J Am Oil Chem Soc (2010) 87:647–657
DOI 10.1007/s11746-010-1541-x

ORIGINAL PAPER

Physicochemical Properties of Lipase-Catalyzed Interesterified Fat Containing α-Linolenic Acid

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Received: 27 August 2008 / Revised: 7 December 2009 / Accepted: 23 December 2009 / Published online: 6 February 2010

Abstract Two substrate blends (8:6:6 and 6:6:9, by weight) of anhydrous butterfat (ABF), palm stearin (PS), and flaxseed oil (FSO) were interesterified by immobilized lipases. The reaction was carried out in the absence of solvent at 60 °C for 24 h in a 1-L tank stirred-batch type reactor. In terms of equivalent carbon number (ECN) of triacylglycerol (TAG), the areas of ECN 36-38 (from FSO) and ECN 48-50 (from PS) decreased during the interesterification while ECN 42–46 increased with increasing reaction time. As interesterification time increased, the decreased enthalpy (ΔH), peak temperature (T_P) and transition range were observed. After short path distillation, interesterified fat (IF) was produced in which α-linolenic acid contents (ALn, mol%) of the 8:6:6 and 6:6:9 IF were 15.7 and 21.7%, respectively. Tocopherol, cholesterol and phytosterol contents in each IF were significantly reduced after short path distillation. In this study, hardness of 6:6:9 IF and 8:6:6 IF were 217 and 800 g/cm^2, respectively. After interesterification, short spacing at 4.6 Å disappeared or weakened, indicating that the predominant polymorphic form had changed from the β form to the desirable crystalline structure of the β' form.

Keywords α-Linolenic acid · Butterfat · Lipase-catalyzed interesterification · Thermal behaviors · Triacylglycerol composition

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Introduction

Butterfat has a wide melting range because it is composed of a myriad of triacylglycerols (TAG) combined with various fatty acid moieties. It imparts buttery aroma, palatability, and texture to the food. However, butterfat has relatively poor spreadability at refrigerator temperatures [1]. If such a characteristic of fats needs to be modified, enzymatic or chemical interesterification, fractionation, hydrogenation and blending can be suggested.

Recently, interesterification has been broadly used for the modification of milk fat [2, 3] in which TAG positional distribution and physical properties of the resulting products were altered. In addition, such a technique imparts improved spreadability and nutritional properties to the fats and oils. Compared with chemical interesterification, enzymatic interesterification has some advantages such as mild reaction condition, low side-products, and high substrate regiospecificity. Furthermore, interesterified fats and oils prepared from hard fat and vegetable oil have been recently applied as a low *trans* alternative because margarine (especially stick or hard margarine) and shortening from partially hydrogenated vegetable fat contain undesirable *trans* fatty acids with ranges from 10 to 40% [4].

Palm stearin (PS) is the solid fraction obtained from palm oil using solvent or dry fractionation. It is widely used in margarine or shortening production due to its relatively high melting point (54 °C) for manipulating physical properties with other low melting oils and fats. For example, Lai et al. [5] performed enzymatic transesterification of a PS:anhydrous milk fat (40:60) mixture. In this study, flaxseed oil (FSO) was used because FSO contains over 50% α-linolenic acid (ALn, omega-3 fatty acid), reducing the risk of cardiovascular disease. According to the report of Vijaimohan et al. [6], FSO supplementation

significantly lowered the body weight, plasma cholesterol, triacylglycerol level, and LDL cholesterol in fed rats.

In this study, anhydrous butterfat (ABF), PS and FSO were used for production of the interesterified fat (IF) containing ALn. Substrate ratios (8:6:6 and 6:6:9, ABF/PS/FSO by weight) for the blends and the interesterification were selected from our previous work [7]. The interesterification was, respectively, performed using Lipozyme RM IM (8:6:6, ABF/PS/FSO by weight) and Novozym SP435 (6:6:9, ABF/PS/FSO by weight) in a 1-L stirred-batch type reactor. During the 24-h reaction, a change in TAG composition was observed along with the melting and crystallization behavior by differential scanning calorimetry (DSC).

After short path distillation, each IF was studied for phytosterol and tocopherols contents, solid fat content (SFC), polymorphism, positional fatty acid composition and texture properties. These IFs are expected to provide some nutritional benefits, and can be used as a spreadable fat containing ALn.

Materials and Methods

Materials

Butterfat was obtained from Murray Goulbum Co-operative Co. Ltd. (Australia) and stored at −30 °C until needed. Organic FSO (Vitamin World Inc., NY) was purchased from a local grocery store without further treatment. PS was a gift from CJ Co. (Seoul, Korea). Lipozyme RM IM (lipase from *Rhizomucor miehei*, immobilized on a macroporous anion exchange resin) and Novozym SP435 (lipase from *Candida antarctica*, immobilized on a macroporous acrylic resin) were provided by Novozymes North America Inc. (Franklinton, NC). Pancreatic lipase, α-, β-, and γ-tocopherol standards (purity 98%), TAG standards (tributyrin, tricaprylin, trilaurin, tripalmitin, triolein and trilinolein, purity 99%) and cholesterol standard were purchased from Sigma–Aldrich Co. (St. Louis, MO). Phytosterol mixture and 5β-cholestan-3β-ol were products of Matreya Inc. (Pleasant Gap, PA). TAG standards were used to obtain the equivalent carbon number (ECN) in reversed-phase HPLC analysis. All solvents and other chemicals for analysis were of the highest available quality or HPLC grade.

Interesterification in a Stirred-Batch Type Reactor

Butterfat was separated from the water phase using a separatory funnel after complete melting at 60 °C. Isolated butterfat was passed through an anhydrous sodium sulfate column to remove residual moisture. Finally, the ABF was filtered in a vacuum using a 0.45-μm membrane filter paper and stored at −30 °C until analysis. The reaction mixture (500 g) of ABF, PS and FSO (8:6:6 and 6:6:9, by weight) was added to a 1-L tank stirred-batch type reactor. The immobilized lipase (50 g, 10% by weight of reaction mixtures) was loaded. The reaction mixture was then stirred at 230 rpm by an impeller (blade length: 9 cm; blade width: 3.3 cm) with a stirrer motor (M Tops MS-3060D, Korea). The height and the diameter of the 1-L tank for the reaction were 11 and 15 cm, respectively. Each immobilized lipase was used for the production of each interesterified fat with an 8:6:6 blend (Lipozyme RM IM) and a 6:6:9 blend (Novozym SP435). The reaction was carried out at 60 °C for 24 h in a solvent-free system. The reactant was withdrawn at the designated reaction time (1, 2, 3, 5, 10 and 24 h), and then filtered with a PTFE syringe membrane filter (25 mm, 0.2 μm, Whatman) before analysis.

Short Path Distillation

The interesterified reactants produced were further purified using a KDL-4 short path distillation unit (UIC Inc., Joliet, IL). The reactants were passed through the short path distillation apparatus kept below 90 mbar vacuum pressure to obtain the refined interesterified fats (IFs) with less than 1% of free fatty acid (FFA). The IFs were separated at 130 °C heating temperature and stored at −30 °C. These refined IFs were used for further analysis for phytosterol and tocopherols contents, solid fat content (SFC), polymorphism, positional fatty acid composition and texture properties.

Water Content, Free Fatty Acid Content, Iodine Value, and Capillary Melting Point

The water contents of ABF, PS, and FSO were determined by the Karl-Fischer titration with a MKC-520 coulometric titrator (KEM, Kyoto, Japan). The melted sample (approximately 0.6 g) was weighed in a syringe, injected into the Karl-Fischer reaction vessel, and then titrated until the equivalence point. Free fatty acid (FFA) content was determined by titration with a 0.1 M sodium hydroxide aqueous solution according to AOCS Official Method Ca 5a-40 [8]. The iodine value (IV) was also determined by AOCS Official Method Cd 1-25 [8]. The capillary melting point was determined by the process described in the AOCS Official Method Cc 1-25 [8]. The melting point was defined as the temperature at which the solidified fat completely melts to a clear liquid.

Fatty Acid Composition Analysis

Methylation for fatty acid composition analysis was carried out at room temperature using 2-*N* potassium hydroxide

solution in MeOH as described previously [9]. Fatty acid methyl ester (FAME) was analyzed using a Hewlett-Packard 6890 Series gas chromatograph (GC) with an auto injector and a flame-ionization detector (Agilent Technology, Little Falls, DE). A fused-silica capillary column (SP^{TM}-2560, 100 m × 0.25 mm i.d., 0.2-μm film thickness, Supelco, Bellefonte, PA) was used. Carrier gas was nitrogen at 1 mL/min with constant mode. 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 programmed from 50 to 150 °C at a rate of 10 °C/min and held for 1 min. The oven temperature was programmed again from 150 to 220 °C at a rate of 4 °C/min and held for 20 min isothermally at 220 °C. The peaks were identified and calibrated with standard fatty acid methyl esters (GLC460 and GLC461, Nu-Check Prep, Inc., Elysian, MN). Fatty acid composition (mol%) of each sample was expressed as the molecular weight percentage. Fatty acid composition at the *sn*-2 position was analyzed by pancreatic lipase followed by isolation of the *sn*-2 MAG (monoacylglycerol) [10]. A sample (5 mg) was weighed in a test tube. Five milliliters of 1 M Tris–HCl buffer (pH 7.6), 1.25 mL of 0.05% (w/v) bile salt solution, 0.5 mL of 2.2% $CaCl_2$ solution, and finally 5 mg of pancreatic lipase were added and vortexed for 1 min. Then, it was incubated at 37 °C for 3 min. For the isolation of the *sn*-2 MAG, thin-layer chromatography (TLC) plates with developing solvent (50:50:1, diethyl ether/hexane/acetic acid, by volume) were used. The separated MAG bands on the TLC plates were scraped, methylated, and analyzed by GC as described above.

Tocopherol, Cholesterol, and Phytosterol Analysis

Saponification for tocopherol, phytosterol and cholesterol analyses was performed using 60% potassium hydroxide solution and 6% pyrogallol ethanol solution [9]. Unsaponifiable compounds were extracted with hexane/ethyl acetate mixture (85:15, by volume) containing 0.05% BHT 3 times. After evaporation under N_2 gas, the dried-unsaponifiable matters were dissolved in 5 mL hexane to prepare proper analytical concentration. Then, the diluted unsaponifiable matter solution was filtered through a 0.45-μm hydrophobic membrane syringe filter. Finally, the diluted solution was used for tocopherol, cholesterol and phytosterol analysis. The tocopherol was quantified using a 1090 Series HPLC system (Agilent Technologies Inc., Palo Alto, CA) composed of a 1046A series fluorescence detector (Agilent Technologies Inc., Palo Alto, CA) and a Zorbax RX-Sil column (4.6 mm ID × 250 mm, 5 μm particle size, Agilent Technologies Inc., Palo Alto, CA). The prepared unsaponifiable-matter solution (20 μL) was injected into the injector port of an analytical HPLC system to obtain the chromatograms. Fluorescence detection of tocopherols was carried out at the 295-nm wavelength for excitation and at the 325-nm wavelength for emission. The separation was performed with isocratic elution of hexane/isopropanol (99.1:0.9, v/v) at a flow rate of 1.5 mL/min [9]. Cholesterol and phytosterol contents were simultaneously analyzed by capillary gas chromatography (GC). Sterol analysis was conducted as described previously using an internal standard (5β-cholestane-3β-ol, 2.5 mg/mL) [9]. The GC system consisted of a 5890 Series II chromatograph (Agilent Technologies Inc., Palo Alto, CA), equipped with a flame-ionization detector and a fused silica capillary column (SAC-5, 30 m × 0.25 mm i.d., Supelco Inc., Bellefonte, PA). The carrier gas was nitrogen with a column flow rate of 1.1 mL/min. The injector was used in split mode with a ratio of 1:21. The injector and the detector temperatures were set at 320 °C. The oven temperature was initially set at 220 °C, and then programmed at 4 °C/min to 300 °C for 10 min.

Triacylglycerol (TAG) Profile by HPLC

The TAG profiles were determined by a reversed-phase HPLC apparatus equipped with a Yonglin SP930D dual pump (Yonglin, Anayang, Korea), a Rheodyne 7125 manual injector and a Yonglin UV830 detector (Yonglin, Anayang, Korea) set at 220 nm. The column was a Nova-Pak® C18 (4 μm particle size, 150 × 3.9 mm, Waters, Milford, MA) and was heated at 30 °C with column oven. The mobile phase was a binary solvent system of acetonitrile (solvent A) and 2-propanol/hexane (2:1, v/v) (solvent B) at a flow rate of 1 mL/min [9]. The samples were dissolved in chloroform (10 mg/mL) with 20 μL tributyrin as an internal standard. TAG standards (tricaprylin, trilaurin, trimyristin, tripalmitin, trilinolein, and triolein) were used for preparing the standard curve and for peak identification of TAG based on the ECN.

Differential Scanning Calorimetry (DSC)

Thermal analysis for melting and crystallization thermograms was obtained using a DSC 2010 Differential Scanning Calorimeter (TA Instruments, New Castle, DE). Calibration was performed with an indium standard (mp 152 °C) and the baseline was obtained with sealed empty aluminum pan as a reference [9]. All samples (5–8 mg) were hermetically sealed in an aluminum pan. Samples were then heated to 80 °C for 10 min to destroy completely previous crystal structure. The samples were cooled from 80 °C at a rate of 10 °C/min to −65 °C for the cooling thermograms. After holding for 10 min, samples were heated again up to 80 °C at a rate of 5 °C/min for the

melting thermograms. Thermograms were analyzed Universal Analysis 2000 Software (Version 3.4C, TA instruments) to gain enthalpy (heat content, ΔH, J/g), transition peak temperature (T_p), and onset temperature (T_{on}) at the transition point which is intersected between extrapolated baseline and extrapolated slop.

Solid Fat Content (SFC) by Pulsed NMR

The solid fat content (SFC) was determined using a low resolution pulsed NMR spectrometer using MARAN-20 (Resonance Instruments Ltd., Oxon, UK) according to AOCS Official Method Cd 16-81 [8]. Prior to the SFC measurement, samples were completely melted at 60 °C for 30 min and then placed at 0 °C for 60 min. Finally, the samples were conditioned for 30 min at each selected 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.

Texture Analysis

The texture of each sample was determined with a TA-XT2 texture analyzer (Stable Micro Systems Ltd., London, UK) after tempering at 4 °C for 12 h. A 45°-angle conical probe was allowed to penetrate into the sample at 1 mm/s to an 8.4-mm depth, and then returned upward at the same speed to the initial position [9]. Hardness was determined as the area of the positive maximum peak or force (g) of the first compression in the sample. Cohesiveness was measured as the area of work during the second compression divided by the area of work during the first compression. Triplicate analyses were performed for each sample.

X-Ray Diffraction (XRD) Spectroscopy

The polymorphic forms of the fat crystals in the interesterified fats (IFs) were determined by an ARL Scintag XDS 2000 (Ecublens, Switzerland) automated diffractometer. The diffractometer was fitted with a scattering angle of 2θ configuration, solid state detector, and a cobalt X-ray tube. The X-ray beam was generated at 40 kV and 40 mA and its wavelength (λ) was 1.7889 nm. Analyzed data were collected from the 2θ range (18–32°). The scan rate was 2.0°/min. The melted samples were placed in rectangular plastic molds and tempered at 4 °C for 12 h after solidification at room temperature. The single spacing of 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 spacing of the β' form was strong at 3.8 and 4.2 Å [11].

Statistical Analysis

Experimental data are expressed as means ± standard deviations. Data were statistically analyzed using a General Linear Model Procedure of SAS Statistical Software (SAS version 8.2, Cary, NC) [12]. Duncan's multiple range test was applied to evaluate the significance of differences between mean at $P < 0.05$.

Results and Discussion

Triacylglycerol (TAG) Profile Changes

The compositional profile of the TAG molecules on each interesterified reactant (8:6:6 and 6:6:9, ABF/PS/FSO) was analyzed to monitor the degree of interesterification. Butterfat contains various different TAGs because of its various fatty acid compositions (i.e., butyric through gadoleic) [13]. In our previous work [9], PS was composed of 59.5 area% of ECN 48 (each 1.8% OOO, 11.4% POO, 24.1% POP, and 22.2% PPP) and 20.2% of ECN 46 (7.2% PLO and 13.0% PLP). Among those TAGs, POP and PPP which led to β polymorphic crystal form were abundantly present in PS [14]. FSO was comprised of two major TAG molecules with 27.7% LnLnLn (ECN 36) and 15.7% LLnLn (ECN 38) [9]. The relative proportion change of ECN on TAG composition of two blends (8:6:6 and 6:6:9, ABF/PS/FSO) during enzymatic interesterification was shown in Fig. 1. After a 1-h interesterification, a larger reduction (59%) of the ECN 36 peak in 8:6:6 interesterified reactant (catalyzed by Lipozyme RM IM) was observed than that (14%) of the ECN 36 peak in 6:6:9 interesterified reactant (catalyzed by Novozym SP435). After a 5-h interesterification, the ECN 36 (peak 1) and the ECN 38 (peak 2) in 8:6:6 reactant (Lipozyme RM IM) were greatly reduced around 87 and 70%, respectively, whereas the the ECN 36 and the the ECN 38 in 6:6:9 reactant (Novozym SP435) decreased about 70 and 40%, respectively, as shown in Fig. 2. These results indicated that Lipozyme RM IM required a shorter reaction time to reach the high interesterification degree than did Novozym SP435 as similarly reported by Rønne et al. [15]. Lipozyme RM IM has been broadly used in tailor-made fat synthesis with high transesterification activity while, Novozym SP435 is markedly useful in ester synthesis [16]. In this study, the decrease in the ECN 36 and ECN 38 peaks was observed by the increase of reaction time while the ECN 42-46 peaks gradually increased (Fig. 1), suggesting that diversified TAG species may be produced during interesterification. Such diversity in TAG species may facilitate β'-crystal formation.

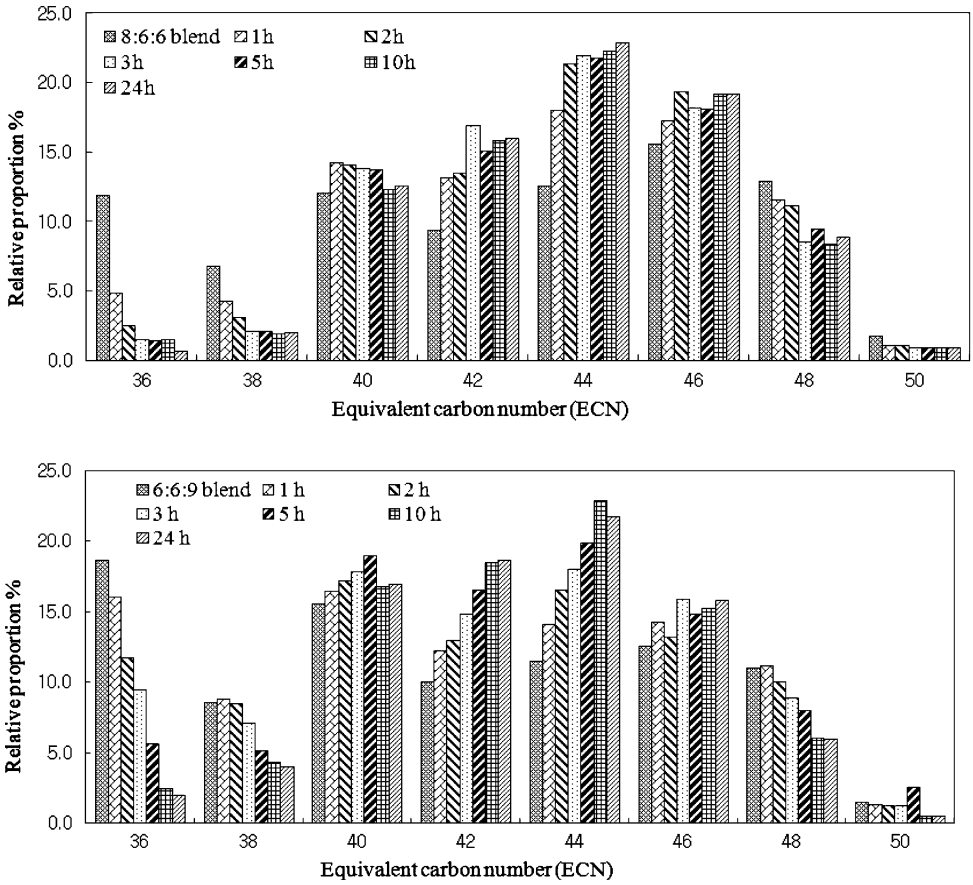

Fig. 1 Equivalent carbon number (*ECN*) change of triacylglycerol composition in the 8:6:6 and 6:6:9 interesterified reactants synthesized for different reaction times in a stirred-batch type reactor

Thermal Behaviors

Melting and crystallization behavior of fats and oils depends on the fatty acid composition in their TAG backbone [17]. For example, vegetable oil composed of various TAG species has broad endothermic peaks. Figure 3 illustrated the isothermal melting (endothermic) and crystallization (exothermic) profiles of 8:6:6 and 6:6:9 blends and interesterified reactants according to reaction time (1, 2, 3, 5, 10 and 24 h). Interesterified reactant with 8:6:6 showed six main melting peaks, namely a, b, c, d, e, and f (Fig. 3a). When the reaction time was increased in 8:6:6 reactant, maximum endothermic and exothermic temperatures of peaks, f and i, were gradually moved to low temperature (Fig. 3a, b).

Thermal characteristics (*Enthalpy* ΔH, *peak temperature* $T_{P,}$ *onset temperature* T_{on}) are presented in Table 1 in which the required enthalpy variation (ΔH, J/g) is the energy (heat, J) needed to melt 1 g of solid fats. Accordingly, the more the ΔH value increase, the more solid it contains. When interesterification time increased, the transition range, $T_{P,}$ and ΔH (J/g) decreased in melting and crystallization thermograms. After 24 h of interesterification, the peak i (T_P, 19.83 °C) in 8:6:6 blend (0 h reaction) of the crystallization thermogram shifted to lower T_P (17.41 °C).

Similarly, five endothermic peaks (j, k, l, m, and n) were mainly observed in 6:6:9 reactant (Fig. 3c). Among them, two small endothermic peaks (j and k) slightly shifted to a higher temperature and almost disappeared as interesterification was prolonged while the broad peak l of 6:6:9 blend (0 h reaction) was narrower and shifted toward a higher temperature as interesterification time increased. In addition, the transition range and ΔH between peaks m and n in 6:6:9 reactant were reduced while exothermic peaks, o and p, were slightly moved to a higher temperature as interesterification was prolonged (Fig. 3d). Also, the transition range, ΔH and T_p of main endothermic peak q gradually decreased (Table 1). Overall, reduced ΔH and transition range in interesterified reactant were observed after a 24-h interesterification.

Fatty Acid Composition and Iodine Value

After studying the thermal behaviors and TAG profile according to the reaction time (1–24 h), the interesterified reactants were refined by short path distillation to produce interesterified fats (8:6:6 and 6:6:9 IFs). The fatty acid composition and iodine value of reaction mixture (ABF, PS and FSO) and IFs are given in Table 2. In ABF, total saturated fatty acids of 74.2% and *trans* fatty acid of 3.6%

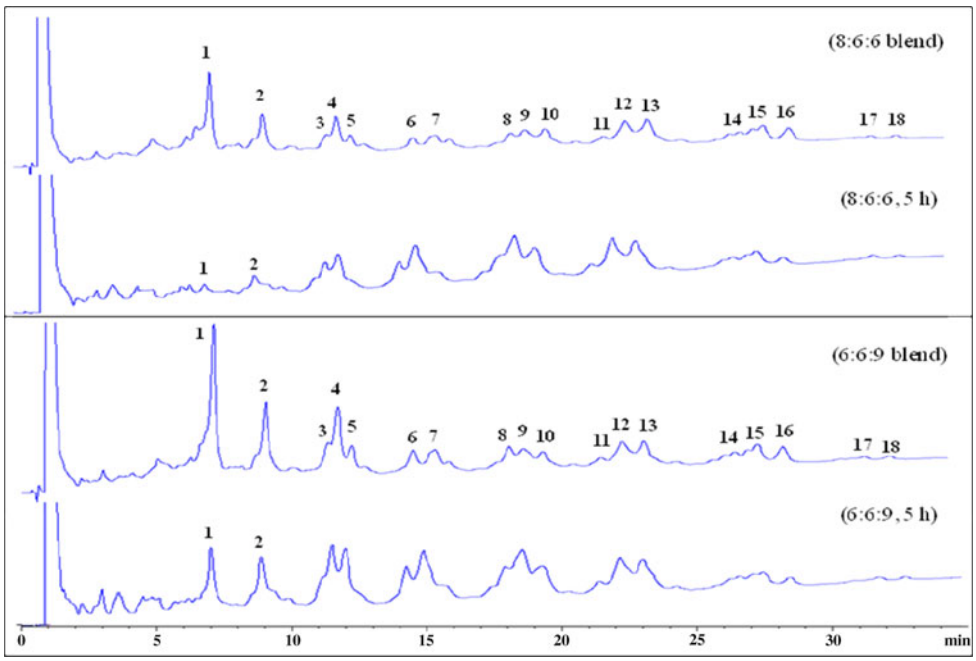

Fig. 2 HPLC-UV chromatograms of triacylglycerols in non-interesterified (*blend*) and interesterified reactants (8:6:6 and 6:6:9, ABF/PS/FSO by weight). Interesterification was conducted for 5 h in a stirred-batch reactor using Lipozyme RM IM (8:6:6 blend) and Novozym SP435 (6:6:9 blend) without short path distillation. Equivalent carbon number (ECN) of each TAG species as follows: Peak *1* (ECN 36); Peak *2* (ECN 38); Peak *3–5* (ECN 40); Peak *6–7* (ECN 42); Peak *8–10* (ECN 44); Peak *11–13* (ECN 46); Peak *14–16* (ECN 48); Peak *17–18* (ECN 50)

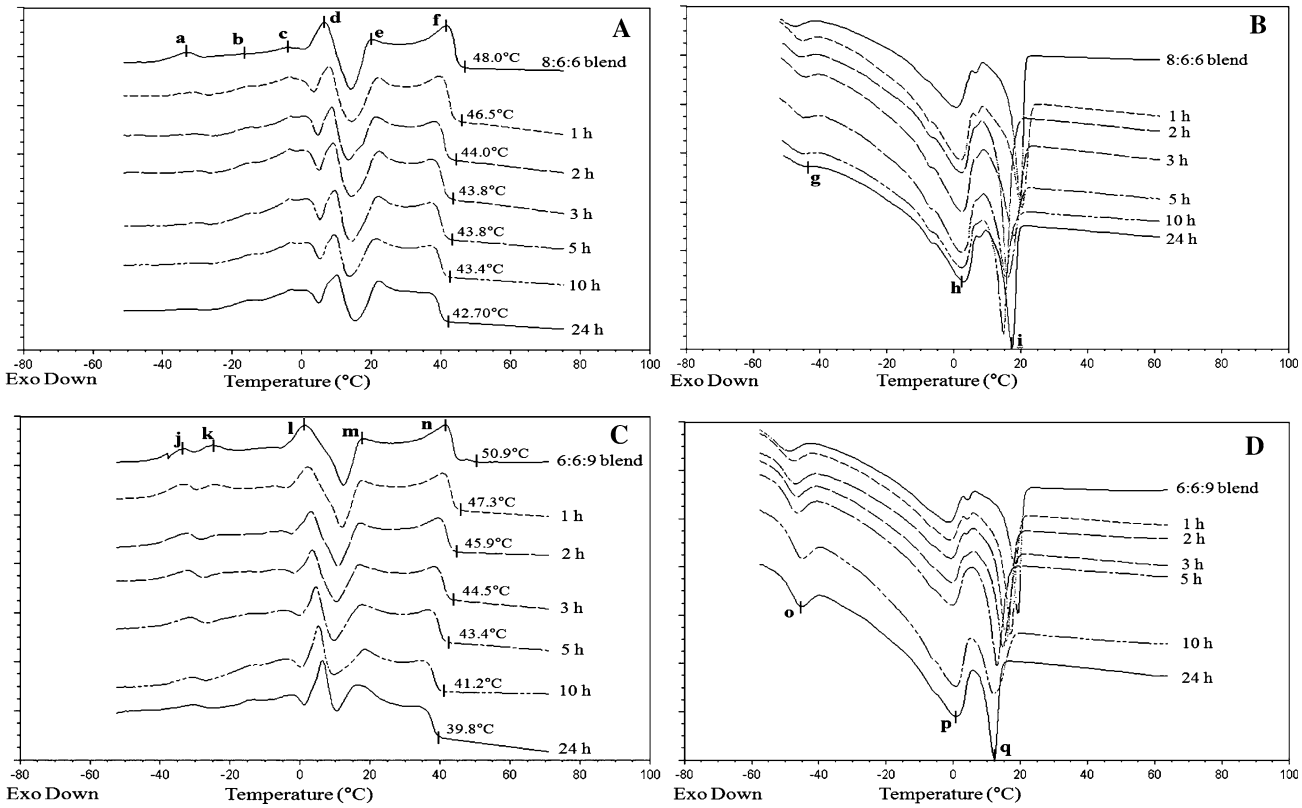

Fig. 3 Differential scanning calorimetry (*DSC*) thermograms showing isothermal melting (**a**, **c**) and crystallization (**b**, **d**) of 8:6:6 and 6:6:9 interesterified reactants according to reaction time. The 8:6:6 and 6:6:9 interesterified reactants (ABF/PS/FSO, by weight) were synthesized at 60 °C for 24 h in a 1-L batch type reactor

were found. Especially, 6.3% lauric, 14.7% myristic, and 31.6% palmitic acid were found among saturated fatty acids (SFAs). Palmitic (63.6%) and oleic acid (24.5%) were main compositional fatty acids in PS while α-linolenic (ALn, 52.7%) and oleic acid (21.3%) were major fatty acids in FSO. The highest iodine value (IV) which indicates the

Table 1 Enthalpy (ΔH) obtained from the melting and crystallization thermograms of interesterified reactants synthesized with Lipozyme RM IM and Novozym SP435

Samples	Reaction time (h)	Melting thermogram				Crystallization thermogram			
		ΔH (J/g)	T_P (°C)	T_{on} (°C)	Range[a] (°C)	ΔH (J/g)	T_P (°C)	T_{on} (°C)	Range (°C)
8:6:6blend	0	52.97	42.05	15.45	33.9	13.50	19.83	21.26	15.1
(ABF/PS/FSO)	1	40.26	39.71	16.19	32.4	12.23	20.22	23.15	16.9
	2	29.74	16.33	16.33	30.5	9.36	15.79	17.49	13.2
	3	35.65	22.27	16.49	29.2	10.72	17.99	21.38	14.1
	5	34.39	21.68	16.08	29.7	10.18	15.80	20.15	13.8
	10	35.37	21.12	15.40	29.7	10.29	14.70	15.56	12.3
	24	32.04	21.90	16.99	27.2	10.10	17.41	19.21	11.2
6:6:9blend	0	51.95	17.76	13.36	38.4	12.63	19.28	20.86	17.5
(ABF/PS/FSO)	1	50.28	17.70	13.11	35.1	13.50	17.10	19.68	16.7
	2	46.37	17.20	11.93	35.1	12.46	15.74	18.60	15.9
	3	41.37	17.12	11.46	34.2	11.61	14.76	18.53	15.0
	5	34.12	17.50	10.53	33.7	9.38	12.99	16.44	14.2
	10	21.14	18.30	11.41	31.2	6.59	12.50	17.50	14.7
	24	18.82	16.80	11.42	29.0	5.90	12.28	14.08	10.4

DSC data parameters: T_p transition peak temperature, T_{on} onset temperature, ΔH (J/g) enthalpy (heat content) calculated from the area under the transition peak of a specific temperature range

[a] Range: temperature difference between onset temperature and offset temperature

Table 2 Positional fatty acid composition (mol%) of substrates and enzymatically interesterified fats (8:6:6 and 6:6:9 IFs)

Fatty acids and others	Substrates			8:6:6				6:6:9			
	ABF	PS	FSO	Blend		IF[a]		Blend		IF	
	Total	Total	Total	Total	*sn*-2	Total	*sn*-2	Total	*sn*-2	Total	*sn*-2
C4:0	2.1	nd	nd	0.8	nd	1.0	nd	0.6	nd	1.0	nd
C6:0	1.7	nd	nd	0.7	nd	0.9	nd	0.5	nd	0.9	nd
C8:0	1.8	Trace	nd	0.7	0.5	0.7	nd	0.5	0.3	0.7	nd
C10:0	4.0	Trace	nd	1.6	1.5	1.3	nd	1.1	1.1	1.4	nd
C12:0	6.3	0.1	nd	2.6	2.6	2.0	1.0	1.8	1.9	1.9	1.0
C14:0	14.7	1.4	nd	6.3	6.3	4.8	4.5	4.6	4.6	4.7	4.5
C16:0	31.6	63.6	5.5	33.4	33.5	36.8	38.8	29.6	28.6	30.8	33.0
C16:1 ω7	1.6	0.1	0.1	0.7	0.8	0.6	0.6	0.5	0.6	0.6	0.7
C18:0	12.0	4.9	4.4	7.6	6.7	6.5	7.0	6.7	5.2	6.1	6.8
C18:1 *trans*	3.6	0.1	nd	1.5	1.3	1.0	1.1	1.1	1.0	1.0	1.2
C18:1 ω9	18.3	24.5	21.3	21.1	22.7	21.6	24.3	21.4	23.5	21.4	23.3
C18:2 ω6	1.1	5.2	16.0	6.8	8.5	7.2	7.3	8.7	11.0	7.9	8.0
C18:3 ω3	1.2	nd	52.7	16.3	15.6	15.7	15.5	22.9	22.2	21.7	21.6
ΣSFA	74.2	70.0	9.9	53.7	51.1	53.9	51.3	45.4	41.8	47.4	45.3
ΣPUFA	22.2	29.8	90.1	46.3	48.9	45.1	48.7	54.5	58.2	51.6	54.7
ΣTFA	3.6	0.1	nd	1.5	1.3	1.0	1.1	1.1	1.0	1.0	1.2
% FFA	0.30	0.10	1.13			0.1				0.3	
Iodine value (IV)	44.1	27.1	184.0			86.3				98.1	
Water content (%)	0.09	0.04	0.02								

nd not detected, *Trace* detection limit less than 0.1, *ΣSFA* The sum of saturated fatty acids, *ΣPUFA* the sum of unsaturated fatty acids, *ΣTFA* the sum of *trans* fatty acids

[a] Each IF was synthesized by Lipozyme RM IM (8:6:6, ABF/PS/FSO by weight) and Novozym SP435 (6:6:9) for 24 h in a stirred-batch type reactor and then refined by short path distillation

unsaturation degree in oils and fats was observed in FSO (IV = 184) due to its approximately 53% ALn content. In addition, water contents (%) of each substrate were 0.02% in FSO, 0.04% in PS, and 0.09% in ABF, respectively.

Fatty acid composition and its positional distribution in TAG species have an effect on the melting point and the physical properties of oils and fats [18]. Accordingly, positional fatty acid composition of the reaction mixtures and their IFs was determined as shown in Table 2. Compared to ABF, total saturated fatty acids in IFs were reduced approximately 27% in 8:6:6 IF and 36% in 6:6:8 IF, respectively, and total *trans* fatty acids in IFs were not more than 1.0%. In addition, ALn contents of 8:6:6 and 6:6:9 IFs were 15.7 and 21.7%, respectively, and similar ALn contents were found at the *sn*-2 positions of each IF. A high content of ALn at the *sn*-2 position is desirable, because not only the carbon chain length and the saturation of fatty acid but also the positional distribution on the TAG species is important to nutritional lipid metabolism. The IV of ABF was 44.1 owing to a high saturated fatty acid content (74.2%). On the other hand, the IV of each IF was 86.3 in 8:6:6 IF and 98.1 in 6:6:9 IF, respectively (Table 2). They showed higher IV values because of the high content of unsaturated fatty acids (45.1% in 8:6:6 IF, 51.6% in 6:6:9 IF), compared to ABF. Also, the free fatty acid (FFA) contents (%) were, respectively, 0.1% in 8:6:6 IF and 0.3% in 6:6:9 IF, resulting from effective short path distillation.

Atherogenicity and Thrombogenicity Index

Lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids are atherogenic and thrombogenic fatty acids which induce platelet aggregation in rats [19]. Therefore, those fatty acids are considered as main promoting factors of coronary heart disease (CHD). Also, the contents of such fatty acids are used to obtain the atherogenicity index (AI) and the thrombogenicity index (TI) [19]. In this study, the AI and TI of original ABF and each IF were calculated from hypercholesteromic fatty acids (C12:0, C14:0, and C16:0) according to Ulbricht and Southgate [19]. The AIs of substrates used were 4.36 in ABF, 2.33 in PS and 0.06 in FSO, respectively (Table 3). The TIs of ABF, PS and FSO were 3.46, 4.37 and 0.03, respectively. FSO showed the lowest AI and TI because it contains approximately 53% ALn, omega-3 fatty acid to protect against CHD. In this result, The AI of each IF (1.29 in 8:6:6 IF and 1.00 in 6:6:9 IF) was much lower than that of ABF (4.35). Particularly, the TI values of IFs were lower (0.68 in 8:6:6 IF and 0.45 in 6:6:9 IF) than that of ABF (3.46) (Table 3). These effects are because of a blending with FSO, not exactly enzymatic interesterification. These results suggest that IF in substitution for butterfat may reduce the hypercholesteromic incidence.

Table 3 Atherogenicity and thrombogenicity index of substrates and enzymatically interesterified fats (8:6:6 and 6:6:9 IFs)

	Atherogenicity index (AI)[b]	Thrombogenicity index (TI)[c]
Substrates		
ABF	4.36	3.46
PS	2.33	4.37
FSO	0.06	0.03
IFs[a]		
8:6:6	1.29	0.68
6:6:9	1.00	0.45

[a] Each IF was synthesized by Lipozyme RM IM (8:6:6, ABF/PS/FSO by weight) and Novozym SP435 (6:6:9) for 24 h in a stirred-batch type reactor and then refined by short path distillation

[b] Atherogenicity index (AI) was calculated as follows [19]: AI = [(C12:0 mol%) + 4 × (C14:0 mol%) + (C16:0 mol%)]/(ΣPUFA mol%)

[c] Thrombogenicity index (TI) was calculated as follows [19]: TI = [(C12:0 mol%) + (C14:0 mol%) + (C16:0 mol%)]/[(0.5 × ΣMUFA mol%) + (0.5 × $\Sigma PUFA_{n-6}$ mol%) + (3 × $\Sigma PUFA_{n-3}$ mol%) + (n-3/n-6)]

ΣUSFA: The sum of unsaturated fatty acids

ΣMUFA: The sum of monounsaturated fatty acids

$\Sigma PUFA_{n-6}$: The sum of n-6 unsaturated fatty acids

$\Sigma PUFA_{n-3}$: The sum of n-3 unsaturated fatty acids

n-3/n-6: The ratio of n-3/n-6 fatty acids

Solid Fat Content, Texture, and Melting Point

Solid fat content (SFC), texture (hardness, cohesiveness and adhesiveness), melting point for predicting the physical properties of original ABF and each IF (8:6:6 and 6:6:9) were determined in Table 4. SFC in fats and oils means the solid-to-liquid ratio at the various temperatures and SFC at the body temperature can predict the texture and plasticity in the final products such as spreadable butterfat and margarine. Compared to SFC (34%) of ABF at 10 °C, relatively low SFCs were observed in 6:6:9 IF (19%) and 8:6:6 IF (27%). When the temperature increased to 30 °C, the SFCs of each IF were 6.9% in 8:6:6 IF and 3.4% in 6:6:9 IF, respectively. At 40 °C, the SFCs of 2.7% (8:6:6 IF) and 1.3% (6:6:9 IF) were observed. These results suggest that each IF can be used for preparing the desirable spreadable fat at refrigeration temperature. Similarly to the results of these SFCs, the melting points of 8:6:6 IF, 6:6:9 IF and ABF were 37, 33 and 34 °C, respectively. Such melting points of IFs were similar to the results from Rousseau and Marangoni [20], in which butterfat-canola oil (80:20) spread was produced by the interesterification reaction.

In TA-XT2 texture analysis, the hardness values of 6:6:9 IF and 8:6:6 IF were 217 and 800 g/cm^2, respectively, suggesting that a satisfactory plastic and spreadable textures of these IFs at 4 °C could be expected while ABF

Table 4 Solid fat content (SFC), texture and melting point of anhydrous butterfat (ABF) and interesterified fats (IFs)

	ABF	8:6:6 IF[A]	6:6:9 IF
SFC (%) at °C			
10	33.94 ± 0.46	26.90 ± 0.58	19.40 ± 0.12
20	12.72 ± 0.46	15.12 ± 1.45	7.83 ± 0.57
30	4.84 ± 0.58	6.91 ± 0.05	3.46 ± 3.46
40	1.32 ± 0.68	2.77 ± 0.77	1.32 ± 0.88
50	nd	0.07 ± 0.10	nd
Textures			
Hardness (g/cm^2)	1768 ± 149^a	800 ± 37^b	217 ± 14^c
Cohesiveness	0.17 ± 0.02^a	0.12 ± 0.004^b	0.11 ± 0.002^c
Adhesiveness (g/s)	1052 ± 116^a	585 ± 52^b	354 ± 44^c
Melting point (°C)	34	37	33

nd not detected

Mean ± SD ($n = 3$ samples)

[a–c] Values in the same row with different superscripts are significantly different ($P < 0.05$)

[A] Interesterified fats (IFs) were synthesized with two weight ratios (8:6:6 and 6:6:9) of ABF, palm stearin (PS) and flaxseed oil (FSO) by Lipozyme RM IM (8:6:6 IF) and Novozym SP435 (6:6:9 IF) for 24 h in a batch type reactor and then refined by short path distillation

showed 1768 g/cm^2 [21]. This phenomenon was due to the high SFA content (74.2%) in ABF whereas a relatively low SFA content (47.4 and 53.9%) was found in each IF. Also, cohesiveness and adhesiveness of IFs were significantly lower than those of the original ABF at 4 °C.

Tocopherol and Sterol Contents

Four tocopherols (α-, β-, γ-, and δ-tocopherol) generally exist in vegetable oils whereas tocotrienols (α-, β-, γ-, and δ-tocotrienol) are abundantly present in cereals (barley and rice bran oil) and palm oil [22]. These tocopherols (natural antioxidants) will enhance the stability of the unsaturated fatty acid against oxidation in oils and fats. The change of tocopherol content in blends, interesterified reactant (before short path distillation), and IF (after short path distillation) is summarized in Table 5. After the interesterification reaction, the total tocopherol content was not significantly different. However, the total tocopherol content was significantly reduced after short path distillation ($P < 0.05$), resulting in 119.2 in 8:6:6 IF and 109.7 μg/g in 6:6:9 IF, respectively (Table 5).

In general, cholesterol is present in animal fats and phytosterol (plant steroid) mainly exists in plant oils. In Table 5, cholesterol and phytosterol contents (β-sitosterol, campesterol and stigmasterol) in blends, interesterified reactant (before short path distillation) and IF (after short path distillation) are compared. According to Fatouh et al. [23], the cholesterol content was 280 mg/100 g in butter oil whereas ABF used in this study contained 109.44 mg/100 g of cholesterol and this content was slightly decreased after interesterification reaction without any significant difference ($P < 0.05$). Similar to tocopherol content, however, the 33–50% cholesterol level significantly

Table 5 The change of phytochemicals of the blend (non-interesterified), the interesterified reactant (before short path distillation), and the interesterified fat (IF, after short path distillation)

Phytochemicals	8:6:6			6:6:9		
	Blend[A]	Interesterified reactant[B]	IF[C]	Blend	Interesterified reactant	IF
Tocopherols (μg/g)						
Alpha	21.7 ± 1.0^a	19.3 ± 1.2^a	16.4 ± 3.5^a	16.7 ± 1.5^a	15.4 ± 1.1^a	10.9 ± 1.2^b
Beta	57.5 ± 0.8^a	53.3 ± 2.1^a	46.9 ± 6.0^a	70.4 ± 2.7^a	67.4 ± 2.1^a	47.6 ± 4.3^b
Gamma	72.7 ± 1.3^a	70.3 ± 1.0^a	55.9 ± 5.5^b	84.1 ± 3.9^a	78.5 ± 1.0^a	51.2 ± 6.9^b
Sum	151.8 ± 1.2^a	142.9 ± 4.4^{a,b}	119.2 ± 15.1^b	171.2 ± 8.2^a	161.4 ± 2.3^a	109.7 ± 12.4^b
Cholesterol (mg/100 g)	56.3 ± 7.9^a	48.6 ± 8.6^{a,b}	24.4 ± 6.3^b	33.5 ± 4.2^a	32.6 ± 1.5^a	21.7 ± 1.9^b
Phytosterols (mg/100 g)						
β-Sitosterol	21.1 ± 3.1^a	18.6 ± 3.3^a	13.4 ± 2.8^a	26.1 ± 7.2^a	23.8 ± 0.2^a	18.3 ± 1.8^a
Campesterol	11.5 ± 0.3^a	9.6 ± 1.5^a	6.1 ± 0.1^a	14.4 ± 0.4^a	12.1 ± 1.2^a	10.4 ± 1.8^a
Stigmasterol	0.14 ± 0.00^a	0.13 ± 0.01^a	0.10 ± 0.02^b	0.21 ± 0.07^a	0.18 ± 0.00^a	0.14 ± 0.02^a
Sum	32.8 ± 3.4^a	28.4 ± 4.7^a	21.0 ± 4.6^a	40.7 ± 7.7^a	38.2 ± 1.7^a	28.9 ± 3.6^a

Values are means ± SD ($n = 2$ samples)

The interesterified fat (IFs) were synthesized for 24 h using Lipozyme RM IM (8:6:6, ABF/PS/FSO by weight) and Novozym SP435 (6:6:9) and refined by short path distillation

[a, b] Values with different letters in the same row of each substrate ratio are significantly different ($P < 0.05$)

[A] Blend: non-interesterified fats of ABF/PS/FSO

[B] Interesterified reactant before short path distillation

[C] IF: interesterified fat after short path distillation

decreased after short path distillation, showing 24.4 mg/100 g in 8:6:6 IF and 21.7 mg/100 g in 6:6:9 IF, respectively. In addition, the total phytosterol contents of 8:6:6 IF and 6:6:9 IF after short path distillation were 21.0 and 28.9 mg/100 g, respectively. It is known that phytosterol disturbs cholesterol absorption in the intestine, and such a nutritional effect makes it a natural cholesterol lowering agent [24].

Polymorphic Form by X-Ray Diffraction (XRD)

The main three polymorphs of fat crystals are α, β', and β forms. Each polymorph has different characteristics as follows; First, the α form is very unstable and has the lowest melting point among the three types of polymorph. Its short spacing is observed at 4.15 Å. Second, the β' form is metastable and has an intermediate melting point. Its two strong short spacings are observed at 3.80 and 4.20 Å. Third, the β form is very stable and has the highest melting point. Its short spacing is observed at 4.60 Å [11]. The desirable polymorphic form of margarine and shortening is the β' form, which exhibits small crystals because large β crystals increase the hardness and decrease the spreadability [25]. The XRD spectra of the polymorphic form in ABF, blends (8:6:6 and 6:6:9) and their IFs was presented in Fig. 4. ABF showed strong two short spacings at 4.2327 Å and at 3.8253 Å. Therefore, its polymorph seems β' form as reported by Ibrahim et al. [14]. Short spacing of non-interesterified blends (8:6:6 blend and 6:6:9 blend) showed spacings at 4.2, 3.8, and 4.6 Å (Fig. 4). After

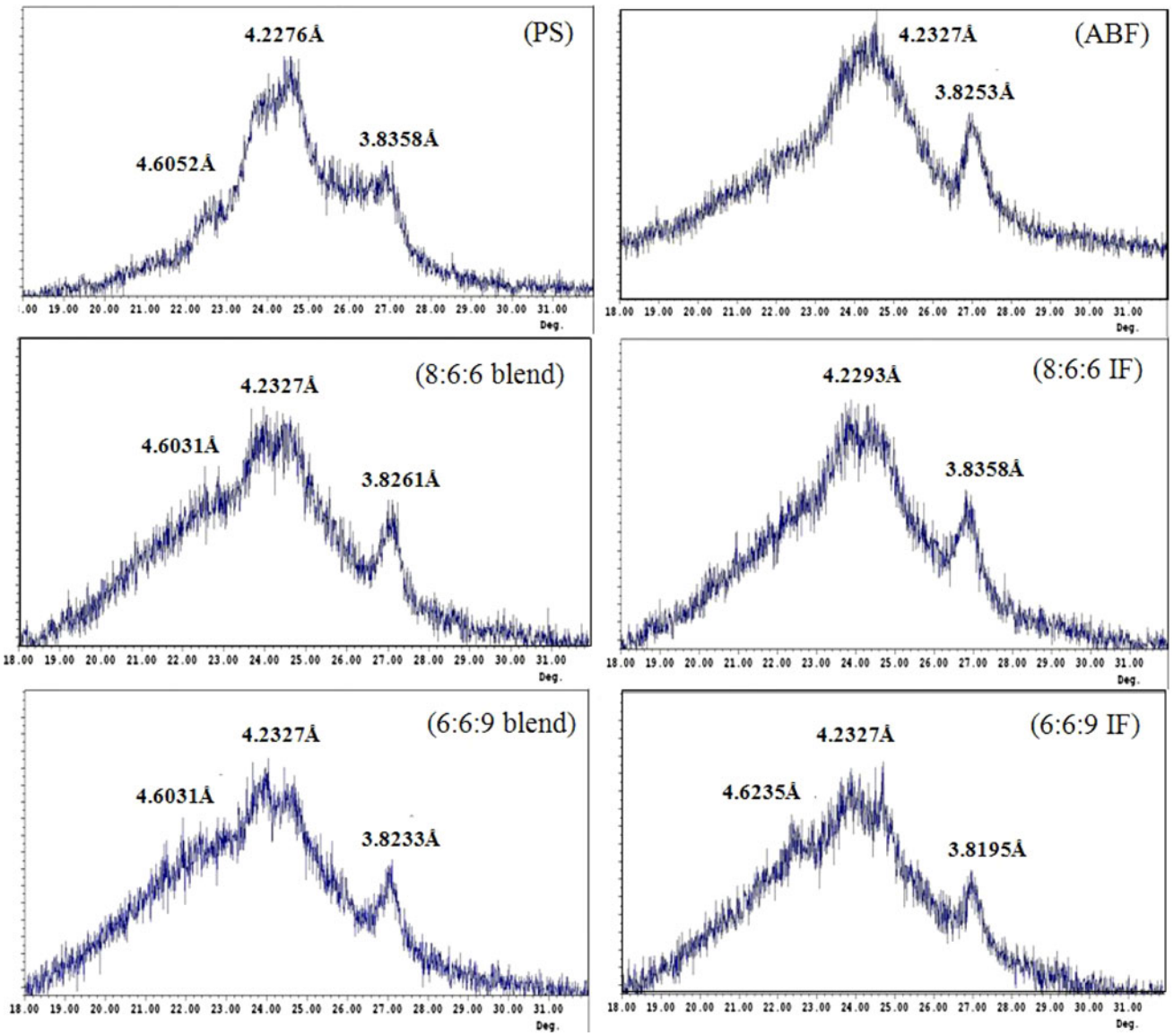

Fig. 4 XRD spectroscopy of palm stearin (*PS*), anhydrous butterfat (*ABF*), 8:6:6 and 6:6:9 blends and their interesterified fats (*IFs*)

interesterification, the short spacing at 4.6 Å disappeared or weakened while short spacing at 4.2 Å became distinctive. This result was similar to that of the polymorphic form (β') of enzymatically transesterified fats of palm stearin and anhydrous milk fat synthesized by Lai et al. [26]. Therefore, the polymorphic form changed from the β form to the β' form after interesterification.

In conclusion, interesterified fats (IFs) containing ALn at around 16–22% were successfully synthesized via a lipase-catalyzed interesterification reaction in a 1-L stirred batch reactor. After interesterification, the thermal properties of the IFs were altered with a decreased transition range, ΔH and T_p. Their spreadability and polymorphic form were improved toward softness and the desirable β' crystal form as margarine stock. These functional hard fats having good properties can be applied as spreadable margarines or butterfat substitutes containing ALn.

Acknowledgments This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries, Republic of Korea.

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