Enzymatic Modification of High-Laurate Canola To Produce Margarine Fat

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Stearic acid was enzymatically transesterified with high-laurate canola using a nonspecific lipase from *Candida antarctica* to produce structured lipids (SL) suitable for margarine application. Stearic acid levels ranged from 10 to 40 wt % of high-laurate canola oil. Differential scanning calorimetry was used to evaluate melting characteristics of the transesterified products. A stearic acid level of 30% was found to best match the melting characteristics of fat extracted from commercially available stick margarine. This SL was used to prepare nonrefrigerated and refrigerated margarine samples. Refrigerated margarine was prepared using 60% SL and 40% canola oil, whereas 100% SL was used for the nonrefrigerated margarine. Slip melting point, solid fat content, and hardness index were determined for all samples. Application of a dynamic temperature step using a dynamic stress rheometer showed complete breakdown of the commercial stick margarine and the experimental refrigerated margarine at ∼30 °C and complete breakdown of the nonrefrigerated margarine at ∼35 °C. Addition of canola oil to the SL improved spreadability at refrigeration temperatures and reduced the hardening effect of lauric acid in the SL. The nonrefrigerated margarine was spreadable at room temperature and exhibited no oil exudation or phase separation.

Keywords: *Acidolysis; differential scanning calorimetry; high-laurate canola oil; margarine; rheology; solid fat content; structured lipid*

INTRODUCTION

Margarine is a water-in-oil emulsion and by U.S. FDA standards of identity must contain at least 80% fat. The aqueous phase consists of water, salt, and preservatives. The fat phase consists of a blend of liquid and solid glycerides, antioxidants, and emulsifiers. In the United States, the solid fat content of margarine is traditionally increased by hydrogenation of liquid oils. Partial hydrogenation isomerizes the double bonds in fats from the cis to the trans configuration (*1*). Several studies with conflicting results have been reported on the health effects of trans fatty acids; although these studies are still controversial, trans fatty acids are widely believed to be implicated in coronary heart disease (*2*−*4*). A trans-free alternative to the use of partial hydrogenation is the use of fully hydrogenated oil interesterified with an unsaturated oil (*1*). Interesterification of highly saturated fats with oils of low saturated fatty acids content is another method used to manufacture zero-trans margarine (*5*). Fats such as palm stearin and lauric oils have been used to produce zero-trans margarine (*6*−*8*). In the early years of wrapped margarine, it was common for lauric oils to be used in the fat blends at levels of up to 40% and higher (*8*). Margarine produced with these oils was harder than the soft margarines, now commonly produced, and was more similar to butter in that respect (*8*). Fluctuating market prices and limited supply in part led to the decreased use of lauric oils in margarine. With the advent of laurate canola, economic and supply concerns may be satisfied. High-laurate canola is a genetically engineered oil that was developed in part to provide a domestic source of oils currently being satisfied by various palm kernel oil fractions. To date, most of the food applications of laurate canola have concentrated on the hydrogenated form of the transgenic oil (*9*), which introduces the controversial trans fatty acids problem. Acidolysis of laurate canola oil with stearic acid will increase its solid fat content without introducing trans fatty acids.

The objective of this research was to synthesize structured lipids (SL) from high-laurate canola oil and stearic acid using a lipase, Novozym 435 from *Candida antarctica*, as the biocatalyst. The resulting SL was then used to prepare trans-free margarine. Physical properties such as slip melting point, hardness index, solid fat content, and rheological properties of the experimental margarine were studied.

MATERIALS AND METHODS

Materials. Refined, bleached, and deodorized (RBD) Laurical canola oil was obtained from Protein Oil Starch Pilot Plant Corp. (Saskatoon, Canada). Ninety-five percent pure stearic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). Immobilized lipase, Novozym 435 from *C. antarctica*, was purchased from Novo Nordisk Biochem North America, Inc. (Franklinton, NC). Lecithin was donated by Riceland Foods, Inc. (Stuttgart, AR). Tenox tertiary butylhydroquinone (TBHQ) was purchased from Eastman Chemical Co. (Kingsport, TN). Organic solvents were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

Acidolysis Reaction. SL synthesis was performed in 125-mL Erlenmeyer flasks in an orbital shaking water bath at 200

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rpm and 55 °C for 24 h. The reaction mixture typically contained 15 g of high-laurate canola oil, 10−80% stearic acid by weight of high-laurate canola oil, and 10% Novozym 435 by total weight of substrates. After the reaction, free fatty acids were removed using the alkaline deacidification method described by Lee and Akoh (*10*).

Differential Scanning Calorimetry (DSC). The melting profile of purified products was determined by DSC on a Perkin-Elmer model DSC7 (Norwalk, CT). The melting profiles of purified products were compared to that of fat extracted from a commercially available stick margarine. Analysis was performed according to the AOCS recommended procedure Cj 1-94 (*11*). Normal standardization was performed with gallium (mp, 29.78 °C) as reference standard. Liquid nitrogen (−196 °C) was used as the coolant.

Large-Scale Synthesis. The acidolysis reaction was carried out in a 1-L capacity stirred tank batch reactor. The substrate consisted of 30% stearic acid by weight of high-laurate canola oil and hexane at 20% of total weight of substrates. The amount of lipase was maintained at 10% (w/w) of total substrates. Temperature was maintained at 55 °C by a circulating water bath, and agitation speed was 200 rpm. The reaction was terminated after 24 h by filtration of the lipase.

Short-Path Distillation. Short-path distillation was carried out with a KDL-4 (UIC Inc., Joliet, IL) unit under the following conditions: heating oil temperature, 185 °C; cooling water temperature, 20 °C; pump vacuum, <1 mmHg; feed rate, maintained at 100 mL/h. The reaction product was passed through the system twice to reduce the free fatty acid percentage to an acceptable level. Free fatty acid (FFA) content was determined according to AOCS Official Method Ca 5a-40 (*12*). Percent free fatty acids was expressed in terms of lauric acid.

Margarine Formulation and Production. Experimental margarine samples were prepared using the following formula: fat, 80.2%; water, 17.5%; salt, 2%; lecithin, 0.3%; TBHQ, 0.008%. An ice-cream maker was used to crystallize the margarine samples. The ice-cream maker featured a double insulated bowl with a liquid refrigerant located between the walls. The bowl was refrigerated overnight to allow for fat crystallization. Lecithin and TBHQ were dissolved in the heated oil phase, and salt was dissolved in the water phase. Both phases were mixed with the automatic mixer until the emulsion crystallized. A commercially available stick margarine was melted down and recrystallized using the same procedure; this sample then served as a control for physical tests performed on the experimental margarine samples.

Slip Melting Point (SMP). SMP was determined using the capillary tube method described in AOCS Method Cc. 3.25 (*13*).

Rheological Analysis. Viscoelastic properties of margarine samples were analyzed using an SR5000 dynamic stress rheometer (Rheometrics Scientific, Piscataway, NJ). Parallel plate geometry was used with a diameter of 40 mm and a gap of 0.5 mm. Temperature control was carried out using a peltier element. Small-amplitude oscillatory experiments were carried out within the linear viscoelastic region of each sample. A stress sweep ranging from 10 to 3000 Pa was used to determine the linear viscoelastic region for each sample. Storage modulus (G') and loss modulus (G'') were measured by an on-line computer for all samples. The temperature was set at 15 °C for refrigerated margarine samples and at 25 °C for nonrefrigerated margarine samples. Oscillatory measurements as a function of temperature were also performed. The experiments were carried out by heating the samples from 5 to 60 °C in steps of 5 °C. A stress of 100Pa, a frequency of 1 Hz, and a gap of 0.5 mm were maintained. At each step the sample was quickly heated to the desired temperature and allowed to equilibrate at that temperature before readings were obtained.

X-ray Diffraction Spectroscopy. Powder X-ray diffraction was used to study the polymorphic forms of fat in the nonrefrigerated and refrigerated margarine samples. An ARL Scintag XDS 2000 (Ecublens, Switzerland) automated diffractometer was used to collect the data. The diffractometer had a 2θ configuration, a solid-state detector, and a cobalt tube as the X-ray source. Generation power for all sample runs was set at 40 kV and 40 mA. The 2θ range used was from 18 to 32°, and the scan rate was 2.0°/min. Samples were melted and poured into rectangular aluminum molds on cold glass plates. They were then allowed to solidify at room temperature and then kept at refrigeration temperatures for 12 h. Short spacings of the major polymorphs are as follows: α, a single spacing at 4.15 Å; β', two strong spacings at 3.8 and 4.2 Å; and β, a very strong spacing at 4.6 and another one usually at 3.85 Å (*14*).

Texture Analysis. Samples were placed in aluminum containers 60 × 12 mm (diameter × depth) and tempered at 4 °C overnight; the texture was evaluated on an Instron Universal Testing Machine (model 5500-C6631, Canton, MA). A flat-ended cylindrical plunger, 60 mm in diameter, was attached to a 50 kg compression load cell fitted in the crosshead of the Instron. Crosshead speed was set at 20 mm/min. The plunger was set to penetrate the sample at a distance of 5 mm. Force deformation curves were obtained during plunger penetration and withdrawal. Refrigerated margarine samples were promptly analyzed on removal from refrigeration temperatures. Instrumental texture profile analysis as defined by Bourne (*15*) was used to analyze the margarine samples. Hardness and cohesiveness were evaluated. Triplicate readings for each sample were obtained.

Fatty Acid Positional Analysis. Positional analysis of the SL and laurate canola was done by ^{13}C NMR spectroscopy. All NMR data were acquired at 20 °C on a Bruker AMX400 spectrometer (400.13 MHz, ^{1}H; 100.62 MHz, ^{13}C). The fat samples were dissolved in deuterated chloroform ($CDCl_3$). ^{13}C chemical shifts were referenced to TMS, via the $CDCl_3$ resonance frequency at 77.0 ppm. Proton decoupling was used during acquisition. The data were recorded with a spectral window at 20 kHz, delay time of 8 s, and 32K data points. The data were zero-filled to 256K data points and line-broadened with 0.1 Hz period to Fourier transformation. A deconvolution program (Bruker xwin-nmr v1.3) was used to calculate the peak areas of overlapped peaks with an assumption of Lorenztian line shape. Integrals of peaks in the spectra were used to determine the fatty acid composition of the samples. The 1,3-acyl and 2-acyl distribution was defined as described by Wollenberg (*16*).

Solid Fat Content (SFC). The SFC was determined using AOCS Official Method Cd 16-81 (*17*). NMR measurements were made using a MARAN-20 pulsed NMR spectrometer (Resonance Instruments Ltd., Oxon, U.K.). The SFC was measured within temperatures ranging from 10 to 45 °C.

Statistics. The Statistical Analysis Systems was used to analyze data (*18*). Significance was determined at $p < 0.05$.

RESULTS AND DISCUSSION

Stearic acid was incorporated into high-laurate canola at various ratios to obtain a product with melting characteristics similar to those of control stick margarine. Figure 1 shows the trend of incorporation of stearic acid for the small-scale transesterification. After incubation with 30% stearic acid, incorporation of stearic acid into high-laurate canola did not increase significantly ($p < 0.05$). Although incorporation increased as percent stearic acid increased, we were interested in making sure that melting profiles as evaluated by DSC were similar to the profile for stick margarine. The melting thermograms for transesterified laurate canola and stearic acid are given in Figure 2. As expected, when stearic acid incorporation increased, peaks shifted from lower melting ranges to higher melting ranges. Peaks also became wider, probably due to an increase in diversity of triacylglycerol (TAG) species and a wider melting range. Thirty percent stearic acid (C) was found to best match the melting characteristics of fat extracted from commercially available stick margarine (H). This

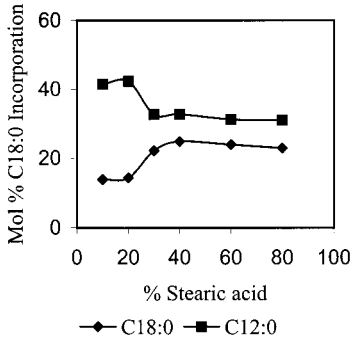

Figure 1. Mole percent incorporation of stearic acid into high-laurate canola during small-scale synthesis. The reaction mixture was incubated at 55 °C for 24 h, and percent stearic acid by weight of high-laurate canola varied from 10 to 80%. Novozym 435 was maintained at 10 wt % of reactants.

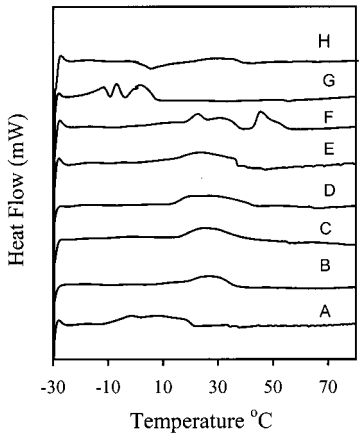

Figure 2. Melting profile of high-laurate canola and SL products from small-scale reaction formed by varying the percentage of stearic acid by weight of high-laurate canola in the substrate mixture: (A) 10%; (B) 20%; (C) 30%; (D) 40%; (E) 60%; (F) 80%; (G) high-laurate canola; (H) commercial margarine. All percentages are of stearic acid by weight of high-laurate canola. Novozym 435 was maintained at 10 wt % of reactants in all reactions.

Table 1. Fatty Acid (Mole Percent) Profile of High-Laurate Canola before and after Stearic Acid Incorporation[a]

fatty acid	before modification	after C18:0 incorporation[b]
12:0	46.99 ± 1.97	32.74 ± 2.93
14:0	3.58 ± 0.53	3.31 ± 0.37
16:0	2.57 ± 0.17	2.27 ± 0.28
18:0	1.25 ± 1.76	22.15 ± 1.26
18:1*n*−9	26.34 ± 0.39	23.39 ± 1.05
18:2*n*−6	14.19 ± 2.37	12.46 ± 0.96
18:3*n*−3	5.08 ± 0.27	3.68 ± 0.32
sum of saturated fatty acids	54.39	60.47
sum of unsaturated fatty acids	45.61	39.53

[a] Means and standard errors of two replicates. [b] Structured lipid prepared by large-scale transesterification.

percentage was then used to produce a scaled-up version of the SL. The SL produced in large scale was purified using short-path distillation, and the percent free fatty acid was reduced to 0.3%. Table 1 shows the fatty acid profile of the purified SL. Stearic acid incorporation appeared to have occurred mostly at the expense of lauric acid.

^{13}C NMR has been used to study the fatty acid distribution of several vegetable oils (*16*, *19*). The carbonyl region can be used to determine the saturated

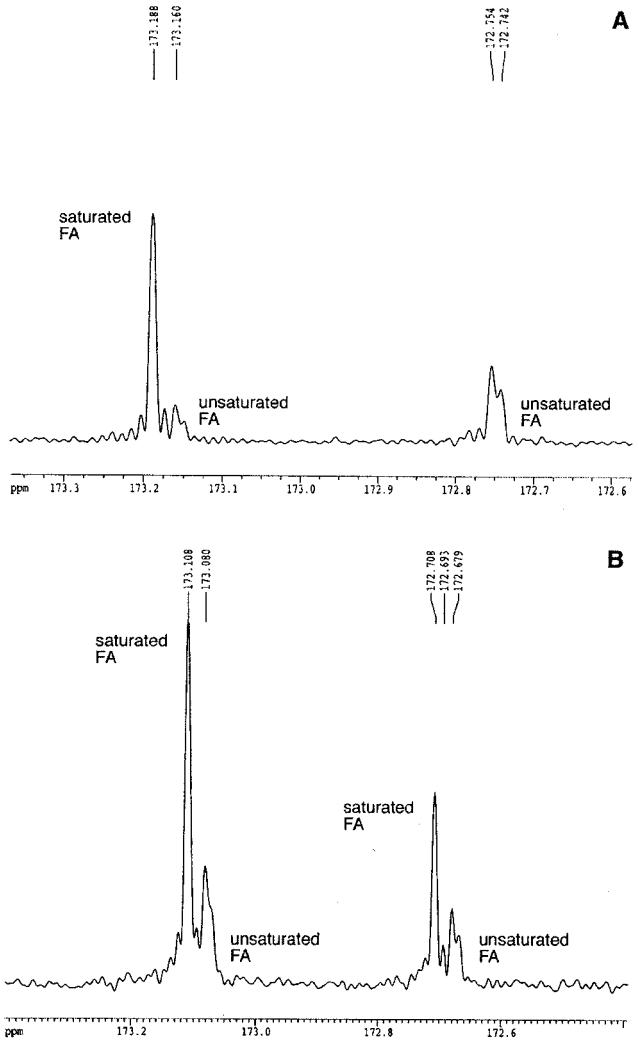

Figure 3. High-resolution ^{13}C NMR spectra of the carbonyl regions of high-laurate canola (A) and structured lipid (B). FA signifies fatty acid. Chemical shifts for saturated FA at the *sn*-1,3 positions are at 173.19 and 173.11 ppm for unmodified laurate canola and SL, respectively. Chemical shifts for unsaturated FA at the *sn*-1,3 positions are at 173.16 and 173.08 ppm for unmodified laurate canola and SL, respectively. At the *sn*-2 position, the chemical shift for saturated FA is at 172.71 ppm for SL. Chemical shifts for unsaturated FA at the *sn*-2 position are at 172.75 and 172.74 ppm for unmodified laurate canola and at 172.69 and 172.68 ppm for SL. Structured lipid was from a large-scale transesterification.

and unsaturated fatty acid contents of the *sn*-1,3 and *sn*-2 positions. Figure 3 shows ^{13}C NMR results for the fatty acid distribution of unmodified laurate canola and SL from large-scale preparation. Chemical shifts for the saturated fatty acid residues at the *sn*-1,3 positions were found at 173.19 and 173.11 ppm for unmodified laurate canola (Figure 3A) and SL (Figure 3B), respectively. At the *sn*-2 position, the chemical shift for saturated fatty acids was at 172.71 ppm for SL. Calgene reported that high-laurate canola should inherently have no saturated fatty acids at the *sn*-2 position (*9*). Results obtained for unmodified laurate canola are consistent with Calgene's claims. The saturated fatty acid content at the *sn*-2 position changed from zero in unmodified laurate canola to 60.81% in SL (Table 2). This increase in saturation at the *sn*-2 position is evidence of the randomization effect of Novozym 435 and the higher amount of stearic acid in the SL. ^{13}C NMR results compare well with total

Table 2. Distribution of Saturated and Unsaturated Fatty Acids in High-Laurate Canola and Structured Lipid by ^{13}C NMR of the Carbonyl Carbons[a,b]

	fatty acid type	*sn*-1,3 positions	*sn*-2 position	total[c,d]
high-laurate canola	saturated	83.5	N/A[e]	55.65
	unsaturated	16.5	100	44.35
structured lipid	saturated	67.48	60.81	65.26
	unsaturated	32.52	39.19	34.74

[a] In mole percent. Structured lipid was from large-scale transesterification. [b] Integrals of peaks in the spectra (Figure 3) give the fatty acid composition of the samples. [c] Total saturated or unsaturated fatty acid in triacylglycerol molecule. [d] Total = (*sn*-1,3 fatty acid × 0.667) + (*sn*-2 fatty acid × 0.333). [e] N/A = not detected.

Table 3. Textural Properties of Experimental Margarine Samples Compared with Properties of Commercially Available Margarine[a]

margarine product	peak force (N)	cohesiveness (no units)
control at 4 °C	2.87 ± 0.51^{a}	0.37 ± 0.11^{a}
60:40 blend at 4 °C	$2.12 \pm 0.36^{a,b}$	0.39 ± 0.18^{a}
100% SL at 4 °C	8.01 ± 1.43^{c}	0.13 ± 0.12^{b}
100% SL at 25 °C	1.04 ± 0.05^{b}	0.36 ± 0.02^{a}

[a] Control, store-bought stick margarine; 60:40 blend, 60:40 (SL/canola oil) margarine; SL, structured lipid. SL was from large-scale transesterification. Means and standard errors of three replicates. Values with the same letter in each column are not significantly different at $p < 0.05$ as determined by Fisher's least significant difference.

fatty acid distribution obtained by gas chromatography shown in Table 1.

Instrumental hardness was used to predict the spreadability of margarine. Hardness has been found to positively correlate with the spreadability of peanut butter (*20*). From Table 3, it can be seen that when margarine made with 100% SL (from large-scale preparation) was stored at refrigeration temperatures, it was considerably harder than the margarine bought from the store as indicated by peak force. This is consistent with the posthardening effect observed in lauric oil margarines under refrigeration conditions (*8*, *21*). One hundred percent SL margarine was softer and spreadable when stored at room temperature. Because margarine made from 100% SL was not spreadable at refrigeration temperatures, we reduced the amount of SL used in the fat blend to 60% to make a soft margarine. Canola oil was used to make up the remaining 40% fat required. Similar recommendations have been made for palm kernel oil incorporation into margarine (*8*). The 60:40 (SL/canola oil) blend showed no significant difference in hardness from the control margarine ($p < 0.05$). Cohesiveness of the margarine samples was also investigated. Brittle foods exhibit very little cohesiveness because there is very little contact with the sample during the second bite, and therefore there is a negligible second area. Cohesiveness in popular terms refers to how brittle, crumbly, or crunchy a food is. One hundred percent SL margarine was brittle and not spreadable after refrigeration, leading to smaller cohesiveness values. The 60:40 blend, control margarine, and 100% SL margarine stored at 25 °C all had similar cohesive values as can be seen in Table 3. We propose that cohesiveness may be used as an indicator of spreadability of margarine, because the more brittle the margarine the less cohesive it is. This would of course be better confirmed with sensory testing.

Addition of canola oil to the SL from large scale synthesis reduced the SMP by ~1 °C, which led to a 4% drop in SFC at equivalent temperatures. SMP was 32.2 °C for the 60:40 blend, 33.3 °C for the SL, and 34.8 °C for the control margarine. Although the SMP value for the SL was lower than that of the store-bought margarine, it was not spreadable at refrigeration temperatures. This may be further evidence of the post-hardening effect in the 100% SL margarine. SMP usually occurs at lower temperatures than the melting point because at their SMP fats still have ~5% solid fat (*22*). The SFC determines the spreadability and mouthfeel of margarine. For margarine to be spreadable at refrigeration temperatures the SFC should not be >32% at 10 °C (*23*). The SFC of the 100% SL and the 60:40 blend was measured at temperatures ranging from 10 to 40 °C and is depicted in Figure 4. Both the 60:40 blend and the 100% SL met the criterion for spreadability at refrigeration temperatures. This supports the assumption that the hardening observed under refrigeration conditions was due to posthardening and not due to the SFC. To eliminate waxy mouthfeel, margarine should have <3.5% solids at temperatures >33 °C or should melt at temperatures below body temperature (*24*). Both experimental margarine samples had SFC values of <3.5%, which should effectively eliminate the waxy mouthfeel. Another important property in margarine is coolness; this is the coldness felt on the tongue when fat crystals melting at nearly the same temperature absorb heat during their dispersion in the mouth (*25*). This aspect is greatly influenced by the difference between the SFC at 15 and that at 25 °C, and the higher the difference the better the cooling effect (*25*). Lauric oils characteristically have sharp melting points, which imparts the pleasant cooling sensation on the tongue (*8*). In this case, both samples had ~12% difference in their SFC at 15 and 25 °C. Nor Aini et al. (*26*) found that a minimum SFC of 7.6% is necessary to maintain a crystal structure. In the SL, the SFC at 30 °C (7.8%) was still sufficient to maintain the crystal structure, whereas that of the 60:40 blend was insufficient. At 25 °C, the 60:40 blend had less than the minimum SFC. This suggests that it may not be stable at room temperature (25 °C).

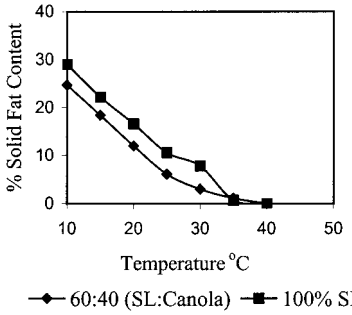

Figure 4. Solid fat contents of structured lipid (SL) and 60:40 (SL/canola oil) blend from large-scale preparation.

Most TAG are polymorphic; that is, they occur in more than one crystal form. The polymorphic form in which TAG exists affects a number of properties such as melting point, stability, and, in the case of margarine, graininess. Chain length diversity appears to play a role in the crystal habit of fats (*21*). Oils such as sunflower oil that consist of a series of closely related homologues are more likely to crystallize in the beta form (β). Lauric oils such as coconut and palm kernel oil usually crystallize in the β' form. Laurate canola has also been shown to crystallize in the β' (*9*). Adding stearic acid to laurate

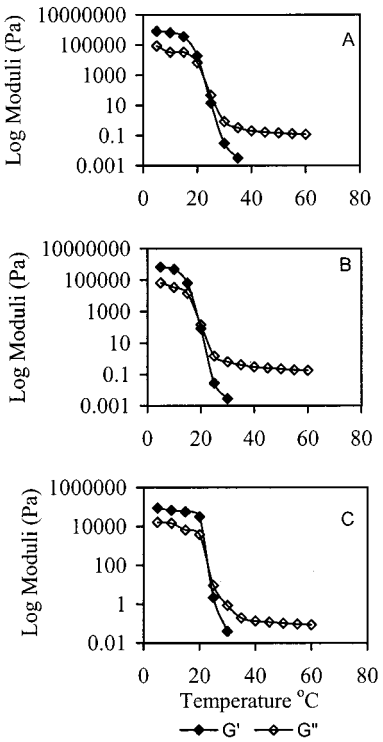

Figure 5. Rheological data, G' and G'' versus temperature: (A) 100% SL; (B) 60:40 (SL/canola oil); (C) commercially available margarine samples. SL was prepared by large-scale transesterification.

canola increases the diversity of TAG species. Laurate canola already contains stearic acid, but the amount is low (1.25%). TAG diversity is also expected to increase because of the randomizing effect of Novozym 435. X-ray diffraction results showed peaks for both the 100% SL and the 60:40 samples at 3.8 and 4.2 Å, both representative of the β' crystal form. Peaks from the 60:40 fat were less intense than those from the 100% SL sample, probably because the sample softened considerably during the X-ray analysis. This indicates that margarine made with either 100% SL or the 60:40 blend would have the desirable crystal size without the undesirable graininess.

The temperature course of structural melting in dynamic oscillation can be used to predict the perceived meltability of spreads (*27*, *28*). Viscoelastic materials have both an elastic component and a viscous component. Storage modulus (G') can be used as a measure of elastic or solid behavior, and loss modulus (G'') values can be used to evaluate viscous or fluid behavior. Storage and loss moduli were measured as functions of temperature. Initially, G' values for the control margarine were lower than those for the 60:40 blend and 100% SL margarine (Figure 5). After 25 °C, the control and 100% SL margarines had similar G' values. This is an indication that at room temperature, the 100% SL margarine and the control margarine may have similar elastic characteristics. After 20 °C, G'' was higher than G' for the 60:40 blend and for the control margarine, whereas for the 100% SL margarine, G'' was greater than G' after 25 °C. Higher G'' values indicate more liquid behavior than solid behavior. Because flow behavior is affected by SFC, we expect SFC to relate to G' values. Storage modulus values were plotted against SFC at equivalent temperatures. Storage modulus values decreased exponentially with decreases in SFC

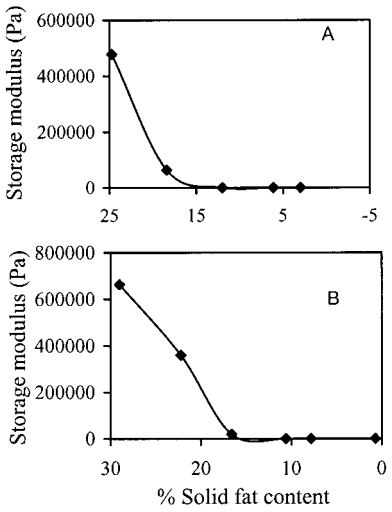

Figure 6. Relationship between storage modulus (G') values and percent solid fat content at equivalent temperatures for (A) 60:40 (SL/canola oil) and (B) 100% SL. The SL was from a large-scale transesterification.

(Figure 6). This was more so for the 60:40 margarine than for the 100% SL margarine. Regression coefficients, r^2, were 0.91 and 0.96 for the 100% SL and the 60:40 blend, respectively, indicating a goodness of fit. The 60:40 margarine melted more quickly than the 100% SL as shown by SFC data, and this may be the reason for its higher r^2 values. Previous studies show that complex moduli also decrease exponentially with an increase in liquid fat content (*29*).

Adding canola oil improved spreadability, reduced SMP, SFC, and the hardness index for the 60:40 margarine, and reduced the posthardening effect observed in the 100% SL margarine.

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