against the position of the glycerol backbone, exhibit high activities at elevated temperatures, and is very robust.

Compositional Analyses of the Fatty Acid Residues and the Free Fatty Acid Content of the Reaction Products

In order to assess the different regiospecificities of these biocatalysts, we determined the positional distributions of the fatty acid residues in the semi-solid fat products obtained with each of these lipases. The reaction conditions were 60:40 (w/w) EVOO to FHPO, 75 °C, 10% (w/w) biocatalyst and 300 rpm. The semi-solid fats produced with these lipases are characterized by similar distributions of saturated and unsaturated FA residues along the glycerol backbone. For the same loading of biocatalyst, Novozym 435 not only produces the most rapid reaction, but also semi-solid fats with higher ratio of unsaturated to saturated FA residues at the *sn*-2 sites (1.1) than the fats obtained with the 1,3-regiospecific enzyme Lipozyme TL IM (1.0). This difference is a factor that must be considered in assessing this technology because of the direct implications of dietary ingestion of saturated fatty acid residues for cardiovascular disease, especially those residues located at the *sn*-2 position. Although Lipozyme RM-IM produces a ratio of unsaturated to saturated FA residues at the *sn*-2 position that is marginally better (1.3) than that obtained with Novozym 435, the former lipase has much lower activity (Fig. 2b).

The FFA contents of the interesterified products were 3.9, 8.4, and 8.9% for Novozym 435, Lipozyme TL, and Lipozyme RM IM, respectively. These results are consis-

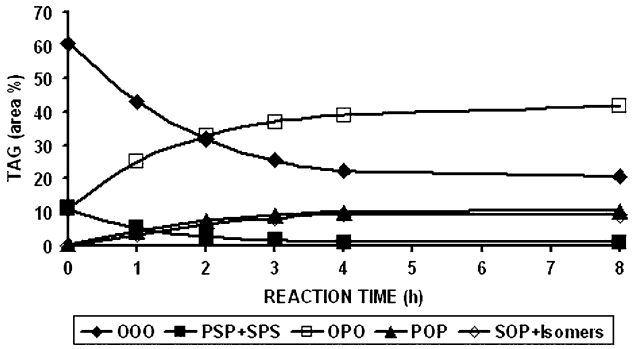

Fig. 1 Time course of the interesterification reaction between EVOO and FHPO. Depletion of the major components of EVOO (OOO) and FHPO (PSP + SPS) and newly formed triacylglycerol species. Reaction conditions: 6 g reaction mixture; 30% (w/w) FHPO; 10% (w/w) Novozym 435 based on the total weight of substrates; 75 °C; orbital shaking at 300 rpm. P Palmitic; O Oleic; S Stearic

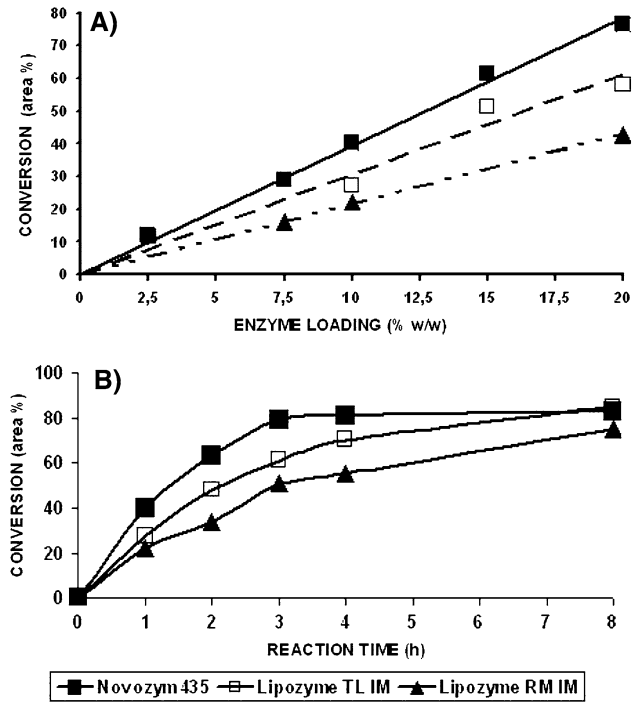

Fig. 2 Effects of enzyme loading and type of biocatalyst. **a** Enzyme loading based on the total weight of substrates. **b** Type of biocatalyst. Reaction conditions: 6 g reaction mixture; 40% (w/w) FHPO; 10% (w/w) biocatalyst (for Fig. 2B) based on the total weight of substrates; 75 °C; orbital shaking at 300 rpm; 1 h reaction time (for Fig. 2A) conversion based on PSP + SPS

tent with the fact that for Novozym 435 the water content and the time required to approach equilibrium are both the smallest of those for the three biocatalysts.

Consequently, from the standpoints of both nutrition and reaction kinetics, Novozym 435 was the preferred biocatalyst. Hence it was utilized in subsequent studies to investigate the relationship between the positional distribution of the fatty acid residues in the semi-solid fat products and the relative proportions of EVOO and FHPO in the starting mixture.

The results of these studies (data not shown) reveal that, for the interesterified products, the ratio of unsaturated to saturated FA residues at the *sn*-2 position is approximately the same or slightly lower than that at the *sn*-1,3 positions, regardless of the relative proportions of the precursor feedstocks with Novozym 435.

The levels of the TAG species containing cholesterolemic saturated fatty acid residues at the *sn*-2 positions of the acylglycerols increased as a direct consequence of increasing the percentage of the FHPO in the original reaction mixture (the ratio of unsaturated to saturated FA residues at the *sn*-2 positions varies from 2.4 to 0.3 for interesterified fats prepared with 80–20% EVOO).

The distribution of FA residues in the semi-solid fats prepared using an initial ratio of EVOO to FHPO of 80:20 contains 16.9% more saturated fatty acid residues than EVOO (30.9 vs. 14.0%). This semi-solid fat also contains 8.5% fewer saturated fatty acid residues than the fat produced in the trial involving 30% FHPO (39.4 vs. 30.9%). However, the product of the trial involving 20% FHPO is 16% lower in total unsaturated fatty acid residues than the original EVOO (69.0 vs. 85.0).

Lower initial levels of FHPO favored formation of TAG species containing only a single saturated fatty acid residue from the original FHPO (namely OPO in Fig. 1). Significantly more TAG species containing two saturated FA were formed when the level of FHPO in the original mixture of reactants was increased. Similar results were observed in previous studies involving IE of a fully hydrogenated soybean oil and TAG containing medium-chain length fatty acid residues [2, 7]. For the trials involving 80% (w/w) of either EVOO or FHPO, self-interesterification of the precursor reagent originally present in excess was very probably occurring along with IE with the other constituents of the original reaction mixture [8].

The FFA contents of three representative physical blends prepared using initial weight ratios of EVOO to FHPO of 30:70, 60:40 and 80:20 were 1.2, 0.8, and 0.6%, respectively, while the FFA contents of the corresponding interesterified products prepared using Novozym 435 as the biocatalyst were 4.2, 3.9, and 2.4%. Lipase-catalyzed interesterification involves hydrolysis of ester bonds in TAG species, followed by re-esterification by a FFA. FFA may be produced via either interesterification or hydrolysis reactions of TAG species. For the systems we studied, the FFA values of the reaction products were higher than the corresponding values prior to reaction. Nonetheless, the low values of the FFA content in all trials indicate that the level of undesirable hydrolysis is very small in the systems of interest.

Distribution of Acylglycerols in the Reaction Products

The NP-HPLC analyses indicated that in absence of any added water, the extent of hydrolysis of triacylglycerols was very low for all the conditions tested. However, there were appreciable amounts of lower acylglycerols present in the two starting materials employed. Consequently, lower glycerides were also present in the reaction products and the composition of the diglycerides were modified as a consequence of the IE reaction (see HPLC-MS analyses below).

For an initial weight ratio of EVOO to FHPO equal to 60:40, the area percentage corresponding to DAG species ranged from 1.3% for a trial with Novozym 435 to 8.3% for a trial with Lipozyme RM IM. For all trials in which Novozym 435 was the biocatalyst, the DAG area percentages were less than 2%. For these analyses the associated conversion values for hydrolysis reactions are defined in terms of the amount (mg) of TAG species converted to DAG species relative to the amount of TAG species originally present. The analyses indicated that the conversions were very low (less than 1% for a weight ratio of EVOO to FHPO of 60:40 and Novozym 435 as the biocatalyst). Thus the extents of the hydrolysis reaction were confirmed to be very low.

Compositional Analyses of the Triacylglycerols (HPLC-MS)

The molecular structures of the TAG species present in both the original fats and the interesterified fats at near equilibrium conditions were determined using reverse phase HPLC/APCI-MS. Identification of the various TAG species was based on the corresponding pseudo-molecular ion and diacylglycerol fragments [9, 10]. The structure of the acylglycerol species and the area percentage (based on the HPLC-ELSD chromatogram) determined for all of the TAG species present in EVOO, FHPO, their corresponding physical blends and the semi-solid fats produced using three different weight ratios of starting materials (20, 40, and 80% by weight of FHPO) are summarized in Table 2.

The enzyme-catalyzed IE reaction between EVOO and the fully hydrogenated fat produced semi-solid fats, which were complex mixtures of TAG species. Some of TAG are species that were not present in the precursor starting materials. The final concentration of those TAG species enriched in saturated FA residues increased with the initial amount of FHPO. Importantly, the reaction mixtures originally containing greater percentages of EVOO yield semi-solid products in which there are higher levels of TAG species that contain only residues of unsaturated fatty acids (for example, LLO isomers, OLO, OOPa isomers, and OOO). Fully saturated species such as SSS, SPS, PSP and PPP are present in increasing proportions in the semi-solid fats obtained in the trials involving the highest percentages of FHPO in the starting material.

Oxidative Stability

Oxidation of unsaturated fatty acid residues is not desirable and leads to rancidity, musty odors and off-flavors. For many applications, steps must be taken to avoid oxidation of the oil. The induction time is used as a measure of the oxidative stability of the semi-solid fat products. The values of the induction time for three representative physical blends (70, 40, and 20 % by weight of FHPO) were 122, 32, and 26 h, respectively. By contrast, the induction times for the corresponding interesterified products obtained with each of the three biocatalysts were 45, 30, and 22 h for Novozym 435; 40, 24, and 20 h for Lipozyme TL IM; and finally, 41, 24, and 17 h for Lipozyme RM IM. At 98 °C when the percentage of FHPO increased, the induction time increased for both the physical blends and the fat products, independent of the biocatalyst employed. The oxidative stability increases when the percentage of TAG containing multiple fully saturated residues increases. In all cases, the stability of the physical blend is higher than that of the reaction products. Similar results were obtained by Lee et al. [11] and Jennings and Akoh [12]. These authors attributed their results to a decrease in the levels of antioxidants accompanying the bioconversion. For each weight ratio of EVOO to FHPO tested, the induction times of the interesterified products are very close to one another, independent of the biocatalyst employed. These results reflect the fact that in all cases similar product compositions were obtained.

DSC Analyses of Reaction Products

The thermal behavior of edible fats is commonly characterized by two physical events, namely melting and crystallization. Generally, melting curves of oil samples contain complex features that are not readily interpretable, such as shoulders not separable from peaks [13]. Melting thermograms of samples corresponding to near-equilibrium reaction conditions for the trials involving weight ratios of EVOO to FHPO ranging from 20:80 to 80:20 are shown in Fig. 3. The changes in the TAG composition of the interesterified blends have concomitant effects on their melting properties. Visual inspection of the thermograms indicated that more peaks associated with high melting point TAG species were observed as the concentration of saturated fatty acid residues in the reaction mixture was increased. In general, the interesterified products melted completely at higher temperatures, and wider melting ranges were observed when the amount of FHPO present initially was increased.

The thermal properties of the various oil samples reflected in the DSC melting and crystallization curves can be characterized in terms of various transition temperatures [14], namely offset (T_{off}) and onset temperatures (T_{on}), respectively. When analyzing the crystallization curves (data not shown), we observed a gradual decrease in the onset-crystallization temperatures corresponding to the presence of lower initial amounts of FHPO. T_{on} values of 26, 26, 25, 22, 17, 15, and 16 °C were obtained from the crystallization thermograms of the samples containing initial weight percentages of FHPO of 80, 70, 60, 50, 40, 30, and 20%, respectively. Analysis of the melting thermograms (Fig. 3) indicated that an increase in the melting offset temperature is related to greater availability of the high melting TAG from the FHPO. The corresponding T_{off} values observed for the trials involving initial FHPO weight percentages of 20, 30, 40, 50, 60, 70, and 80% were 44, 45, 46, 48, 51, 51, and 51 °C. The corresponding temperature range ($T_{off} - T_{on}$) exhibited concomitant increases when the amount of highly saturated TAG initially present in the samples was increased. Temperature ranges of 24, 25, 25, 26, 26, 28, 29, and 30 °C were observed for the transesterified fats that initially contained 80, 70, 60, 50, 40, 30, and 20% (w/w) FHPO, respectively.

Inspection of the various melting thermograms revealed that the melting ranges observed were smaller when larger amounts of FHPO were present initially. This observation can be related to the relatively low melting points corresponding to the TAG comprising the EVOO. When larger amounts of FHPO were used, the reaction products exhibited shorter melting ranges that shifted to increasingly higher temperatures as the initial FHPO increased. This change in the melting profile can be largely attributed to the presence of highly saturated TAG species.

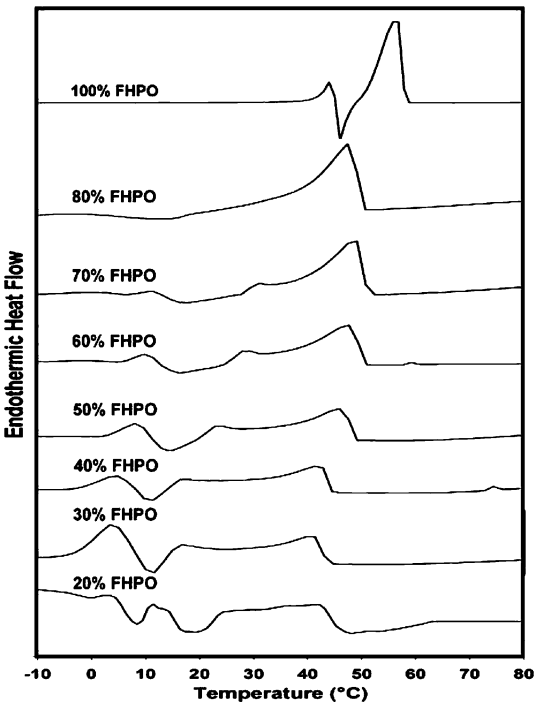

Fig. 3 DSC melting profiles of the precursor reagents and the interesterification products. Reaction conditions: 10 g reaction mixture; 10% (w/w) Novozym 435 based on the total weight of substrates; 75 °C; orbital shaking at 300 rpm; 4 h reaction time

For example, the interesterified products formed from the mixtures that initially contained 20% EVOO exhibited a very broad melting profile, ranging from 0 to 55 °C (Fig. 3). These peaks can be largely attributed to TAG species in which some saturated fatty acid residues have been substituted for some of the primary unsaturated residues present in the TAG of the native EVOO in reactions that produce new TAG species such as SOS, OSP isomers, POP, OPO, OOO, PLP, PPaO isomers, LOP isomers, and OLO, as determined from HPLC-MS analyses of the products. The samples of the products corresponding to initial EVOO contents of 50% or greater exhibited melting ranges starting at 2–15 °C. These samples melted completely at temperatures close to 50 °C. When analyzing the corresponding thermograms, a characteristic fusion region between –5 and 15 °C can be clearly identified. The presence of TAG species containing at least two unsaturated residues (e.g., LLP, LPL, LLO, LOL) in the samples corresponding to initial EVOO contents of 50% (w/w) or greater are primarily responsible for the observed shift in the melting profiles of these samples to lower temperatures. In the melting curve obtained for the precursor FHPO, the peak corresponding to the melting point of tristearin (71–73 °C) does not appear to be significant because of the small amount of this TAG present in this precursor (6.26%).

The melting endotherm of the 60:40 physical blend of EVOO and FHPO exhibits a major peak at 56 °C that corresponds to one observed in the endotherm of pure FHPO. This melting temperature can be attributed to the presence, at similar levels, of different TAG species containing palmitic and stearic acid residues that are also present in FHPO. Moreover, a small melting region at temperatures from 0 to 10 °C was also observed, presumably from components of the EVOO. By contrast, the melting curves for products of the interesterification reactions catalyzed by Lipozyme TL IM and Lipozyme RM IM were very similar, exhibiting major melting transitions near 15 and 32 °C. The lengths of the corresponding melting ranges (from 10 to 46 °C) were also similar. The melting curve of the fat blend modified using Novozym 435 showed a more pronounced transition at 46 °C, presumably resulting from a higher level of a high melting TAG fraction comprised of TAG species such as the isomers of SOS, OSP POP, OPO, PLP, LOP, and OLO, as determined from

HPLC-MS analyses. When the three different lipases were used to effect interesterification of the 60:40 reaction mixture, minimal or no differences were observed in the values of parameters such as T_{on}: –2 to –4 °C, and T_{off}: 48–50 °C.

Solid Fat Contents of the Reaction Products

Solid fat contents (SFC) of the seven samples subjected to DSC analyses were determined at different temperatures (Fig. 4). The SFC profile (the percent of the shortening, which is solid at various temperatures) is responsible for many important characteristics of fats, including their physical appearance, some organoleptic properties, and the spreadability. This profile also influences the melting properties of a specific fat and the plasticity or physical consistency of an edible product. The variation of the solid fat content with temperature and the sharpness of the melting range, together with other factors such as the crystal morphology, determine the range within which a fat can be considered plastic [14, 15].

Once the SFC curve is obtained, the temperature range within a given fat can be considered as plastic (retain its consistency) can be obtained by determining those temperatures within which the SFC values lie in the range 15–35%. SFC values of less than 32% at 10 °C are essential for good spreadability. The SFC values at temperatures between 4 and 10 °C determine the spreadability of the product at refrigeration temperatures. At temperatures between 33 and 38 °C, the SFC values influence "mouth feel" or waxy sensations that will be exhibited by the fat [15, 16].

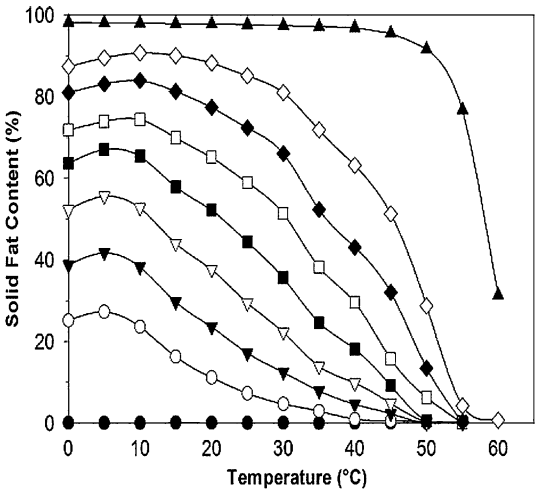

Fig. 4 Solid fat contents of the precursor reagents and the interesterification products. *Filled circles* Extra virgin olive oil, *open circles* 20% FHPO, *inverted filled triangles* 30% FHPO, *inverted open triangles* 40% FHPO, *filled squares* 50% FHPO, *open squares* 60% FHPO, *filled diamonds* 70% FHPO, *open diamonds* 80% FHPO, *filled triangles* FHPO. Reaction conditions: 10 g reaction mixture; 10% (w/w) Novozym 435 based on the total weight of substrates; 75 °C; orbital shaking at 300 rpm; 4 h reaction time

Inspection of Fig. 4 indicates that harder fats, characterized by high SFC values, were obtained from the starting mixtures containing high contents of saturated fatty acid residues (from the FHPO). The SFC of our products increased as the initial amount of saturated TAG species increased; this result is consistent with the report of Petrauskaite et al. [17]. In general, the final products corresponding to those samples with lower initial contents of FHPO can be considered as plastic over wider temperature ranges. The large amount of unsaturated residues present in these samples, primarily oleic acid residues is the factor leading to the extended range of plasticity of these interesterified mixtures. The highly saturated fat products were those in which the initial weight percentage of FHPO was large. These products were characterized by relatively short plastic ranges at relatively high temperatures (above 45 °C). The temperature ranges corresponding to the plastic ranges for the trials involving 20, 30, 40, 50, 60, 70, and 80% (w/w) FHPO were 16, 15.2, 12.6, 11.8, 8.8, 6, and 2.6 °C.

Comparison of our SFC results with those obtained by Lai et al. [18] for the IE products of palm stearin and palm kernel oil revealed that for similar weight ratios of substrates our fats were softer at all temperatures. High SFC values at refrigeration temperatures were observed for the interesterified products obtained from mixtures containing large amounts of the hard fat precursor. This behavior can be attributed to the presence of high-melting TAG species (mainly SPS, PSP, OSP isomers, PPP and residual amounts of SSS from the FHPO) in the interesterified product samples. The interesterified samples obtained from trials involving more than 50% FHPO exhibited high SFC and melted at temperatures above 50 °C. Potential uses include coatings, fillings, and high stability frying fats.

The samples corresponding to low initial concentrations of FHPO (30 and 40% by weight) were characterized by SFC profiles similar to those previously reported for stick margarines by DeMan and Blackman [19]. The differences in the SFC values observed for the samples obtained with 30 and 40% FHPO can be attributed to differences in the concentrations of TAG species containing two saturated fatty acid residues, namely POP and OSP isomers, and to residual amounts of unreacted TAG residues present in the original FHPO (PSP and SPS).

On the other hand, the low concentrations of saturated residues present in the TAG constituting the products obtained for the trials involving 20% FHPO are reflected in the low SFC values and the corresponding observed melting range. For these trials, the solid fat content of the product was always less than 30%, and this mixture was

almost completely melted at 45 °C. The SFC of the final products obtained from the initial mixtures containing low amounts of FHPO were comparable to those of tub margarines.

For the 60:40 physical blend of EVOO and FHPO at temperatures above 15 °C, the SFC values are greater than those for the corresponding interesterified products, regardless of the biocatalyst employed in their preparation. Only at temperatures between 0 and 15 °C was the physical blend softer than the modified fats. The SFC values of the interesterified products prepared with the three lipases were very similar, but the product prepared with Novozym 435 was marginally harder. The plastic ranges of the physical mixture and the interesterified products were approximately 16 °C (from 32 to 48 °C) and 12 °C (from 20–22 to 32–34 °C), respectively.

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