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Production and Physicochemical Properties of Functional-Butterfat through Enzymatic Interesterification in a Continuous Reactor

JUNG-AH SHIN,† CASIMIR C. AKOH,‡ AND KI-TEAK LEE*,†

Department of Food Science and Technology, Chungnam National University, Daejeon 305-764, Republic of Korea, and Department of Food Science and Technology, The University of Georgia, Food Science Building, Athens, Georgia 30602-7610

Modified-butterfat (MBF) was synthesized with four blends (8:6:6, 6:6:8, 6:6:9, and 4:6:10, by weight) of anhydrous butterfat (ABF), palm stearin (PS) and flaxseed oil (FSO) through enzymatic interesterification in a continuous packed-bed reactor. Flow rate effect of 3, 5, 8 and 10 mL/min on enzymatic interesterification was investigated. By increasing the enzyme contact time with substrates (decreased flow rates), not only did melting and crystallization points shift to lower temperature but also the equivalent carbon number, ECN 36−38 from FSO decreased. Further all reactions were performed at flow rate of 5 mL/min (contact time 140 min) in a continuous reactor packed with 150 g of Lipozyme RM IM. After short path distillation, α-linolenic acid composition (%) of 8:6:6, 6:6:8, 6:6:9, and 4:6:10 MBFs were 16, 21, 23 and 25%, respectively. The contents of ECN 36−38, and ECN 48−50 decreased in the blends and MBFs for each substrate ratio. ECN 42−46 in the newly produced TAG increased. Melting points of MBFs were 38 °C (8:6:6), 35.5 °C (6:6:8), 34 °C (6:6:9), and 32 °C (4:6:10). MBFs interesterified with FSO contained phytosterols (17−36 mg/100 g) and tocopherols (116−173 μg/g). The products of 8:6:6, 6:6:8, 6:6:9 and 4:6:10 MBFs were softer (69, 88, 80, and 92%, respectively) than pure butterfat at refrigeration temperature. The polymorphic form changed from β form (blends) to desirable crystalline structure of β' form (MBFs). Crystal morphology of MBFs also changed and was composed of small spherulites of varying density.

KEYWORDS: Butterfat; continuous packed-bed reactor; enzymatic interesterification; flaxseed oil; physicochemical properties; polarized light microscopy; polymorphism

INTRODUCTION

The process for the modification of natural fats and oils (tailor-made fat) includes interesterification (enzymatic or chemical), fractionation, hydrogenation, and blending. After interesterification, triacylglycerol (TAG) profile, fatty acid position on TAG, melting, and crystallization behaviors can be changed. Interesterification can be used to add the desirable nutritional and physicochemical properties to traditional fats and oils for use in functional foods, margarine and shortening, cocoa butter-like fats, spreads, cooking and baking fats, and human milk fat substitutes.

Lipase-catalyzed interesterification has several advantages, such as easy recovery, mild reaction conditions, fewer side products (diacylglycerols, monoacylglycerols, and free fatty acids), and reaction specificity (substrate and positional specificity and stereospecificity) (*1*, *2*). These enzymatic interesterification reactions can be performed in a batch type reactor or a continuous packed-bed reactor. Modification of fats using a continuous reactor on an industrial scale may lead to low side reactions (acyl migration) and economic operation (*3*). For example, enzymatic interesterification using medium chain TAG and fish oil for the production of structured lipid (SL) was carried out in a packed-bed reactor (*4*), and SL was synthesized through enzymatic acidolysis of palm olein and caprylic acid in a bench-scale packed bed bioreactor (*5*).

Palm stearin is a solid fraction obtained from fractionation of palm oil. Palm stearin, as a hard fat source, has been widely used to produce zero-*trans* fat with desirable physical properties by interesterifying with liquid oils (*6*−*8*). In some cases fully hydrogenated soybean oil (melting point: approximately 80 °C) can be used for producing hard fat (*9*). But it is proper to produce a hard-type of margarine and shortening because of its high amount of stearic acid (approximately 85%). Therefore, palm stearin instead of fully hydrogenated soybean oil was used to provide the desirable spreadability. Although butterfat has desirable physical properties such as a buttery flavor, palatability,

* To whom correspondence should be addressed at Chungnam National University, Department of Food Science and Technology, 220 Gung-dong, Yuseong-gu, Daejeon 305-764, Republic of Korea (South). Telephone: +82-42-821-6729. Fax: +82-42-822-6729. E-mail: ktlee@cnu.ac.kr.

† Chungnam National University.

‡ The University of Georgia.

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Table 1. Fatty Acid Composition (mol %) and Chemical Properties of Modified-Butterfat (MBF) Enzymatically Interesterified in a Continuous Packed Bed Reactor

				MBFs[a] (ABF:PS:FSO, by weight)			
fatty acids and others	ABF	PS	FSO	8:6:6	6:6:8	6:6:9	4:6:10
$C_{4:0}$	3.93	nd[b]	nd	1.24	0.96	0.88	0.58
$C_{6:0}$	2.88	nd	nd	1.10	0.81	0.77	0.50
$C_{8:0}$	2.12	0.02	nd	0.85	0.63	0.59	0.40
$C_{10:0}$	4.39	0.01	nd	1.71	1.29	1.24	0.80
$C_{12:0}$	6.24	0.13	nd	2.47	1.86	1.79	1.16
$C_{14:0}$	14.06	1.37	0.06	5.88	4.54	4.35	2.94
$C_{16:0}$	29.57	63.57	2.81	33.32	30.97	30.18	27.22
$C_{16:1}$ ω7	2.03	0.10	0.07	0.84	0.65	0.63	0.44
$C_{18:0}$	11.04	4.87	4.34	6.82	6.12	5.96	5.50
$C_{18:1}$ *trans*	3.34	0.06	nd	1.30	0.99	0.95	0.62
$C_{18:1}$ ω9	17.89	24.54	21.41	20.62	21.03	20.83	21.38
$C_{18:2}$ ω6	1.04	5.16	16.33	7.67	8.75	8.78	13.73
$C_{18:3}$ ω3	1.09	nd	54.98	16.19	21.41	23.06	24.74
∑SFA[c]	74.24	69.97	7.21	53.38	47.17	45.74	39.09
∑USFA[d]	21.39	29.89	92.79	45.32	51.84	53.31	60.29
∑TFA[e]	3.34	0.06	nd	1.30	0.99	0.95	0.62
AI[f]	4.30	2.31	0.03	0.39	0.26	0.24	0.14
%FFA (palmitic)	0.26	0.11	1.13	0.38	0.50	0.63	1.02
iodine value	44.1	27.1	195.5	90.1	100.9	104.1	116.8

[a] All MBFs were synthesized with anhydrous butterfat (ABF), palm stearin (PS) and flaxseed oil (FSO) and catalyzed by Lipozyme RM IM. [b] Not detected. [c] ∑SFA: The sum of saturated fatty acids. [d] ∑USFA: The sum of unsaturated fatty acids. [e] ∑TFA: The sum of *trans* fatty acids. [f] Atherogenicity index (AI) was calculated as follows: AI = [($C_{12:0}$ mol%) + 4 × ($C_{14:0}$ mol%) + ($C_{16:0}$ mol%)]/(∑USFA mol%), ∑USFA was calculated except *trans* fatty acids in lipids.

and creamy mouth feel, it has several disadvantages such as poor spreadability at refrigerator temperature and high content of saturated fatty acid and cholesterol because it is derived from animal sources (*10*, *11*). Flaxseed oil contains over 50% α-linolenic acid ($C_{18:3}$, omega-3 fatty acid). According to Vijaimohan et al. (*12*), supplementation of α-linolenic acid (ALn) as a precursor of plasma eicosapentanoic acid (EPA, $C_{20:5}$) levels significantly decreased the body and liver weight, plasma cholesterol level, low-density lipoprotein (LDL)-cholesterol level, and TAG level in a high fat diet fed to rats. Accordingly ALn will be expected as a desirable source of omega-3 fatty acid due to its relatively less unsaturation than EPA or docosahexaenoic acid (DHA, $C_{22:6}$) against oxidation.

The objective of this work is to produce functional modified-butterfat (MBF) with improved physical properties (spreadability) and nutritional properties through enzymatic interesterification using palm stearin (PS), flaxseed oil (FSO), and anhydrous butterfat (ABF). This reaction was carried out in a continuous packed-bed reactor. The interesterification degree based on substrates flow rate (contact time) was monitored by analyzing the melting and crystallization behavior by DSC, and the TAG composition by reversed-phase HPLC. After purification through short path distillation, the physicochemical properties of the MBFs synthesized with different substrate ratios were investigated.

MATERIALS AND METHODS

Materials. Flaxseed oil (Vitamin World Inc., NY) and palm stearin were purchased from a local grocery store. Butter obtained from Murray Goulbum Co-operative Co. Ltd. (Australia) was stored at −30 °C. Lipozyme RM IM (150 IUN/g of catalytic activity with a bulk density of 350−450 kg/m^3, a particle size of 0.2−0.6 mm, and a water content of 2−3%) from *Rhizomucor miehei* was purchased from Novozymes North America Inc. (Franklinton, NC). Lipozyme RM IM immobilized on a macroporous anion exchange resin by adsorption was an sn-1,3 specific lipase (EC 3.1.1.3). The phytosterol mixture and 5β-cholestan-3β-ol were products of Matreya Inc. (Pleasant Gap, PA). Tocopherols (α-, β-, and γ-tocopherols) and cholesterol standards were purchased from Sigma-Aldrich Co. (St. Louis, MO). Standards of fatty acid methyl esters (GLC-461) were purchased from Nu-Check (Elysian, MN). All solvents (*n*-hexane, 2-propanol, acetonitrile, and ethanol) and other chemicals used were high-performance liquid chromatography (HPLC) grade.

Preparation of Anhydrous Butterfat. Butter melted completely at 60 °C was separated from water using a separatory funnel. To remove residual moisture, the butterfat layer was passed through an anhydrous sodium sulfate column. The anhydrous butterfat (ABF) was filtered under a vacuum using 0.45 μm membrane filter paper. Finally, ABF was flushed with nitrogen gas and stored at −30 °C until the next analysis.

Interesterification in a Continuous Packed-Bed Reactor. The interesterification of anhydrous butterfat (ABF), palm stearin (PS), and flaxseed oil (FSO) (8:6:6, 6:6:8, 6:6:9, and 4:6:10, by weight) was performed in a continuous packed-bed reactor (length: 40 cm; inner diameter: 4.25 cm) equipped with a peristaltic pump. Immobilized Lipozyme RM IM (150 g) enzyme was soaked using soybean oil by stirring at 60 °C for 30 min. The water and air from the enzyme slurry were removed using a vacuum pump. The enzyme slurry was poured slowly into the column, which had 4 cm of sponge gourd at the bottom. The column was surrounded with a heating double jacket. That was connected to a water bath for maintaining the reactor temperature at 60 °C. Thus, the whole system was held at 60 °C. The selected blends (8:6:6 and 6:6:9 blends) were pumped into the reactor at volumetric flow rates of 3, 5, 8, and 10 mL/min. The contact time (T_c) was calculated using substrate volume (V_s) and the flow rate (V_f) as $T_c = V_s/V_f$. This equation was obtained from the residence time (contact time) and the void fraction, where the contact time $T_c = V(\omega/V_f)$. In this equation, V is the enzyme bed volume and ω the void fraction, which was calculated as $\omega = V_s/V$ (*3*, *13*). In conclusion, the four blends were run continuously at the selected flow rate of 5 mL/min, showing a contact time of 140 min.

Differential Scanning Calorimetry (DSC). A DSC 2010 differential scanning calorimeter (TA Instruments, New Castle, U.S.A.) was used for melting and crystallization profiles (*14*). The instrument was calibrated with an indium standard (mp 152 °C) and the baseline was obtained with a sealed empty aluminum pan as a reference. Samples weighing 5−8 mg were hermetically sealed in an aluminum pan. Samples were melted at 80 °C for 10 min to remove completely the previous crystal structure. These were cooled from 80 to −65 °C at a rate of 10 °C/min for the cooling thermograms. After holding for 10 min, samples were heated again to 80 °C at a rate of 5 °C/min for the melting thermograms. Data analysis was performed with software provided with the DSC.

Table 2. Equivalent Carbon Number (ECN) of Triacylglycerol in the 6:6:9 MBF and 8:6:6 MBF Synthesized at Different Flow Rates in a Continuous Packed-Bed Reactor[a]

		ECN of triacylglycerols (area %)																		
		ECN <36	36	38	40			42		44			46			48			50	
MBFs	flow rate	peak no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
6:6:9	blend	10.5	18.7	8.6	4.0	7.7	3.9	3.9	6.1	4.5	4.4	2.7	1.6	5.7	5.2	3.3	4.7	3.0	1.0	0.5
	10 mL/min	10.8	7.1	6.9	4.3	6.6	6.0	7.8	8.7	9.8	5.6	3.6	2.0	7.5	4.7	3.1	3.7	1.4	0.2	0.2
	8 mL/min	8.8	4.5	6.9	5.1	5.9	6.1	9.4	9.5	11.1	5.7	3.1	2.5	8.4	4.8	3.0	3.4	1.1	0.6	0.2
	5 mL/min	12.4	5.0	4.7	3.2	7.1	9.7	5.9	10.2	6.0	8.8	5.3	1.5	7.6	5.4	2.4	3.4	1.0	0.3	0.3
	3 mL/min	8.4	2.7	5.8	4.4	5.6	7.2	8.4	11.0	11.9	7.6	4.4	2.2	8.6	5.0	2.8	2.8	0.7	0.2	0.2
8:6:6	blend	16.8	11.9	6.8	2.7	5.6	3.9	2.7	6.7	4.1	4.3	4.2	1.9	6.7	7.0	4.2	5.6	3.1	1.2	0.6
	10 mL/min	10.8	5.0	3.5	3.4	4.8	6.5	7.0	9.2	11.1	5.9	4.7	2.7	9.4	6.9	3.1	3.6	1.6	0.4	0.3
	8 mL/min	10.3	2.7	4.2	3.5	3.8	6.1	8.6	9.2	13.9	5.6	4.1	3.3	10.4	6.3	3.5	3.3	0.8	0.2	0.2
	5 mL/min	14.1	2.4	2.0	2.3	4.9	8.8	4.6	11.6	6.1	8.5	6.5	2.0	9.5	8.3	2.9	3.9	0.9	0.5	0.4
	3 mL/min	11.8	2.2	4.1	2.9	3.6	7.0	6.9	11.8	10.6	6.3	5.2	2.4	10.0	7.2	3.0	3.4	0.7	0.6	0.3

[a] Modified-butterfat (MBF) was synthesized using 6:6:9 (anhydrous butterfat/palm stearin/flaxseed oil, by weight) and 8:6:6 mixtures at different flow rates. Flow rate means enzyme contact time to substrates: 10 mL/min (70 min), 8 mL/min (87.5 min), 5 mL/min (140 min), and 3 mL/min (233 min). Chromatogram showing TAG profiles of each peak in MBFs was illustrated in **Figure 4**.

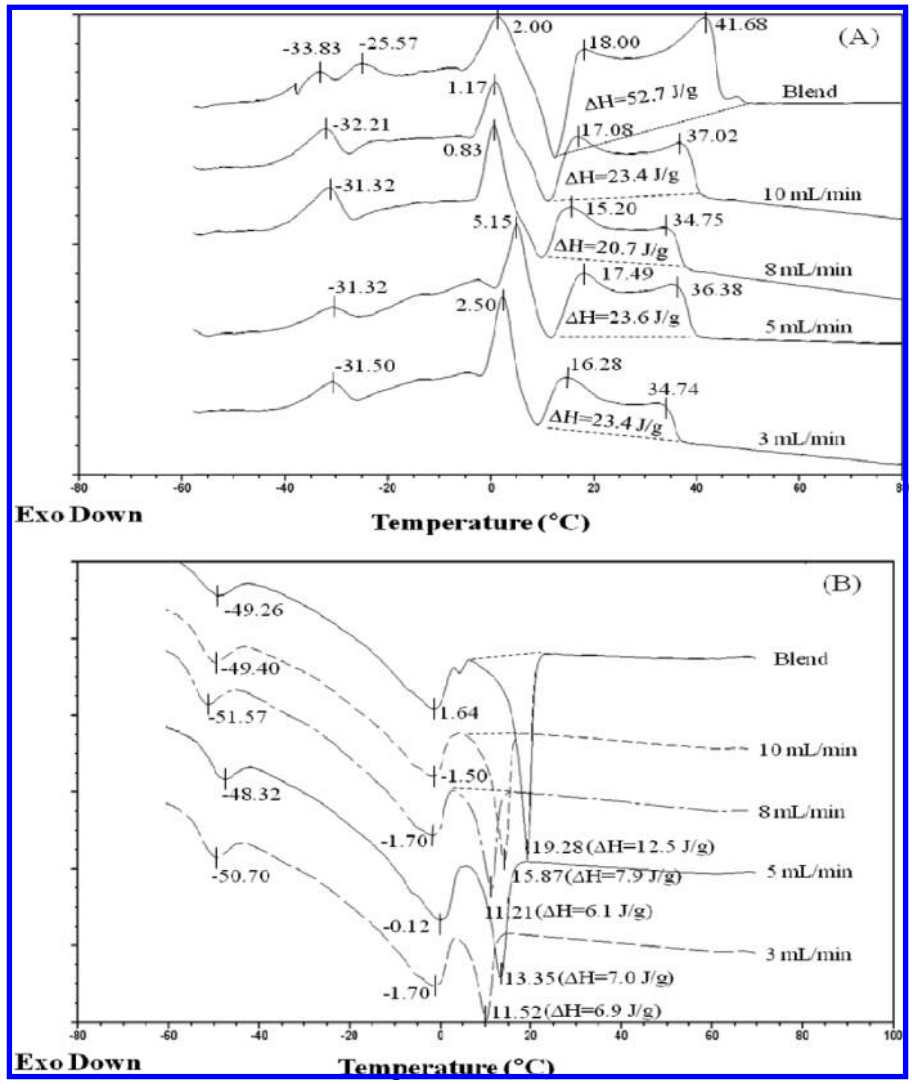

Figure 1. Differential scanning calorimetry (DSC) thermograms of modified-butterfat (MBF) with an enzymatically interesterified ABF/PS/FSO mixture (6:6:9, w/w/w) according to flow rate: blend (0 mL/min), 10 mL/min (contact time 70 min), 8 mL/min (87.5 min), 5 mL/min (140 min), and 3 mL/min (233 min). (**A**) Melting curves. (**B**) Crystallization curves. The MBF was synthesized using Lipozyme RM IM (150 g) in a continuous packed-bed reactor.

Short Path Distillation. The free fatty acids (FFA) as a byproduct in the MBFs synthesized after interesterification were removed using a KDL-4 short path distillation unit (UIC Inc., Joliet, IL). The sample was passed through the distillation apparatus under the following conditions: holding temperature, 25 °C; heating oil temperature, 145 °C; cooling water temperature, 15 °C; and vacuum pressure, <100 mbar.

Triacylglycerol Analysis by HPLC. The samples (10 mg) were dissolved in 1 mL of chloroform with 20 μL of tributyrin as an internal standard. The injection volume was 10 μL. The TAG profiles of the blends and the MBFs were determined with reversed-phase HPLC equipped with a Yonglin SP930D dual pump (Yonglin, Anayang, Korea), a Rheodyne (Cotati, CA), a 7125 manual injector, and a Yonglin

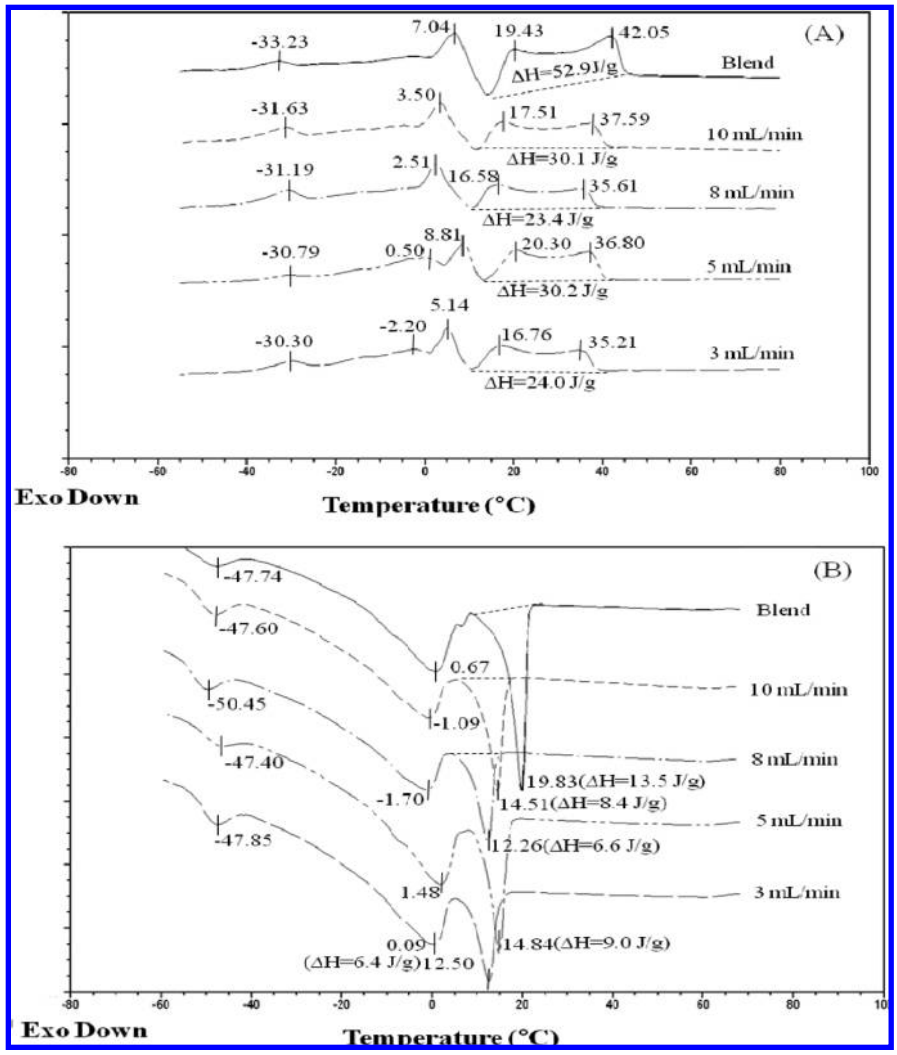

Figure 2. Differential scanning calorimetry (DSC) thermograms of modified-butterfat (MBF) with an interesterified ABF/PS/FSO (8:6:6, w/w/w) mixture according to flow rate: blend (0 mL/min), 10 mL/min (contact time 70 min), 8 mL/min (87.5 min), 5 mL/min (140 min), and 3 mL/min (233 min). (**A**) Melting curves. (**B**) Crystallization curves. The MBF was synthesized in a continuous reactor packed with Lipozyme RM IM (150 g).

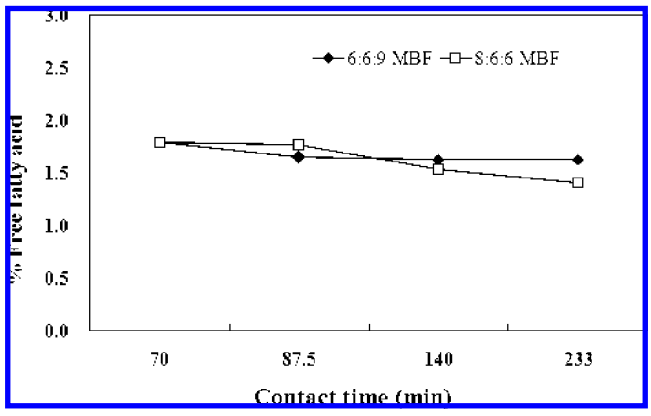

Figure 3. Free fatty acid content changes in 6:6:9 MBF and 8:6:6 MBF according to contact time (different flow rates) during interesterification. Contact time (flow rate): 70 min (10 mL/min), 87.5 min (8 mL/min), 140 min (5 mL/min), and 233 min (3 mL/min).

UV830 detector (Yonglin, Anayang, Korea). A Nova-Pak C18 column (4 μm particle size, 150 mm × 3.9 mm, Waters, Milford, MA) was used. The UV absorbance detector was set at 220 nm. The column oven was 35 °C to separate TAG molecules clearly. The mobile phase was a binary solvent system of acetonitrile (solvent A) and 2-propanol/hexane (2:1, by volume) (solvent B) at a flow rate of 1 mL/min (*4*). A linear gradient of solvent B from 20 to 46% over 45 min was applied, followed by 100% solvent B over 6 min and holding for 6 min and then reversed to initial solvent A for equilibration of the system. The total run time was 65 min. Tricaprylin, trilaurin, trimyristin, tripalmitin, trilinolein, and triolein were used to create a standard curve and for peak identification of TAG based on equivalent carbon number (ECN).

Fatty Acid Composition Analysis. Each sample (100 mg) was weighed in a 25 mL screw-capped test tube, and 3 mL of hexane was added to dissolve the sample and 1 mL of 2 N potassium hydroxide in MeOH was added. The sample and chemicals were mixed vigorously with a vortex exactly for 1 min, and then it was methylated at room temperature for 20 min. After 20 min, heptadecanoic methyl ester as an internal standard and 1 mL of deionized water were added into the test tube, and the solution was mixed again. The hexane and water layers were distinctly separated. The upper layer (hexane) was taken and passed through an anhydrous sodium sulfate column to remove residual moisture. The fatty acid composition of the blends and the MBFs was analyzed using a 6890 Series gas chromatograph (GC) with an autoinjector and flame-ionization detector (Agilent Technology, Avondale, PA). A fused-silica capillary column (SP-2560, 100 m × 0.25 mm i.d. × 0.2 μm film thickness, Sulpelco, Bellefonte, PA) was used. The carrier gas was nitrogen at 1 mL/min. The split ratio was 1:50. The injector and detector temperatures were set at 250 and 260 °C, respectively. The column oven temperature was isothermal at 50 °C for 5 min and was programmed from 50 to 150 °C at the rate of 10 °C/min and held for 1 min. The oven temperature was programmed again from 150 to 220 °C at the rate of 4 °C/min and held for 20 min

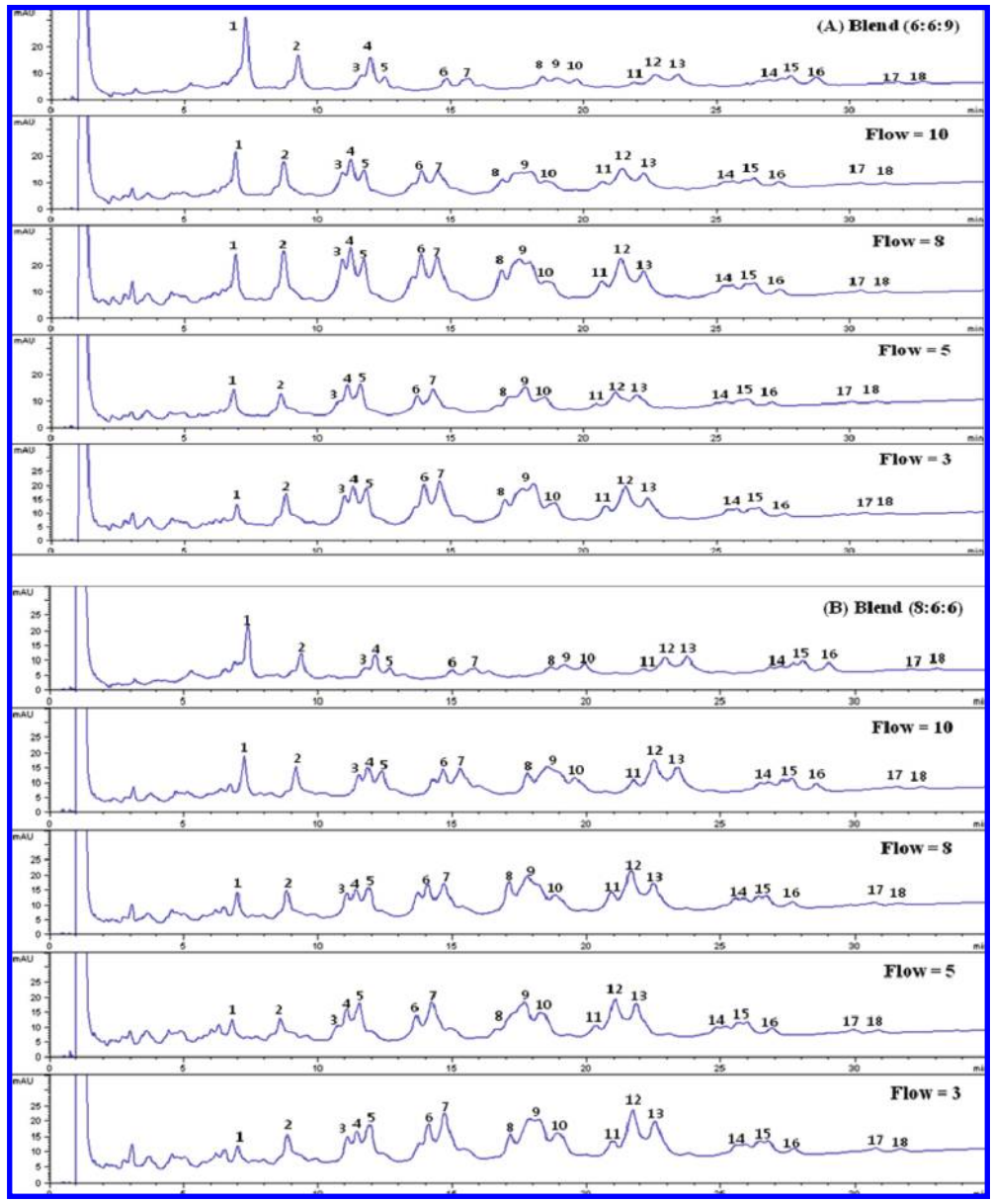

Figure 4. Triacylglycerol profile changes of a ABF/PS/FSO mixture (6:6:9 (**A**) and 8:6:6 (**B**), by weight ratio) according to flow rate: blend (0 mL/min), 10 mL/min (70 min contact time), 8 mL/min (87.5 min), 5 mL/min (140 min), and 3 mL/min (233 min). Peak 1 (ECN 36); peak 2 (ECN 38); peaks 3−5 (ECN 40); peaks 6−7 (ECN 42); peaks 8−10 (ECN 44); peaks 11−13 (ECN 46); peaks 14−16 (ECN 48); peaks 17−18 (ECN 50).

isothermally at 220 °C. The peaks of fatty acid methyl esters (FAMEs) were identified and calibrated with standard fatty acids. The fatty acid composition (mol %) of each sample was expressed as a molecular weight percentage.

Saponification. Each homogenized sample (1 g) was placed into a screw Teflon-lined cap tube with 5 mL of ethanol containing pyrogallol (6%, w/v). After immediately agitating the tube to avoid agglomeration, 1.25 mL of 60% potassium hydroxide in distilled water was added and the tube was flushed with N_2 gas for 1 min to prevent oxidation of unsaponifiable matters. Saponification was carried out at 70 °C for 30 min in a shaking water bath. Saponification was performed according to Eitenmiller et al. (*15*). After completing saponification, the tube was cooled in an ice bath, and 5 mL of sodium chloride solution (2%, w/v) was added. Unsaponifiable compounds were extracted from the reactant using 2.5 mL of solvent (hexane/ethyl acetate, 85:15, v/v) containing 0.05% BHT. The extracting solvent was evaporated under N_2 gas and 5 mL of hexane was added into the dry residue to make an appropriate analytical concentration. Finally, the diluted unsaponifiable matter solution was filtered through a 0.45 μm hydrophobic membrane syringe filter. The diluted solution was used for the determination of tocopherol, cholesterol, and phytosterol content in the blends and the MBFs.

Tocopherol Analysis. The tocopherols of butterfat blends and modified-butterfats (MBFs) were analyzed using a normal phase HPLC system (Hewlett-Packard model 1090 series, Agilent Technologies Inc., Palo Alto, CA) with a fluorescence detector (Hewlett-Packard model 1046A series, Agilent Technologies Inc., Palo Alto, CA) and a Zorbax RX-Sil column (4.6 mm i.d. × 250 mm, 5 μm particle size, Agilent Technologies Inc., Palo Alto, CA) (*15*). The prepared unsaponifiable-matter solution of 20 μL was injected into the analytical HPLC system. Flouorometric detection of tocopherols was performed at 295 nm wavelength for excitation and at 325 nm wavelength for emission. The standards of α-, β-, and γ-tocopherol (purity 98%) were purchased from Sigma (St. Louis, MO). Stock solutions of 10, 25, 50, 100, and 250 μg/mL concentration for quantification of α-, β-, and γ-tocopherols in samples were

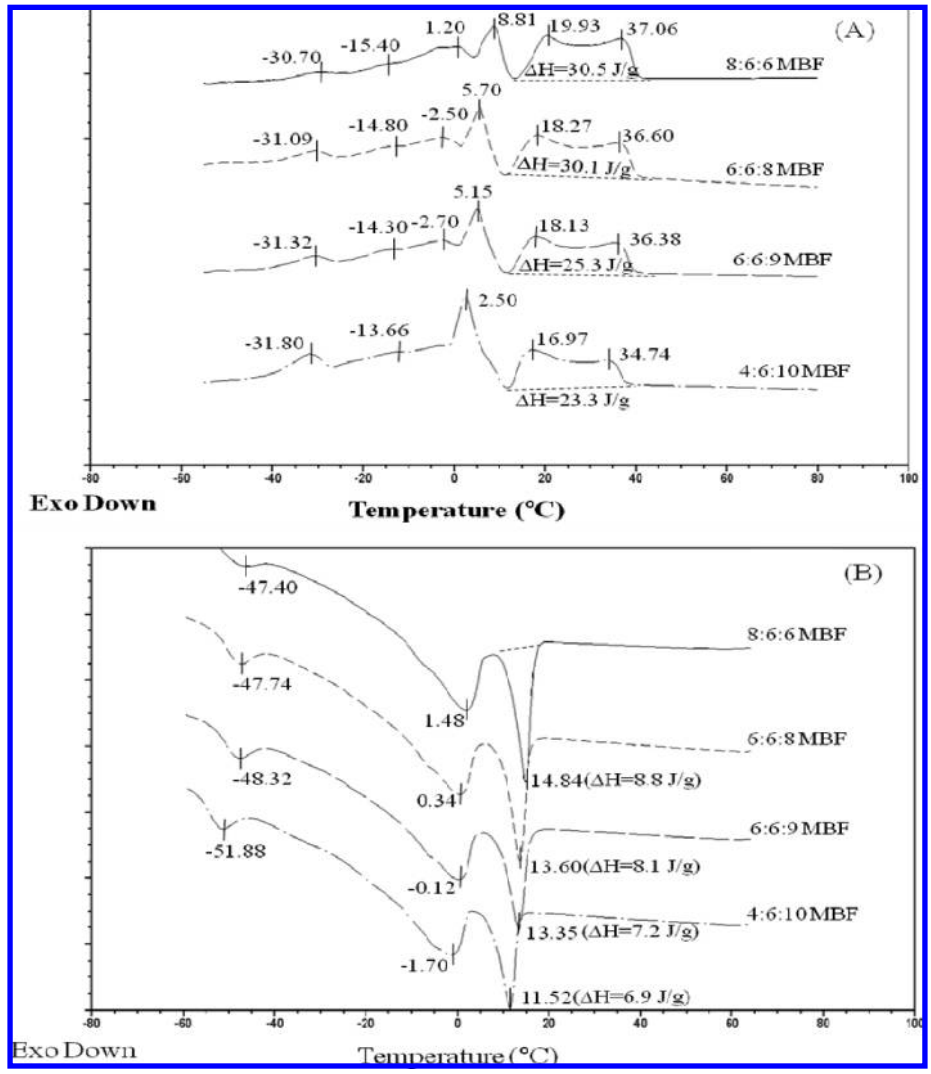

Figure 5. Melting and crystallization thermograms of modified-butterfat (MBF) interesterified with 8:6:6, 6:6:8, 6:6:9, and 4:6:10 (ABF/PS/FSO, by weight) mixtures at a flow rate of 5 mL/min (140 min contact time). The MBF was synthesized in a continuous reactor packed with Lipozyme RM IM (150 g).

Table 3. Solid Fat Content (SFC) and Melting Point of Anhydrous Butterfat (ABF) and Modified-Butterfats (MBFs) by Pulsed NMR at Different Temperatures

	SFC[a] (%) at °C					
sample	10	20	30	40	50	melting point (°C)
ABF	33.94 ± 0.46	12.72 ± 0.46	4.84 ± 0.58	1.32 ± 0.68	nd[b]	34
MBF 8:6:6[c]	24.17 ± 0.36	12.56 ± 0.52	5.83 ± 0.99	3.34 ± 0.67	0.14 ± 0.20	38
MBF 6:6:8	18.82 ± 0.36	10.31 ± 0.52	3.88 ± 0.99	3.25 ± 0.67	nd	35.5
MBF 6:6:9	16.83 ± 1.94	9.48 ± 2.42	3.48 ± 0.60	2.01 ± 0.86	nd	34
MBF 4:6:10	10.70 ± 1.31	5.81 ± 2.51	2.71 ± 2.84	1.18 ± 1.08	nd	32

[a] Solid fat contents (SFCs) were means ± SD in duplicate analyses. [b] Not detected. [c] MBF 8:6:6 was synthesized by enzymatic interesterification with different weight ratios of anhydrous butterfat (ABF), palm stearin (PS), and flaxseed oil (FSO) in a continuous reactor.

respectively prepared and injected. The isocratic mobile phase contained 0.9% isopropanol in *n*-hexane (J.T. Baker Chemical Co., Phillipsburg, NJ) at a flow rate of 1.5 mL/min.

Cholesterol and Phytosterol Analysis. Cholesterol and phytosterol contents in the blends and the MBFs were simultaneously analyzed by capillary gas chromatography (GC). Twenty microliters of 5β-cholestane-3β-ol (2.5 mg/mL) as an internal standard was added to a test tube containing 1 mL of the unsaponifiable matter. Two microliters of this solution was injected into the GC. A Hewlett-Packard 5890 series II chromatograph (Agilent Technologies Inc., Palo Alto, CA) equipped with a flame-ionization detector (FID) and a fused silica capillary column (SAC-5, 30 m × 0.25 mm i.d., Supelco Inc., Bellefonte, PA) was used. Nitrogen was the carrier gas at a column flow rate of 1.1 mL/min. The split mode in the injector was a ratio of 1:21. The injector and the detector temperatures were set at 320 °C. The oven temperature was programmed from 220 °C at rate of 4 °C/min to 300 °C, and then held for 10 min. For the quantification of cholesterol and phytosterol, 1 mL of 5β-cholestane-3β-ol (50 μg/mL in hexane) as an internal standard was added into each test tube containing 45, 90, 227, 454, and 909 μg of cholesterol, and added into test tubes containing 71, 142, 284, 568, 1136, and 2271 μg of phytosterol mixture, respectively. External curves were acquired, and then the cholesterol and phytosterol contents in samples were quantified in milligrams per 100 g using the external standard curve and an internal standard.

Iodine Value and Free Fatty Acid Content. Iodine values (IV) of each sample were measured by AOCS Official Method Cd 1-25 (*16*).

Table 4. Equivalent Carbon Number (ECN) of Triacylglycerol Molecule in Anhydrous Butterfat (ABF), Palm Stearin (PS), and Flaxseed Oil (FSO)[a]

	ABF		PS				FSO			
peak no.	ECN	area %	peak no.	ECN	area %	species[b]	peak no.	ECN	area %	species
1−4	<36	27.4			9.8	DAG, etc.[c]			8.0	DAG, etc.
5−7	36	0.8	1	42	1.3	unknown	1	36	27.7	LnLnLn
8−11	38	2.1	2	46	7.2	PLO	2	38	15.7	LLnLn
12−14	40	6.6	3	46	13.0	PLP	3, 4	40	24.5	
15−17	42	9.8	4	48	1.8	OOO	5, 6	42	12.5	
18−20	44	18.5	5	48	11.4	POO	7, 8	44	9.2	
21−24	46	24.9	6	48	24.1	POP	9, 10	46	2.3	
25−28	48	9.2	7	48	22.2	PPP	11	48	0.2	
29, 30	50	0.8	8	50	0.8	SOO				
31, 32	52	0.1	9	50	3.7	POS				
			10	50	3.9	PPS				

[a] Chromatogram showing TAG profiles of each peak in ABF, PS, and FSO was illustrated in **Figure 6**. [b] Triacylglycerol (TAG) species: P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Ln, linolenic acid. [c] Diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acid (FFA).

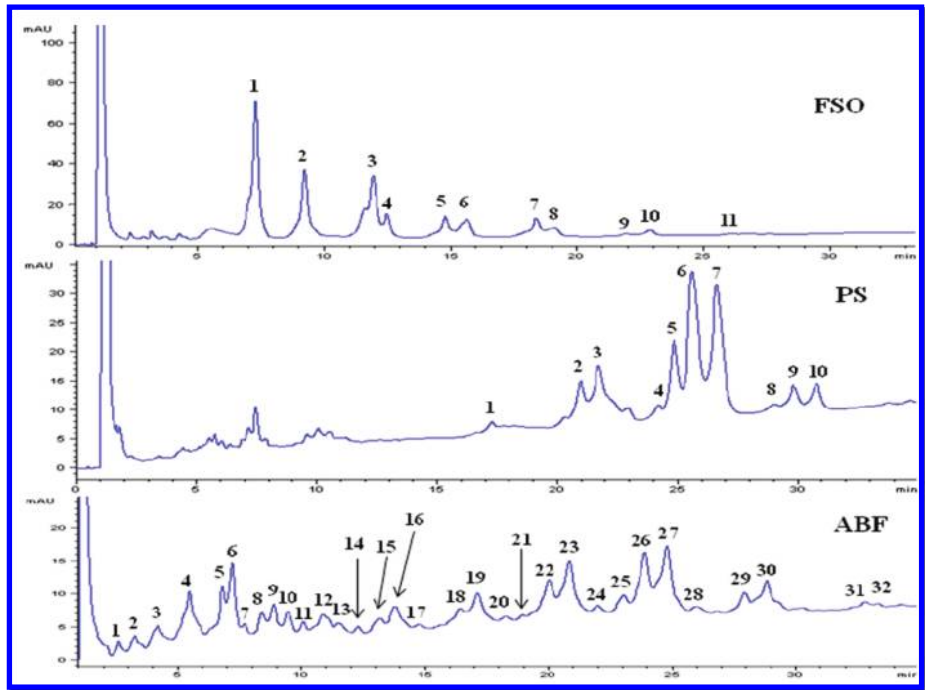

Figure 6. Triacylglycerol profiles of anhydrous butterfat (ABF), palm stearin (PS), and flaxseed oil (FSO). The ECN of each peak is illustrated in **Table 4**.

Table 5. Equivalent Carbon Number (ECN) of Triacylglycerol Composition in Noninteresterified Blends (Blend) and Interesterified Modified-Butterfats (MBFs) in a Continuous Reactor[a]

		ECN of triacylglycerols (area %)																			
		ECN <36	36	38	40			42		44			46			48			50	
MBFs	flow rate	peak no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
8:6:6	blend	16.8	11.9	6.8	2.7	5.6	3.9	2.7	6.7	4.1	4.3	4.2	1.9	6.7	7.0	4.2	5.6	3.1	1.2	0.6
	5 mL/min	14.1	2.4	2.0	2.3	4.9	8.8	4.6	11.6	6.1	8.5	6.5	2.0	9.5	8.3	2.9	3.9	0.9	0.5	0.4
6:6:8	blend	10.7	18.7	8.4	3.0	7.3	3.6	3.7	6.6	3.9	4.1	3.3	1.4	6.0	5.2	3.7	5.4	3.1	1.3	0.7
	5 mL/min	13.0	4.7	4.8	2.9	6.8	9.0	5.3	10.2	5.8	9.1	5.3	1.6	7.9	5.7	2.6	3.7	1.2	0.3	0.3
6:6:9	blend	10.5	18.7	8.6	4.0	7.7	3.9	3.9	6.1	4.5	4.4	2.7	1.6	5.7	5.2	3.3	4.7	3.0	1.0	0.5
	5 mL/min	12.4	5.0	4.7	3.2	7.1	9.7	5.9	10.2	6.0	8.8	5.3	1.5	7.6	5.4	2.4	3.4	1.0	0.3	0.3
4:6:10	blend	5.3	21.5	11.0	4.0	10.0	4.7	4.8	6.3	5.1	4.5	2.2	1.3	4.9	3.5	2.4	4.2	2.7	0.9	0.5
	5 mL/min	10.0	5.0	5.1	4.2	7.7	8.4	7.4	10.6	8.0	9.4	5.2	1.6	6.9	4.2	2.4	2.8	0.7	0.2	0.2

[a] The MBFs were synthesized using four substrate blends such as 8:6:6, 6:6:8, 6:6:9, and 4:6:10 (ABF/PS/FSO, by weight). The interesterification was carried out in a continuous packed-bed reactor at a flow rate of 5 mL/min (contact time 140 min).

The free fatty acid (FFA) contents of samples defined as the percentage by weight of free acid groups existing in oils and fats were measured by AOCS Official Method Ca 5a-40 (*17*).

Pulsed Nuclear Magnetic Resonance (pNMR). A low resolution pulsed NMR spectrometer, MARAN-20 (Resonance Instruments Ltd., Oxon, U.K.) was used to determine the solid fat content (SFC) in samples. Primary samples were completely melted at 60 °C for 30 min and then placed at 0 °C for 60 min. Next, each sample was conditioned for 30 min at each chosen measuring temperature of 10, 20, 30, and 40 °C. Pure olive oil (Costa d'oro S.P.A., Italy) was used as the reference oil. The SFC was calculated according to AOCS Official Method Cd 16-81 (*18*).

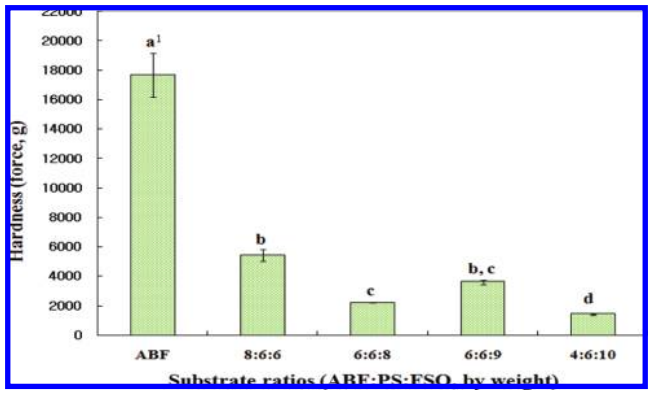

Figure 7. Hardness of anhydrous butterfat (ABF) and modified-butterfats (MBFs) interesterified with different substrate ratios after 12 h at 4 °C (*1*). The same letters on the bar graph are not significantly different ($p < 0.05$).

Texture Analysis. The hardness of each sample was determined after tempering at 4 °C for 12 h using a TA-XT2 texture analyzer (Stable Micro Systems Ltd., London, U.K.). Triplicates were performed for each sample. An angle 45° conical probe penetrated into the sample at 1 mm/s to a 8.4 mm depth and then returned upward at the same speed to the initial position. Hardness was measured as the area of the positive maximum peak or force (g) required to attain a given deformation.

X-ray Diffraction (XRD) Spectroscopy. The samples (about 0.6 g) were completely melted and placed into rectangular plastic molds. Samples were tempered at 4 °C for 12 h after solidifying at room temperature. The polymorphic forms (α-, β'-, and β-form) of each sample were determined by ARL Scintag XDS 2000 (Ecublens, Switzerland) automated diffractometer. The diffractometer was fitted with a scattering angle of 2θ configuration, a solid state detector, and a cobalt X-ray tube. The X-ray beam was generated at 40 kV and 40 mA. The X-ray wavelength (λ) was 1.7889 nm. Data were collected in the 2θ range 18−32°, and the scan rate was 2.0°/min. The single spacing of the α-form was at 4.15 Å, and two strong spacings of the β form were at 4.6 and 3.85 Å. The short spacings of the β' form were strong at 3.8 and 4.2 Å (*19*). The relative intensity of the β' and β crystal form in samples was determined at 4.2 and 4.6 Å.

Polarized Light Microscopy (PLM). For removal of the crystal memory, the samples were completely melted in an oven at 70 °C for 20 min. A small amount (approximately 10 μL) of the melted sample was placed on a glass microscope slide and then covered with a cover glass slip. The prepared slides were cooled at 20 °C for 30 min. The fat crystal morphology was analyzed under isothermal conditions with a polarized light microscope (Leica DMLB upright microscope, Wetzlar, Germany). The photomicrograph of the crystal was taken at 400× magnification.

Statistical Analysis. Analysis was carried out in triplicate, and the variance of results was performed using the General Linear Model Procedure of SAS Statistical Software (SAS version 8.2, Cary, NC) (*20*). Multiple comparisons of means were conducted by Duncan's multiple range test to evidence the significant difference at $P < 0.05$.

RESULTS AND DISCUSSION

Fatty Acid Composition and Chemical Properties. The modified-butterfats (MBFs) were synthesized by lipase-catalyzed interesterification with different weight ratios of anhydrous butterfat (ABF), palm stearin (PS), and flaxseed oil (FSO) as substrates to produce *trans*-free and functional spreadable hard fat. The interesterification was carried out in a packed-bed continuous reactor. The fatty acid composition (mol %) and chemical properties of the substrates and the MBFs are tabulated in **Table 1**. After a short path distillation process to remove free fatty acids (FFAs), the MBFs synthesized showed FFA values less than 1. ABF contained a high amount of saturated fatty acids (74.2%), including butyric acid ($C_{4:0}$, 3.93%). The total saturated fatty acids (∑SFA) content of PS consisting mainly of palmitic acid ($C_{16:0}$, 63.57%) and oleic acid ($C_{18:1}$, 24.54%) was 69.97%. After enzymatic interesterification, the ∑SFA contents of the MBFs synthesized with 8:6:6, 6:6:8, 6:6:9, and 4:6:10 blends (ABF/PS/FSO, by weight) were reduced to 53.38%, 47.17%, 45.74%, and 39.09%, respectively. Their α-linolenic acid (ALn) ($C_{18:3}$, omega-3 fatty acid) contents were 16.19%, 21.41%, 23.06%, and 24.74%, respectively. The increase in FSO content by weight ratio of blends led to a slight increase in ALn, as indicated in **Table 1**.

The atherogenicity index (AI) was calculated with lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), and $C_{16:0}$ as suggested by Ulbricht and Southgate (*21*). Generally, $C_{12:0}$, $C_{14:0}$, and $C_{16:0}$ have been considered primary risk factors for coronary heart disease. Originally, ABF had the highest AI (4.3) while FSO showed the lowest AI (0.03) due to the high amount of total unsaturated fatty acids (∑USFA, 92.79%). Each AI of synthesized MBFs was lower than that of pure ABF as follows: 0.39 in 8:6:6 MBF, 0.26 in 6:6:8 MBF, 0.24 in 6:6:9 MBF, and 0.14 in 4:6:10 MBF.

The measured iodine values (IV), which explains the degree of unsaturation in lipids, of 8:6:6, 6:6:8, 6:6:9, and 4:6:10 MBFs were 90.1, 100.9, 104.1, and 116.8, respectively. Lower AI and higher IV values in MBFs than those for pure ABF occurred because of the increased FSO content, which has a high amount of ALn in MBF blends. Therefore, the nutritional properties of functional MBFs with omega-3 fatty acids ($C_{18:3}$, 16−25%) can be improved.

Flow Rate Effects on Interesterification. The flow rate effect (3, 5, 8, and 10 mL/min) was investigated with two modified-butterfat (MBF) blends (6:6:9 and 8:6:6, ABF:PS:FSO) in a continuous packed-bed reactor. As flow rate increased (3, 5, 8, and 10 mL/min), the calculated enzyme contact time decreased (233, 140, 87.5, and 70 min). The effects of flow rate on melting and crystallization behaviors of 6:6:9 MBF and 8:6:6 MBF are illustrated in **Figures 1** and **2**, respectively. A broad and big endothermic (melting) peak and small peaks at 41.68, 18.00, 2.00, −25.57, and −33.83 °C were obtained in the 6:6:9 blend (**Figure 1**A). After interesterification, as the flow rate decreased (increased resident or contact time with enzyme), the broad endothermic peaks (between 18.00 to 41.68 °C) shifted to lower temperature. The required heat energy (ΔH) to melt the endothermic peak in the 6:6:9 blend decreased from 52.7 J/g (blend) to 23.4 J/g (3 mL/min). The sharp exothermic peak at 19.28 °C (blend) was moved to lower temperature at 11.52 °C (3 mL/min) (**Figure 1**B). The required ΔH in the exothermic peak at 19.28 °C was reduced from 12.5 J/g (blend) to 6.9 J/g (3 mL/min).

A broad endothermic peak and small peaks in the 8:6:6 blend were observed at 42.05, 19.43, 7.04, and −33.23 °C (**Figure 2**A). In the melting thermogram after interesterification, the broad peak between 19.43 and 42.05 °C in the 8:6:6 blend shifted to lower temperatures (16.76 and 35.21 °C) in 8:6:6 MBF (3 mL/min). Its required ΔH also decreased from 52.94 J/g in the 8:6:6 blend (blend) to 24.0 J/g (3 mL/min). In the crystallization thermogram after interesterification, the sharp peak at 19.83 °C (blend) moved to lower temperature and its required ΔH was reduced from 13.5 J/g (blend) to 6.4 J/g (3 mL/min) (**Figure 2**B). These results indicate that decreased flow rate (longer contact time) induced a higher interesterification degree in our reaction system.

The change in free fatty acid content during interesterification in 6:6:9 MBF and 8:6:6 MBF is shown in **Figure 3**. Byproducts such as diacylglycerol (DAG), monoacylglycerol (MAG), and

Table 6. Polymorphic Form and Short Spacings (Å) of Noninteresterified and Interesterified Modified-Butterfats (MBFs) Determined by X-ray Diffraction

		short spacings (Å)[a]				
	substrate ratios	4.6	4.2	3.8	relative content[b] (β'/β)	polymorphic form
noninteresterified	8:6:6	4.6031m	4.2327s	3.8261w	56:44	$\beta' > \beta$
	6:6:8	4.6052s	4.2293m	3.8275w	44:56	$\beta' < \beta$
	6:6:9	4.6031s	4.2327m	3.8233w	48:52	$\beta' < \beta$
	4:6:10	4.6256s	4.2327m	3.8275w	45:55	$\beta' < \beta$
interesterified (MBF)[c]	8:6:6	4.6052m	4.2293s	3.8275w	56:44	$\beta' > \beta$
	6:6:8	4.6011m	4.2327s	3.8247w	54:46	$\beta' > \beta$
	6:6:9	4.6072m	4.2327s	3.8275vw	95:5	$\beta' \gg \beta$
	4:6:10	nd[d]	4.365s	3.8289vw	100:0	β'

[a] Intensity of diffraction spacings: v, very; w, weak (lower intensity); m, medium (medium intensity); s, strong (highest intensity). [b] Relative content of β': the β crystal form was obtained from the intensities at 4.2 and 4.6, respectively, β' and β. [c] Enzymatically interesterified MBFs were synthesized with different substrate ratios (8:6:6, 6:6:8, 6:6:9, and 4:6:10, ABF/PS/FSO, by weight) in a packed-bed continuous reactor. [d] Not detected.

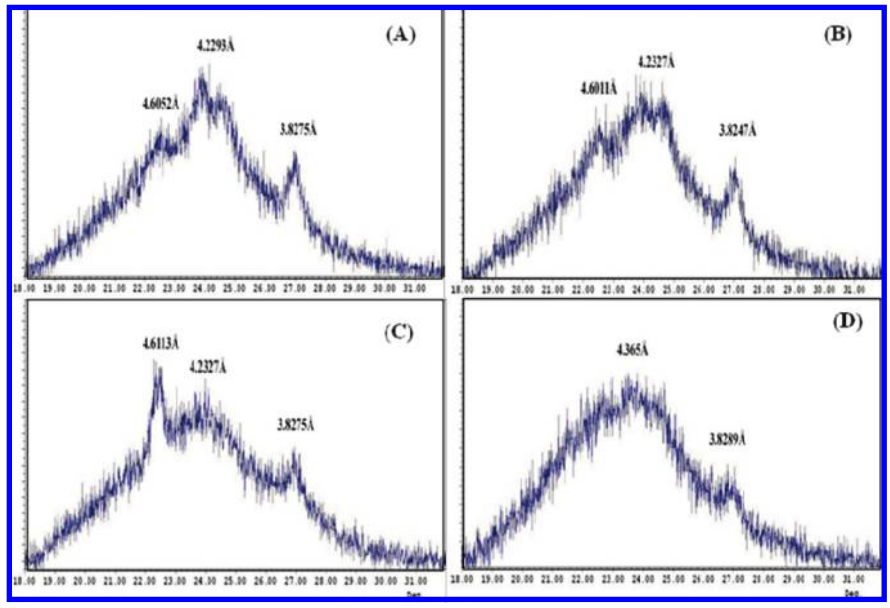

Figure 8. XRD spectroscopy of modified-butterfat (MBF) interesterified with 8:6:6, 6:6:8, 6:6:9, and 4:6:10 (ABF/PS/FSO, by weight): (**A**) 8:6:6 MBF; (**B**) 6:6:8 MBF; (**C**) 6:6:9 MBF; (**D**) 4:6:10 MBF. The MBF was synthesized in a continuous reactor packed with Lipozyme RM IM (150 g).

free fatty acid (FFA) can be generated in final products during an interesterification reaction. The free fatty acid value was slightly decreased with an increase in the contact time of the enzyme with substrates (decreased flow rate). In 6:6:9 MBF, there was no significant difference between 140 min (5 mL/min) and 233 min (3 mL/min) contact times.

Equivalent carbon number (ECN) variation of TAG molecules according to different flow rates for 6:6:9 MBF and 8:6:6 MBF are summarized in **Table 2** and **Figure 4**. ECN 36 (LnLnLn, peak 1) and ECN 38 (LLnLn, peak 2) that originated from flaxseed oil (FSO) rapidly decreased in the blend due to reduced flow rate in both 6:6:9 MBF and 8:6:6 MBF. The areas of ECN 36−38 in 5 mL/min and 3 mL/min were similar to each other, as shown in **Table 2** and **Figure 4**. Also, the ECN 42−46 increase after interesterification showed no difference at 5 mL/min and 3 mL/min. In conclusion, decreased flow rate (longer contact time) increased the interesterification reaction, but there was no significant difference on the reaction degree between 5 mL/min (140 min) and 3 mL/min (233 min). Thus, we set the flow rate at 5 mL/min (140 min) for the next enzymatic interesterification.

Melting and Crystallization Behavior. The melting and crystallization curves of MBFs synthesized with four blends (8:6:6, 6:6:8, 6:6:9, and 4:6:10, ABF:PS:FSO, by weight) are presented in **Figure 5**. As FSO increased in weight percentage in MBF blends, one sharp peak (8.81 °C) and a big sharp peak between 19.93 and 37.06 °C in 8:6:6 MBF slightly moved toward lower temperature, as illustrated in **Figure 5**A. The required energies (ΔH) in each big sharp peak of MBFs were 30.5 J/g in 8:6:6 MBF, 30.1 J/g in 6:6:8 MBF, 25.3 J/g in 6:6:9 MBF, and 23.3 J/g in 4:6:10 MBF. The ΔH decreased with an increase in FSO content in MBF blends. Among the melting thermograms of MBFs from 8:6:6 to 4:6:10, two small peaks (−30.70 and −15.40 °C) shifted to higher temperature and a broad peak at 1.20 °C disappeared. In the crystallization profiles (**Figure 5**B), MBFs had three sharp exothermal (crystallization) peaks. A broad exothermic peak at 1.48 °C in 8:6:6 MBF shifted to lower temperature (−1.70 °C in 4:6:10 MBF) with increase in FSO content. Furthermore, a sharp exothermic peak shifted from 14.84 °C (8:6:6 MBF) to 11.52 °C (4:6:10 MBF). Their ΔH also decreased, ranging from 8.8 J/g to 6.9 J/g as the FSO content increased. These results showed that melting and crystallization properties of functional MBFs after enzymatic interesterification were changed by rearrangement of fatty acids on the glycerol backbone.

Solid Fat Content (SFC) and Melting Point. In **Table 3**, the SFCs and melting points of anhydrous butterfat (ABF) and modified-butterfats (MBFs) enzymatically interesterified are tabulated. The melting point of palm stearin (PS) used in this study was 54 °C. The melting points of 8:6:6 MBF, 6:6:8 MBF, 6:6:9 MBF, 4:6:10 MBF, and ABF were 38, 35.5, 34, 32 and 34 °C, respectively. These MBFs products showed a melting

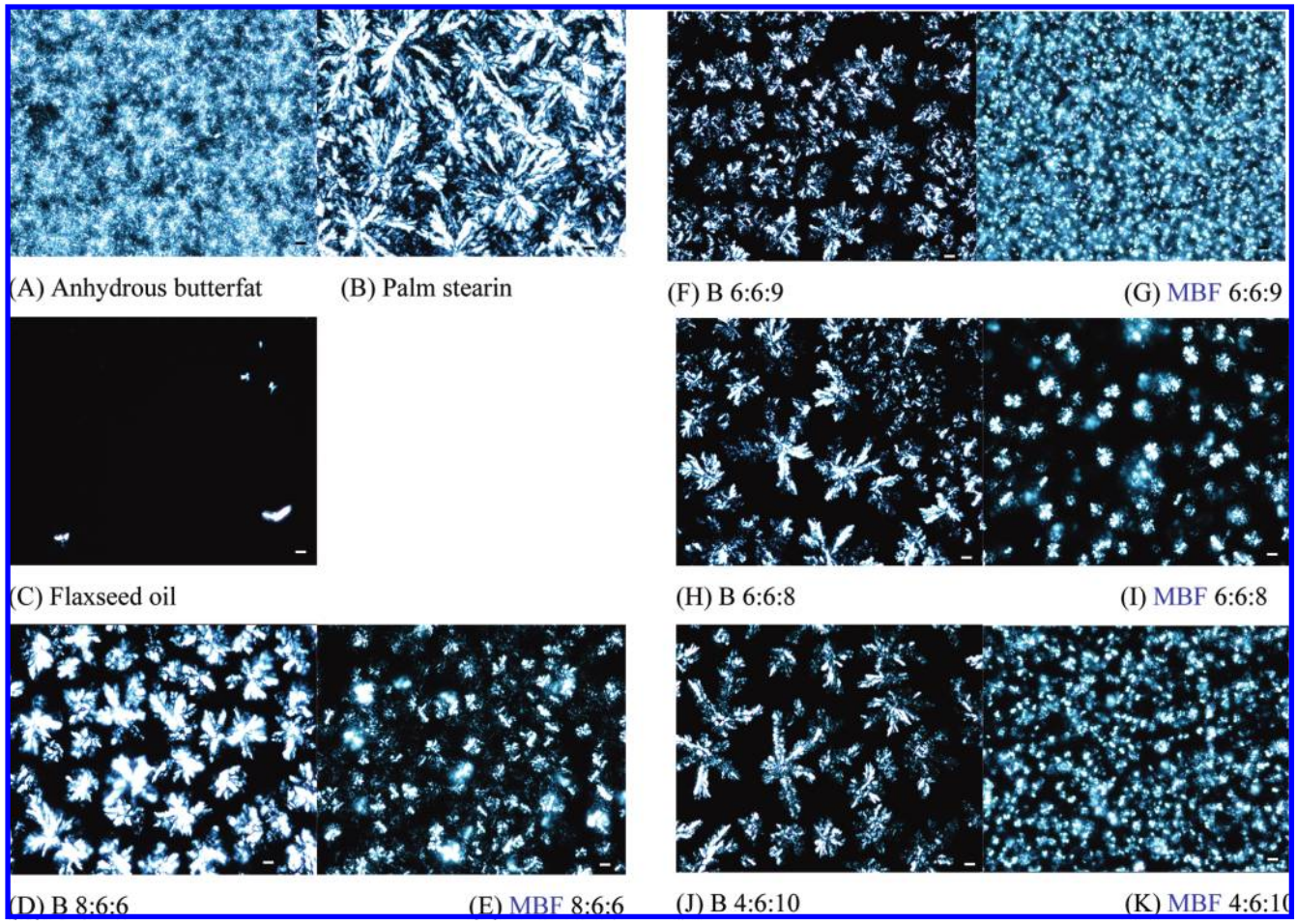

Figure 9. Polarized light microscopy photomicrographs of anhydrous butterfat (ABF), palm stearin (PS), and noninteresterified (blend; B) and enzymatically interesterified (MBF) butterfats after tempering for 30 min at 20 °C; scale bar = 100 μm.

Table 7. Total Tocopherol Content of the Noninteresterified (Blend) and the Interesterified Modified-Butterfats (MBFs) in a Continuous Packed-Bed Reactor

samples		tocopherols (μg/g) α	β	γ	γ tocotrienol	sum
anhydrous butterfat (ABF)		29.11 ± 1.6	nd[a]	nd	nd	29.11 ± 1.6
flaxseed oil (FSO)		10.51 ± 2.1	214.08 ± 2.9	204.22 ± 7.1	nd	428.81 ± 2.0
palm stearin (PS)		19.02 ± 1.3	nd	3.91 ± 0.8	9.42 ± 0.3	32.35 ± 2.5
8:6:6[b]	blend	21.67 ± 1.0[a]	57.45 ± 0.8[a]	72.70 ± 1.3[a]	nd	151.82 ± 11[a]
	before	21.13 ± 1.0[a]	55.67 ± 0.5[a]	71.85 ± 2.0[a]	nd	148.60 ± 3.5[a]
	after	15.10 ± 0.5[b]	46.71 ± 0.4[b]	54.44 ± 0.9[b]	nd	116.25 ± 1.0[b]
6:6:8	blend	18.27 ± 0.4[a]	68.81 ± 0.1[a]	85.71 ± 6.2[a]	nd	172.78 ± 5.5[b]
	before	17.34 ± 0.8[a]	68.59 ± 3.0[a]	70.98 ± 2.1[b]	nd	156.9 ± 15.9[a,b]
	after	15.12 ± 1.4[a]	67.59 ± 1.8[a]	66.64 ± 0.4[b]	nd	149.36 ± 3.7[b]
6:6:9	blend	16.67 ± 1.5[a]	70.43 ± 2.7[a]	84.10 ± 3.9[a]	nd	171.20 ± 8.2[a]
	before	15.02 ± 0.8[a]	70.31 ± 0.9[a]	71.53 ± 2.3[b]	nd	156.90 ± 0.6[a,b]
	after	12.06 ± 0.2[b]	70.21 ± 0.4[a]	68.16 ± 0.5[b]	nd	150.43 ± 0.3[b]
4:6:10	blend	11.96 ± 0.7[a]	86.58 ± 0.6[a]	114.49 ± 2.1[a]	nd	213.03 ± 0.8[a]
	before	11.16 ± 0.5[a]	77.22 ± 3.5[b]	109.30 ± 4.8[a,b]	nd	197.70 ± 1.8[a]
	after	10.28 ± 0.4[a]	63.86 ± 3.4[c]	98.86 ± 5.3[b]	nd	173.00 ± 8.4[b]

[a] Not detected. [b] The enzymatic interesterification was carried out with the blends (blend) of ABF/PS/FSO (by weight). The tocopherol contents before short path distillation (before) and after short path distillation (after) of the MBFs were compared with the blends. The tocopherol values are mean ± SD in duplicate analyses. These values with the different letters in the same column within the same substrate ratio are significantly different ($P < 0.05$).

range between 32−38 °C and are suitable for application in spreadable margarine or butterfat-based fat. Their melting range is similar to the melting point of commercial margarines reported by Karabulut and Turan (*22*). The SFC of ABF was 33.94% at 10 °C. The SFCs of MBFs interesterified with 8:6:6, 6:6:8, 6:6:9, and 4:6:10 (ABF/PS/FSO, by weight) blends were respectively 24.17%, 18.82%, 16.83%, and 10.7% at 10 °C. The SFC should be less than 32% at 10 °C for desirable spreadability at refrigeration temperature according to Lida and Ali (*23*). Therefore, MBFs synthesized were more spreadable than the original ABF.

Triacylglycerol (TAG) Profiles. The equivalent carbon numbers (ECNs) of TAG molecules in anhydrous butterfat (ABF), palm stearin (PS), and flaxseed oil (FSO) are shown in **Table 4**. TAG molecules can be separated by ECN or partition number (PN), which increase with increased carbon chain length

Table 8. Cholesterol and Phytosterol Contents of the Noninteresterified (Blend) and the Interesterified MBFs in a Continuous Packed-Bed Reactor[a]

sample		cholesterol (mg/100 g)	phytosterols (mg/100 g)			
			β-sitosterol	campesterol	stigmasterol	sum
anhydrous butterfat (ABF)		109.44 ± 10.70	nd	nd	nd	nd
flaxseed oil (FSO)		nd	76.43 ± 4.26	48.25 ± 3.72	0.54 ± 0.04	125.22
palm stearin (PS)		nd	4.26 ± 1.25	nd	nd	4.26
8:6:6	blend	56.32 ± 7.94[a]	21.14 ± 3.08[a]	11.50 ± 0.33[a]	0.14 ± 0.00[a]	32.77 ± 3.4[a]
	before	45.36 ± 3.46[a]	17.01 ± 1.54[a,b]	10.00 ± 2.77[a,b]	0.16 ± 0.02[a]	27.17 ± 4.3[a,b]
	after	27.70 ± 2.80[b]	11.40 ± 1.51[b]	5.86 ± 0.98[b]	0.08 ± 0.01[b]	17.34 ± 2.5[b]
6:6:8	blend	34.64 ± 0.94[a]	24.18 ± 1.12[a]	11.87 ± 0.68[a]	0.16 ± 0.01[a]	35.94 ± 1.7[a]
	before	34.62 ± 5.30[a]	21.28 ± 4.16[a]	9.73 ± 0.06[a]	0.13 ± 0.01[a]	31.13 ± 6.0[a]
	after	20.96 ± 0.33[b]	14.38 ± 0.59[b]	4.80 ± 1.05[b]	0.07 ± 0.02[b]	19.24 ± 0.5[b]
6:6:9	blend	33.50 ± 4.19a	26.10 ± 7.18[a]	14.36 ± 0.44[a]	0.21 ± 0.07[a]	40.66 ± 7.7[a]
	before	28.81 ± 4.21[a,b]	19.24 ± 2.88[a]	9.28 ± 1.66[b]	0.16 ± 0.01[a]	28.68 ± 4.6[a,b]
	after	21.42 ± 2.27[b]	16.66 ± 1.62[a]	7.40 ± 0.59[b]	0.12 ± 0.01[a]	23.03 ± 2.2[b]
4:6:10	blend	23.75 ± 0.53[a]	28.85 ± 2.90[a]	16.98 ± 1.71[a]	0.26 ± 0.00[a]	46.08 ± 4.6[a]
	before	19.61 ± 0.15[a]	28.89 ± 0.07[a]	16.03 ± o.60[a,b]	0.20 ± 0.01[b]	45.12 ± 0.7[a]
	after	13.54 ± 1.78[b]	24.44 ± 1.97[a]	12.03 ± 1.62[b]	0.15 ± 0.02[c]	35.90 ± 0.5[b]

[a] The cholesterol and phytosterol values are mean ± SD in duplicate analyses. Values with different letters in the same column within the same substrate ratio are significantly different ($P < 0.05$). nd = not detected. The before and the after stand for the modified-butterfats (MBFs) before short path distillation (before) and after short path distillation (after). The MBFs were synthesized with four blends (blend) of ABF/PS/FSO (by weight).

and decrease with increase in double bond number in the esterified fatty acid chain. ABF contained many TAG peaks because of its various fatty acid compositions from butyric acid to linolenic acid, as summarized in **Table 1** and **Figure 6**. The TAG species in PS and FSO were identified with standard samples and from previous studies (*24*−*26*). PS consists of mainly three ECNs as follows: ECN 46 (7.2 area % PLO and 13.0% PLP), ECN 48 (1.8% OOO, 11.4% POO, 24.1% POP, and 22.2% PPP) and ECN 50 (0.8% SOO, 3.7% POS, and 3.9% PPS). The main two peaks of TAG species in FSO were LnLnLn (ECN 36, peak 1), 27.7 area %, and LLnLn (ECN 38, peak 2), 15.7%. The ECN of MBFs produced in a continuous packed-bed reactor operated at a flow rate of 5 mL/min (contact time 140 min) is shown in **Table 5**. The areas of ECN 36 (LnLnLn) and ECN 38 (LLnLn) from FSO were reduced from the blends to the MBFs (5 mL/min). After interesterification at 5 mL/min flow rate, ECN 48−50 derived from PS also slightly decreased while ECN 42−46 increased in all MBFs. Generally, diversified TAG species from the rearrangement of TAG profiles after enzymatic interesterification tend to form β′ type crystals (*26*).

Hardness. Figure 7 shows the hardness in original anhydrous butterfat (ABF) and interesterified modified-butterfats (MBFs), as evaluated by the texture profile analyzer (TPA). Hardness measured using a cone penetrometer was obtained from the maximum penetration force in a texture profile of samples. Hardness associated with the polymorphic form and crystal size shows the relative ratio of solid crystal to liquid oil (*10*). The hardness and the spreadability of butter were considered as one of the important properties in consistency and quality evaluations. But butter has poor spreadability at refrigerator temperature because various TAG species of butter crystallized in a limited temperature range due to the high content of solid fat (*10*). Therefore, some vegetable oils such as canola oil (*27*) and corn oil (*28*) are added to improve the spreadability and to strengthen the nutraceutical function of butterfat. The hardness in interesterified MBFs significantly decreased compared to that of the original ABF. Among MBFs, 4:6:10 MBF was significantly the lowest in hardness due to enriched FSO content and decreased hard fat content. There was no significant difference in hardness between 6:6:8 MBF and 6:6:9 MBF. The 8:6:6 MBF, 6:6:8 MBF, 6:6:9 MBF, and 4:6:10 MBF were softer (more spreadable) than the original ABF (69%, 88%, 80%, and 92%, respectively). In conclusion, more spreadable hard-fats were successfully produced through enzymatic interesterification of ABF, FSO, and PS blends in a continuous reactor operated at 5 mL/min (contact time 140 min). The obtained MBFs may be suitable for spreadable margarine or butterfat substitutes.

Polymorphism. The three representative polymorphic forms of fat crystals are known as the α, β′, and β forms. The α form (hexagonal) present in chilled milk fats (oil-in-water emulsion) is the least stable and has a short spacing at 4.15 Å (0.415 nm). The β form (triclinic) found in cocoa butter crystal in confectionery is the most stable and has a strong short spacing at 4.6 Å. The β′ form (orthorhombic) displayed in margarine and shortening is metastable and has two strong short spacings at 3.8 and 4.2 Å (*29*). Normally, the melting point of the crystal forms in fats is on the order of the α < β′ < β form (*30*). Most margarine and shortening prefer the β′ form with small crystals. The formation of noticeable β form in margarine products induces a sandy mouth feel with increased crystal size, increased hardness, and decreased spreadability (*31*).

The polymorphic form of noninteresterified and interesterified butterfats measured by X-ray diffraction is summarized in **Table 6**. X-ray diffraction spectroscopy of four MBFs (8:6:6 MBF, 6:6:8 MBF, 6:6:9 MBF, and 4:6:10 MBF) was shown in **Figure 8**. The original anhydrous butterfat (ABF) showed two short spacings at 4.2327 and 3.8253 Å, respectively. Therefore, its polymorph was predominantly β′ form, as suggested by Ibrahim et al (*32*). In TAG profiles of palm stearin (PS), the total area % of POP and PPP (mainly β-tending crystals) and total area % of PLO, POO, and PPS (β′-tending crystals) were respectively 46.3% and 22.5%. This result suggests that PS tends to form the β crystal more readily than the β′ crystal. In X-ray diffraction, the short spacing of PS showed not only at 4.2276 and 3.8358 Å but also at 4.6052 Å, indicating the β polymorph.

In the blends, their polymorphism showed predominantly β form crystals except 8:6:6 blends. The 8:6:6 blend showed a β′ form crystal because of containing a high amount of ABF with the abundance of asymmetrical and diverse TAGs (*33*). After interesterification, polymorphisms of all modified-butterfats (MBFs) were the desirable β′ form crystals with stronger 4.2 Å and weaker 4.6 Å short spacing (**Figure 8**).

Fat Crystal Network. The β crystal form was shown to be more stable and harder in texture than the β′ crystal form. This is because it has a crystal size over 50−100 μm while the β′ crystal form has a crystal size within a 5 μm maximum in length (*34*). Polarized light microscopy (PLM) photomicrographs of

substrates, the noninteresterified blends, and the interesterified MBFs are illustrated in **Figure 9**. The fat crystal size of anhydrous butterfat (ABF), containing the β' crystal form, was small in size and approximately 100 μm in diameter. Palm stearin (PS), with the β crystal form, had a much larger and complex crystal structure. Typically, a dark background in PLM micrographs indicates a noncrystalline substrate (liquid oils), and a white color spectrum indicates a crystalline structure in fat.

In the blends, the fat crystal appeared much coarser and larger with more complex crystalline structures originating from PS. These fat crystal sizes would provide a sandy mouth feel in margarine fats. After interesterification, the fat crystal size in modified-butterfats (MBFs) was smaller with 100 μm and agglomerated into spherical crystals of varying density like the typical β' form (tiny needle-like agglomerates).

Tocopherol Content. Tocopherols (Tc), a natural antioxidant mainly present in fruit and vegetable oil, belong to vitamin E with α-, β-, γ-, and δ-Tc that contain saturated phytol side chains based on the chromanol ring (*35*). Among those tocopherols, α-Tc is biologically the most active and γ-Tc is considered the best antioxidant. Tocotrienols (Tc3), isomers of tocopherol, exist in cereals and palm oils and are classified as α-, β-, γ-, and δ-Tc3. Tc3 contain a double bond in the side chain. The variations in tocopherol content in MBFs during the interesterification process and purification process (before and after short path distillation) are shown in **Table 7**. The total tocopherol contents of anhydrous butterfat (ABF), flaxseed oil (FSO), and palm stearin (PS) used as substrates were 29.11 (α-Tc), 428.81, and 32.35 μg/g, respectively. FSO, a vegetable oil, predominantly contained β-Tc 214.08 μg/g and γ-Tc 204.22 μg/g. PS, from palm oil, contained α-Tc 19.02 μg/g and γ-Tc3 9.42 μg/g. During enzymatic interesterification, the total tocopherol contents in four MBFs (8:6:6, 6:6:8, 6:6:9, and 4:6:10) were not significantly different between the blends and the interesterified MBFs (before, nonpurified). However, the total tocopherol contents in four MBFs (after, purified) significantly decreased during the short path distillation process ($P < 0.05$). The loss in tocopherol content during short path distillation was similar to reports related to the purification of structured lipid by short path distillation (*36*, *37*). Higher FFA content in raw material and higher evaporate temperature led to higher losses of tocopherol homologues (*37*). The total tocopherol content of each purified 8:6:6, 6:6:8, 6:6:9, and 4:6:10 MBF was 116.25, 149.36, 150.43, and 173.00 μg/g, respectively. The highest amount of tocopherol was in 4:6:10 MBF. The homologues β-Tc and γ-Tc were abundant in the four final products.

Cholesterol and Phytosterol Contents. Cholesterol and phytosterol are present in animal fats and vegetable oils, respectively. Phytosterols (plant sterols), like β-sitosterol, campesterol, and stigmasterol, are less efficiently absorbed than cholesterol even though their structures are similar to the structure of cholesterol. β-Sitosterol is the least absorbed. Therefore, excess phytosterols play an important part in preventing atherosclerosis by blocking cholesterol absorption (*38*). Cholesterol and phytosterol contents of anhydrous butterfat (ABF) obtained from animal sources, and palm stearin (PS) and flaxseed oil (FSO) from plant sources are shown in **Table 8**. Milk and milk products contain cholesterol that ranges from 148 to 369 mg/100 g fat according to Seçkin et al. (*39*). The cholesterol content in the ABF used was 109 mg/100 g of fat. This value is lower than the cholesterol level in dairy products and in butter oil reported by Fatouh et al. (*40*). On the whole, the cholesterol level was not significantly different between the blends (blend) and the nonpurified MBFs (before). But there was a significant difference between the nonpurified MBFs (before) and the purified MBFs (after). The cholesterol content in MBFs decreased (25−40%) during the short path distillation process. The highest cholesterol level was in 8:6:6 MBF, containing a high amount of ABF. The total phytosterol of FSO was 125.22 mg/100 g, mainly β-sitosterol (76.43 mg/100 g) and campesterol (48.25 mg/100 g).

Phytosterol contents as well as the cholesterol level in each MBF ratio slightly decreased during the interesterification reaction, but there was no significant difference. After short path distillation, the phytosterol levels in four MBFs were significantly reduced to 22−47% from the blends. After short path distillation, the total phytosterol contents of purified 8:6:6 MBF, 6:6:8 MBF, 6:6:9 MBF, and 4:6:10 MBF were 17.34, 19.24, 23.03, and 35.90 mg/100 g, respectively.

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LITERATURE CITED

(1) Lee, K. T.; Akoh, C. C. Structured lipids: Synthesis and applications. *Food Rev. Int*. **1998**, *14*, 17–34.
(2) Kim, E. J.; Yoon, S. H. Recent progress in enzymatic production of structured lipids. *Food Sci. Biotechnol*. **2003**, *12*, 721–726.
(3) Xu, X.; Balchen, S.; Høy, C. E.; Adler-Nissen, J. Production of specific-structured lipids by enzymatic interesterification in a pilot continuous enzyme bed reactor. *J. Am. Oil Chem. Soc*. **1998**, *75*, 1573–1579.
(4) Xu, X. B.; Porsgaard, T.; Zhang, H.; Adler-Nissen, J.; Høy, C. E. Production of structured lipids in a packed-bed reactor with *Thermomyces lanuginosa* lipase. *J. Am. Oil Chem. Soc*. **2002**, *79*, 561–565.
(5) Lai, O. M.; Low, C. T.; Akoh, C. C. Lipase-catalyzed acidolysis of palm olein and caprylic acid in a continuous bench-scale packed bed bioreactor. *Food Chem*. **2005**, *92*, 527–533.
(6) Alim, M. A.; Lee, J. H.; Shin, J. A.; Lee, Y. J.; Choi, M. S.; Akoh, C. C.; Lee, K. T. Lipase-catalyzed production of solid fat stock from fractionated rice bran oil, palm stearin, and conjugated linoleic acid by response surface methodology. *Food Chem*. **2008**, *106*, 712–719.
(7) Jirasubkunakorn, W.; Bell, A. E.; Gordon, M. H.; Smith, K. W. Effects of variation in the palm stearin: palm olein ratio on the crystallization of a low-*trans* shortening. *Food Chem*. **2007**, *103*, 477–485.
(8) Siew, W. L.; Cheah, K. Y.; Tang, W. L. Physical properties of lipase-catalyzed interesterification of palm stearin with canola oil blends. *Eur. J. Lipid Sci. Technol*. **2007**, *109*, 97–106.
(9) Otero, C.; López-Hernandez, A.; García, H. S.; Hernández-Martín, E.; Hill, C. G. Continuous enzymatic transesterification of sesame oil and a fully hydrogenated fat: effects of reaction conditions on product characteristics. *Biotechnol. Bioeng*. **2006**, *94*, 877–887.
(10) Wright, A. J.; Scanlon, M. G.; Hartel, R. W.; Marangoni, A. G. Rheological properties of milkfat and butter. *J. Food Sci*. **2001**, *66*, 1056–1071.
(11) Precht, D. Cholesterol content in European bovine milk fats. *Nahr./Food*. **2001**, *45*, 2–8.
(12) Vijaimohan, K.; Jainu, M.; Sabitha, K. E.; Subramaniyam, S.; Anandhan, C.; Devi, C. S. S. Beneficial effects of alpha linolenic acid rich flaxseed oil on growth performance and hepatic cholesterol metabolism in high fat diet fed rats. *Life Sci*. **2006**, *79*, 448–454.
(13) Laudani, C. G.; Habulin, M.; Knez, Ž.; Porta, G. D.; Reverchon, E. Immobilized lipase-mediated long-chain fatty acid esterification in dense carbon dioxide: bench-scale packed-bed reactor study. *J. Supercrit. Fluids* **2007**, *41*, 74–81.
(14) Lee, J. H.; Kim, M. R.; Kim, I. H.; Kim, H.; Shin, J. A.; Lee, K. T. Physicochemical and volatile characterization of structured lipids from olive oil produced in a stirred-tank batch reactor. *J. Food Sci*. **2004**, *69*, 89–95.

(15) Chun, J.; Lee, J.; Ye, L.; Exler, J.; Eitenmiller, R. R. Tocopherol and tocotrienol contents of raw and processed fruits and vegetables in the United States diet. *J. Food Compos. Anal*. **2006**, *19*, 196–204.
(16) *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th ed.; AOCS Press: Champaign, IL, 1998; Method Cc 1-25.
(17) *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th ed.; AOCS Press: Champaign, IL, 1998; Method Ca 5a-40.
(18) *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th ed.; AOCS Press: Champaign, IL, 1998; Method Cd 16-81.
(19) Lee, J. H.; Akoh, C. C.; Lee, K. T. Physical properties of *trans*-free bakery shortening produced by lipase-catalyzed interesterification. *J. Am. Oil Chem. Soc*. **2008**, *85*, 1–11.
(20) SAS (Statistical Analysis System Institute). *The SAS System for Windows*, Release 8.02; SAS Institute: Cary, NC, 2001.
(21) Ulbricht, T. L. V.; Southgate, D. A. T. Coronary heart disease: seven dietary factors. *Lancet* **1991**, *338*, 985–992.
(22) Karabulut, I.; Turan, S. Some properties of margarines and shortenings marketed in Turkey. *J. Food Compos. Anal*. **2006**, *19*, 55–58.
(23) Lida, H.; Ali, A. R. M. Physico-chemical characteristics of palm-based oil blends for the production of reduced fat spreads. *J. Am. Oil Chem. Soc*. **1998**, *75*, 1625–1631.
(24) Bergqvist, M. H. J.; Kaufmann, P. A multivariate optimization of triacylglycerol analysis by high-performance liquid chromatography. *Lipids* **1993**, *28*, 667–675.
(25) Lonchampt, P.; Fanni, J.; Linder, M.; Parmentier, M. Molecular interaction of triglycerides on a modified silica (Kieselguhr G): a thermodynamical approach by surface tension calculation and DSC measurements. *Eur. J. Lipid Sci. Technol*. **2001**, *103*, 576–582.
(26) Chu, B. S.; Ghazali, H. M.; Lai, O. M.; Man, Y. B. C.; Yusof, S. Physical and chemical properties of a lipase-transesterified palm stearin/palm kernel olein blend and its isopropanol-solid and high melting triacylglycerol fractions. *Food Chem*. **2002**, *76*, 155–164.
(27) Rousseau, D.; Marangoni, A. G. The effects of interesterification on physical and sensory attributes of butterfat and butterfat-canola oil spreads. *Food Res. Int*. **1999**, *31*, 381–388.
(28) Rodrigues, J. N.; Gioielli, L. A. Chemical interesterification of milkfat and milkfat-corn oil blends. *Food Res. Int*. **2003**, *36*, 149–159.
(29) deMan, J. M. X-ray diffraction spectroscopy in the study of fat polymorphism. *Food Res. Int*. **1992**, *25*, 471–476.
(30) Ghotra, B. S.; Dyal, S. D.; Narine, S. S. Lipid shortenings: a review. *Food Res. Int*. **2002**, *35*, 1015–1048.
(31) Widlak, N.; Hartel, R.; Narine, S. Crystallization and solidification properties of lipids. In *Polymorphism and Texture of Fats*; deMan, J. M., deMan, L., Eds.; AOCS Press: Champaign, IL, 2001; pp 225−235.
(32) Ibrahim, N. H.; Idris, N. A.; Abdullah, A.; Said, M.; Dian, N. L. H. M.; Man, Y. C. Polymorphism of anhydrous milk fat as affected by the addition of palm stearins. *J. Oil Palm Res*. **2006**, 58–64.
(33) D'Souza, V.; deMan, J. M.; deMan, L. Short spacings and polymorphic forms of natural and commercial solid fats: A review. *J. Am. Oil Chem. Soc*. **1990**, *67*, 835–843.
(34) Stauffer, C. E. *Fats and oils*; Eagan Press: St. Paul, MN, 1996; pp 1−14.
(35) Chun, J.; Lee, J.; Yea, L.; Exler, J.; Eitenmiller, R. R. Tocopherol and tocotrienol contents of raw and processed fruits and vegetables in the United States diet. *J. Food Compos. Anal*. **2006**, *19*, 196–204.
(36) Akoh, C. C.; Moussata, C. O. Characterization and oxidative stability of enzymatically produced fish and canola oil-based structured lipids. *J. Am. Oil Chem. Soc*. **2001**, *78*, 25–30.
(37) Xu, X.; Jacobsen, C.; Nielsen, N. S.; Heinrich, M. T.; Zhou, D. Purification and deodorization of structured lipids by short path distillation. *Eur. J. Lipid Sci. Technol*. **2002**, *104*, 745–755.
(38) Thompson, G. R.; Grundy, S. M. History and development of plant sterol and stanol esters for cholesterol-lowering purposes. *Am. J. Cardiol*. **2005**, *96*, 3D−9D.
(39) Seçkin, A. K.; Gursoy, O.; Kinik, O.; Akbulut, N. Conjugated linoleic acid (CLA) concentration, fatty acid composition and cholesterol content of some Turkish dairy products. *LWT−Food Sci. Technol*. **2005**, *38*, 909–915.
(40) Fatouh, A. E.; Singh, R. K.; Koehler, P. E.; Mahran, G. A.; Metwally, A. E. Physical, chemical and stability properties of buffalo butter oil fractions obtained by multi-step dry fractionation. *Food Chem*. **2005**, *89*, 243–252.

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