Tailoring the Textural Attributes of Butter Fat/Canola Oil Blends via *Rhizopus arrhizus* Lipase-Catalyzed Interesterification. 1. Compositional Modifications

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Butter fat/canola oil blends ranging from 100% butter fat (w/w) to 60:40% butter fat/canola oil were enzymatically interesterified with an *sn*-1,3 specific lipase from *Rhizopus arrhizus* in a liquid/solid two-phase system. Interesterification progress was monitored by following the changes in the relative proportions of 50-carbon triacylglycerols (TAGs) to 38-carbon TAGs (50/38 ratio) as a function of reaction duration. Most of the changes in TAGs occurred within the first 24 h of reaction. As the proportion of oil in the blend increased, greater changes in TAG composition became apparent. The interesterification reaction was optimal at 0.35% water content (w/w). The initial rate of hydrolysis was linearly dependent on initial water content within the range 0−0.55% water (w/w). Free fatty acids released ranged from 3 to 4.5% after 96 h of interesterification. The predominant free fatty acids were oleic acid (18:1) and palmitic acid (16:0). The lipase displayed little activity toward butyric acid (4:0) and caproic acid (6:0) residues. Monoacylglycerol levels ranged from 1.4 to 2.4% and diacylglycerol levels from 1.5 to 2.0% in all blends.

Keywords: *Rhizopus arrhizus; butter fat; canola; interesterification; lipase-catalyzed; oil*

INTRODUCTION

Lipid interesterification can be performed with either enzymatic or chemical catalysts. Chemical interesterification has been commercially viable since the 1940s to improve the spreadability and baking properties of lard (Weiss et al., 1961; Wiedermann et al., 1961) and is in use today for the production of *trans*-free margarines (List et al., 1977). However, during the chemical modification of butter fat, the flavor profile is adversely affected (Weihe, 1961). Lipase catalysis has certain advantages over chemical catalysis such as milder reaction conditions and regiospecificity (Macrae, 1985), which may be less harmful to butter flavor.

Unlike chemical interesterification, many more factors must be taken into consideration when enzymatic interesterification is performed. These factors include the choice of lipase (e.g., for specificity considerations), method of immobilization and support material, environmental conditions (temperature, pH, and water content, etc.), and bioreactor characteristics. Of key importance is the role of water content. In a water-rich environment, hydrolysis predominates, whereas at low water content, ester formation predominates (Yamane, 1987). Interesterification requires a balance of these two reactions to be effective.

Previous investigators have shown that lipases can substantially modify the composition of butter fat with lipase-catalyzed interesterification, which has been studied in a variety of systems and for different purposes. Christensen and Hølmer (1993) synthesized a human milk fat substitute for infant formulas by interesterification of butter fat with eicosapentanoic acid (EPA), docosahexanoic acid (DHA), oleic acid and linoleic acid, catalyzed by immobilized *Mucor miehei* lipase on anion-exchange resin. Oba and Witholt (1994) enzymatically interesterified butter fat and oleic acid in isooctane with immobilized *Rhizopus oryzae* lipase on controlled-pore glass to improve the nutritional properties of butter. Reyes et al. (1994) reported that lipase from *Pseudomonas cepacia*, a nonselective lipase, immobilized onto hydrophobic polypropylene powder was effective in catalyzing interesterification between butter fat and octanoic acid. Safari and Kermasha (1994) and Pabai et al. (1995) interesterified butter fat in microemulsions to evaluate fatty acid specificity of commercial lipases. Kalo and co-workers have predominantly used Celite, a hydrophilic support, as a lipase carrier for butter fat interesterification and have studied the physical and chemical properties of the modified butter fat (Kalo, 1988; Kalo et al., 1986a,b, 1987a,b, 1989a,b, 1990).

Lipase from *Rhizopus arrhizus* (RAL) has been used in a variety of systems. Bell et al. (1981) used RAL in organic solvent for continuous hydrolysis of olive oil. Wisdom et al. (1987) performed laboratory- and pilot-scale studies with RAL to interesterify shea olein and shea oil. During this study, RAL was chosen due to its lack of activity toward short-chain fatty acids, such as butyric and caproic acid (Sémériva and Dufour, 1972).

The impetus behind this study is the production of a cold-spreadable butter fat via blending with a vegetable oil and enzymatic interesterification. The primary objective is to evaluate the tailoring potential of lipase as a butter texture modifier. In the first of this two-

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part study, the constitutional modifications induced in triacylglycerols (TAG), diacylglycerols (DAG), monacylglycerols (MAG), and free fatty acids (FFA) will be discussed. The physical, crystal, and rheological properties will be discussed in the following paper.

EXPERIMENTAL PROCEDURES

Source Material. Butter fat was obtained from a local creamery, stored at −20 °C until needed, and vacuum-dried in a rotary evaporator to remove residual moisture. Canola oil was purchased from a local grocery store and used without further treatment. RAL (*sn*-1,3 specific) was purchased from Sigma-Aldrich (Mississauga, ON). Lipase activity was standardized to 4×10^6 units/mL (1 unit will hydrolyze 1.0 μequiv of fatty acid from a TAG in 30 min at pH 7.7 and 37 °C). The support used, Accurel EP100 polypropylene powder, was a gift from Akzo Chemicals (Onernburg, Germany). Unless otherwise noted, all chemicals were obtained from Fisher Scientific (Toronto, ON).

Blend Preparation. Liquefied butter fat (∼60 °C) and canola oil were mixed in proportions ranging from 100% butter fat to 60:40% butter fat/canola oil in 10% increments (w/w). Non-interesterified is abbreviated NIE and enzymatic interesterification, EIE. Blends are identified as BF (butter fat) and 90:10 to 60:40 [90:10% to 60:40% (w/w) butter fat/canola oil blend].

Lipase Immobilization. Polypropylene powder (1 g) was prewet with 2−3 volumes of absolute (100%) ethanol (Commercial Alcohols, Toronto, ON) for 2 h with stirring and then washed with 2 volumes of ethanol and filtered under reduced pressure with a Millipore filter apparatus (filter pore size = 8 μm). The powder was then mixed with constant stirring with a 25 mL solution containing 2.3 mg of lipase (4.3×10^5 units/mg) until immobilization was complete. The reaction was performed at room temperature. Changes in free protein concentration were determined using the Coomassie Blue binding protein assay (Bio-Rad Laboratories, Mississauga, ON) (Bradford, 1976) using bovine serum albumin as the standard in the calibration curve. Following immobilization, the support was washed with 0.05 M, pH 7.0, Tris buffer (Sigma-Aldrich Canada) to remove loosely bound enzyme and then freeze-dried overnight (18 h).

Optimization of Reaction Conditions. Reactions were carried out in water-jacketed vials using a circulating water bath. Mixtures (10 g of 80:20 blend) were prepared according to parameter levels dictated by a four-factor response surface design (RSD) with the following parameters: pH (6−8), temperature (40−60 °C), initial water content (0.1−1.0%), and support load (0.5−2.5%). Reactions were performed in the absence of any added solvent. Following analysis, the effects of water content were further examined in the range (0−0.55%) to optimize the reaction conditions. In this case, the substrate was butter fat.

Interesterification Scaleup. The interesterification reaction for all blends was carried out in 50 g batches in a dry bath with constant stirring at the optimized reaction conditions. At prescribed intervals (0−96 h), samples were withdrawn from the reaction mixture, filtered to separate the immobilized enzyme from the product, and immediately stored (−18 °C) until analysis.

Support Stability. Stability was evaluated by measuring the evolution of the 50/38 ratio (see below). Fifty gram batches of the 80:20 blend were enzymatically interesterified for 24 h. Prior to reuse, the loaded support was washed in hexane between each run and dried to remove traces of solvent and freeze-dried overnight to remove traces of moisture.

TAG Profile. The TAG composition (carbon number) of blends was determined by gas−liquid chromatography (GLC) using a Shimadzu GC-8A (Tokyo, Japan). From a 1% (v/v) solution of liquefied sample in isooctane, 3 μL aliquots were injected into the GLC at 350 °C. Runs were performed from 270 to 340 °C at 5 °C/min and then isothermally for 10 min at 340 °C. A 60 cm glass column was packed with 3% OV-1 on 80/100 mesh Supelcoport (Supelco Canada, Mississauga, ON) with high-purity nitrogen as the carrier gas (BOC Gases, Guelph, ON). The flame ionization detector was set at 350 °C. Flow rates were as follows: nitrogen, 60 mL/min; hydrogen, 50 mL/min; and air, 500 mL/min. Response factors, determined with tricaprin, tripalmitin, and tristearin, were evaluated at 0.95−1.05 for all species.

The ratio of the relative proportions of 50-carbon species to 38-carbon species (50/38) was used to measure the evolution of TAG species. A similar ratio (38/50) has been used to follow chemical interesterification of butter fat, during which the 50-carbon species increase the most, whereas the 38-carbon species decrease the most. Non-interesterified butter fat has a 50/38 ratio of ∼0.9, whereas fully randomized butter fat has a theoretical ratio of ∼2.3 (Huyghebaert et al., 1994).

Free Fatty Acids (FFA) Profile. The AOCS official method Ca 5a-40 was used for determination of total FFA. FFAs were expressed as percent oleic acid (w/w). During the scaled up reactions, FFA profiles were determined using the methylation method of Williams and Macgee (1983). A gas−liquid chromatograph (Shimadzu GC-8A, Tokyo, Japan) with a 2 m glass column packed with Silar 9CP on Chromosorb W (80/100 mesh) (Chromatographic Specialties, Brockville, ON) was used. Runs were performed from 80 to 190 °C at 8 °C/min and then isothermally for 14 min at 180 °C. High-purity nitrogen was the carrier gas (BOC Gases), and the flame ionization detector and injector were set at 240 °C. Flow rates were as follows: nitrogen, 15 mL/min; hydrogen, 50 mL/min; and air, 500 mL/min. External fatty acid standards used for peak identification and response factors included AOCS No. 5 oil reference standard (Sigma-Aldrich Canada), methyl butyrate (Eastman Kodak Co., Rochester, NY), and methyl caproate (K&K Laboratories, Plainview, NY). Margaric acid (17:0) was used as internal standard. The proportion of each free fatty acid (percent weight) was calculated according to the following equation

$$(\%\ \text{wt FFA})_A = \frac{RF_A[\text{wt (mg)}_{IS}](\text{peak area})_A}{[\text{wt (mg)}_A](\text{peak area})_{IS}}$$

where A = sample A, FFA = free fatty acids, RF = response factor, and IS = internal standard.

Fatty Acid Composition. The total fatty acid profile of the non-interesterified blends was determined using the method of Bannon et al. (1984). The same instrumental setup as for the FFA analysis was used, except that runs were performed from 80 to 210 °C at 6 °C/min and then isothermally at 210 °C for 9 min. Detector and injector temperatures were set at 230 °C.

Analysis of MAG and DAG. Identification and concentration of MAG and DAG were evaluated via GLC−FID using the method of Goh and Timms (1985). With butter fat, however, DAGs eluted at the same time as small TAGs. For DAG separation, thin-layer chromatography (TLC) plates (Whatman 150 Å silica gel plates, Fisher Scientific, Toronto, ON) were used following the method of Becker et al. (1993). DAGs were expressed as diolein and MAGs as monoolein.

Sample Measurements and Statistical Analysis. Triplicate analyses were performed and the averages reported for all compositional analyses. The statistical analysis was performed using the SAS General Linear Methods (GLM) procedure (SAS Institute, 1994). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Immobilization. The lipase used in this study was immobilized via adsorption. For the lipase adsorption

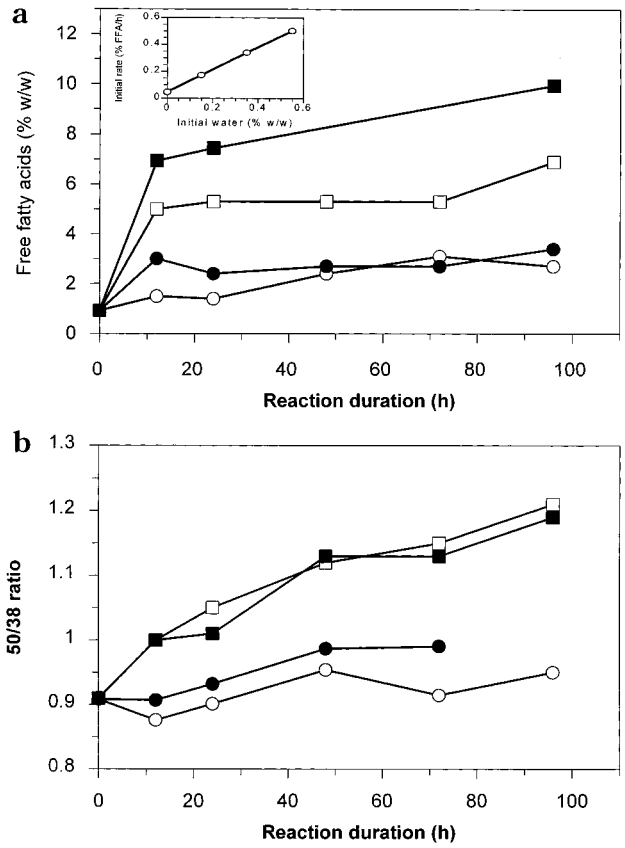

Figure 1. (a) Progress curve of FFA release in butter fat as a function of water content and reaction duration (inset shows initial rate of FFA formation); (b) progress curve of the 50/38 ratio in butter fat as a function of water content and reaction duration: 0% (w/w) water (○); 0.15% (w/w) water (●); 0.35% (w/w) water (□); 0.55% (w/w) water (■).

process, the support and lipase solution (0.092 mg/mL) were placed in a beaker with stirring to promote contact. Within 2 h, the amount of protein in solution had dropped by over 90%, presumably adsorbed onto the polypropylene support. This support was chosen because hydrophobic supports have been found to enhance interesterification activity over hydrophilic supports (Brady et al., 1988; Ruckenstein et al., 1993; Reyes et al., 1994).

Reaction Condition Optimization. In this study, a four-factor second-order response surface design with temperature, pH, water content, and enzyme loading as parameters was carried out to estimate optimal reaction conditions. Water content and enzyme loading both had significant effects ($P < 0.05$) on hydrolysis activity, whereas pH and temperature did not ($P > 0.05$). No parameter had any significant effect on the evolution of the 50/38 ratio ($P > 0.05$). On the basis of this information, the pH was fixed at 7, the temperature at 50 °C, and the enzyme at 2% (w/w). Further analysis of the effect of water content on the enzymatic interesterification was undertaken.

Water content was optimized to catalyze interesterification while minimizing FFA release. Figure 1a shows the effects of initial water content (0−0.55% w/w) at four different water concentrations on fatty acid release as a function of interesterification duration. The lipase was inactive in anhydrous systems. Gradual addition of water led to a positive linear response in the initial rate of FFA production [Figure 1a (inset)]. Others have shown similar patterns. Kalo (1988) and Kalo et al.

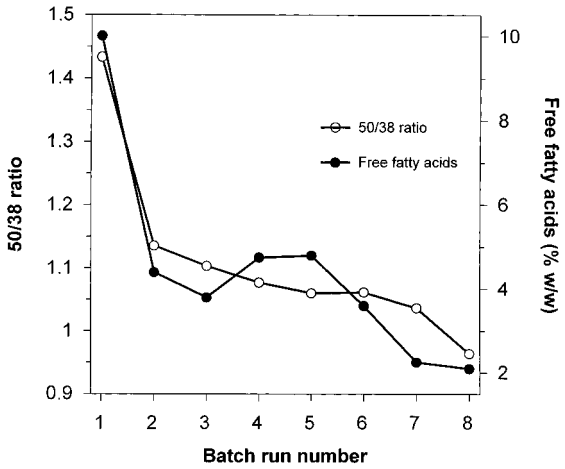

Figure 2. Evolution of the 50/38 ratio and total FFA (measured by titration) as indicators of the immobilized enzyme support efficiency: 50/38 ratio (○); % FFA (●). Batch runs were performed for 24 h with 0.35% added water.

(1989b) performed butter fat interesterification for 6 days with immobilized *sn*-1,3 specific lipases (*Aspergillus niger* and *Mucor miehei*) at 0.375% water content and obtained similar proportions of FFAs (~3.3−4.5%) as in this study.

The effect of water content on the relative rate of interesterification was gauged by following the evolution of the 50/38 carbon species ratio as a function of water content and interesterification duration (Figure 1b). As with the FFA results, little difference existed between the 50/38 ratios of butter fat containing either 0 or 0.15% (w/w) water, both 50/38 ratios being at ~0.95. Addition of 0.35% (w/w) water led to a large increase in the interesterification rate. At an initial water content of 0.55% (w/w), the transesterification rate was similar to that of 0.35% (w/w) water. The optimum value obtained in this study is very similar to others in the literature. For example, Oba and Witholt (1994) found that maximal interesterification of milk fat and oleic acid in isooctane was obtained at 0.3% (w/w) water in the system.

Support Stability Study. For evaluation of immobilized lipase stability, the immobilized lipase (2% w/w) was repeatedly used to interesterify samples (50 g) of an 80:20 butter fat/canola oil blend for 24 h intervals until the immobilized catalyst showed little or no activity. Results of the effectiveness of immobilization are shown in Figure 2. The evolution of the 50/38 ratio and FFA production were used to evaluate efficiency. The reaction was performed eight times before the catalyst was spent. Likewise, for the FFA evolution, there was a sharp drop from batch 1 to batch 2. Following the eighth use of the immobilized catalyst, there was little FFA production. These results indicated that immobilization was not as effective as in other studies with batch reactors (Mojovič et al., 1993; Svensson et al., 1992). Total run time of the present system was ~168 h before substantial inactivation of the support. Between each 24 h reaction, the support was washed with hexane to remove substrate and dried to remove residual hexane. Analyses indicated that enzyme leaching was the cause of the reduced interesterification activity as weakly bound lipases were washed away into the reaction media or by the hexane. Although it is a simple method, immobilization via adsorption is not as efficient as other modes of im-

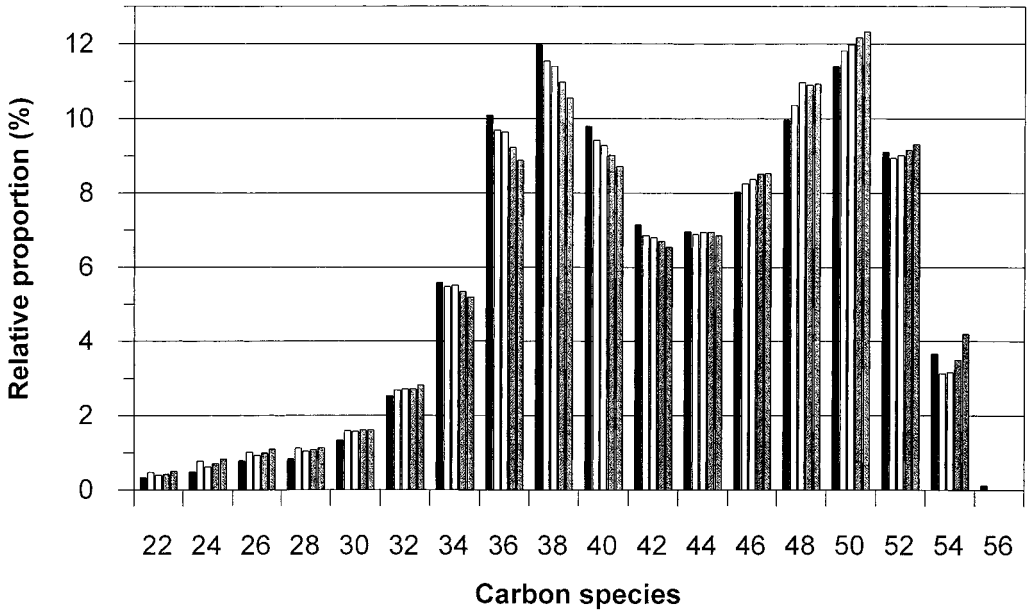

Figure 3. Relative proportion of carbon species in butter fat as a function of interesterification duration. Bars in each grouping represent, from left to right, NIE and 6, 12, 24, and 96 h.

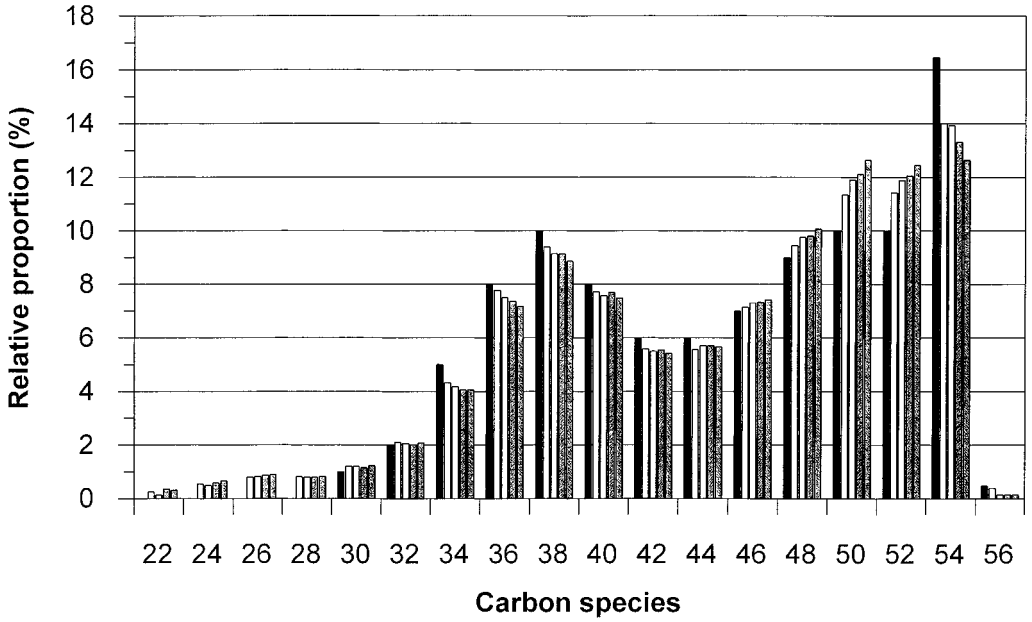

Figure 4. Relative proportion of carbon species in the 80% butter fat/20% canola oil blend as a function of interesterification duration. Bars in each grouping represent, from left to right, NIE and 6, 12, 24, and 96 h except the four groupings at left, which show no NIE.

mobilization, such as covalent attachment or entrapment, due to leaching problems.

TAG Evolution. Butter fat TAGs can be broken down into two main families (Figure 3)—carbon species 34−42 and 46−54. As interesterification progressed, TAG carbon species 34−42 and 54 decreased while species 46, 48, 50, and 52 increased. Carbon species 44 was the midpoint and did not change noticeably. Most of the TAG changes occurred within the first 24 h. The trends in carbon species modifications resulting from EIE mimicked those of chemically interesterified butter fat, although with smaller relative changes (Rousseau et al., 1996; Kalo et al., 1986a).

The previously mentioned carbon species behaved similarly regardless of the proportion of butter fat replaced by oil. As the proportion of oil in the blend increased, greater changes in TAG composition, as exemplified by the changes in the 80:20 blend (Figure 4), became apparent. Generally, a sharper drop in the 54-carbon species led to a larger increase in 48−52-carbon species. The large drop in 54-carbon species in the blends is attributed to the loss of TAGs containing unsaturated 18-carbon fatty acids in canola oil, which represent the majority of its fatty acid makeup. As shown by Kalo et al. (1986b), following interesterification of a blend of butter fat solid fraction and rapeseed oil, there was a drop in trisaturates. This is due to the shuffling of unsaturated fatty acids found in the oil, which take the place of saturated fatty acids on butter fat TAGs. The relative changes in TAG species are shown in Figure 5.

FFAs and Partial Acylglycerols. The level of FFAs in butter fat reached a maximum at 6 h of interesterification (~4.5%) and then stabilized at ~3% (after 96

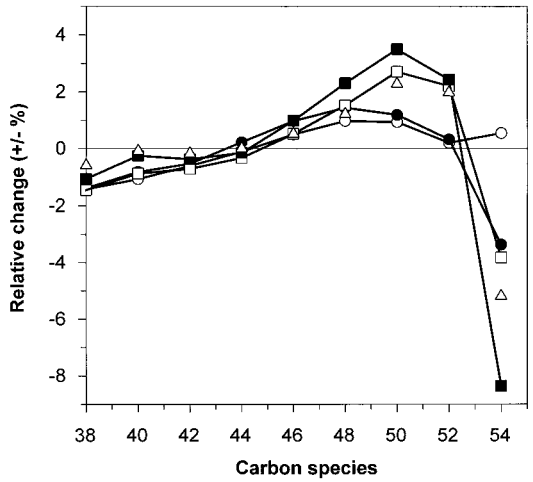

Figure 5. Relative changes in the proportion of carbon species in all blends following 96 h of enzymatic interesterification: BF (○); 90:10 (●); 80:20 (□); 70:30 (■); 60:40 (△).

h), whereas FFAs reached ~4.5% after 12 h of EIE and remained constant for all blends. For each blend, within 3 h of interesterification, the majority of FFAs had been released and consisted of 14:0, 16:0, 18:0, and 18:1, which correspond to the main fatty acids found in the blends (Figure 6). The proportion of the main fatty acids released was dependent on the initial proportion of butter fat and canola oil. As the amount of oil, primarily composed of unsaturated fatty acids, increased in the blend, so did the amounts of unsaturated FFAs released.

Comparison of the relative proportion of component fatty acids in the blends compared with the FFAs released during enzymatic interesterification can indicate whether RAL displayed any kind of fatty acid specificity. The lipase did not substantially hydrolyze short-chain fatty acids (butyric and caproic acid), as shown in Figure 7 for butter fat, yet was active toward long-chain fatty acids, such as oleic and stearic acid, in agreement with former work (Sémériva and Dufour, 1972). Results indicated that myristic acid (14:0) was not preferentially hydrolyzed. This may be due to the high proportion of 14:0 at the *sn*-2 position (Nawar, 1996) and indicates that acyl migration was low.

Table 1. Proportions of MAGs and DAGs in Blends [Butter Fat to 60:40 (w/w) Butter Fat/Canola Oil] following 24 h of Enzymatic Interesterification

blend	MAGs (% w/w)	DAGs (% w/w)
100	1.50	1.54
90:10	1.68	2.00
80:20	2.44	1.93
70:30	1.38	1.78
60:40	1.39	1.50

The proportion of DAGs and MAGs was constant after 24 h of interesterification for all substrates (Table 1). The amount of DAGs was similar to or lower than amounts found in other studies (Mohamed et al., 1993; Kalo et al., 1986b). DAGs present consisted of carbon species 24−36. With increasing proportion of oil in the blend, the relative proportion of longer chained DAGs increased due to the presence of higher amounts of di-18-carbon DAGs. Likewise, MAG analysis revealed that the relative amount of MAGs with 18-carbon fatty acids increased as the proportion of oil increased.

In this study we have investigated the effects of lipase-catalyzed interesterification of butter fat and butter fat/canola oil blends. The results show that (i) TAG species of butter fat and butter fat/canola oil are substantially modified as a result of interesterification, with greater changes in composition as the proportion of oil is increased; (ii) an optimized process with respect to water content minimizes production of undesirable side reactions (free fatty and partial acylglycerol formation); and (iii) the lipase specificity is not altered by immobilization.

This study indicates that enzymatic interesterification is a viable tool to modify the compositional properties of butter fat and butter fat/canola oil blends. The changes in the physical, microstructural, and rheological properties in butter fat/canola oil blends resulting from enzymatic interesterification catalyzed by RAL are discussed in the following paper.

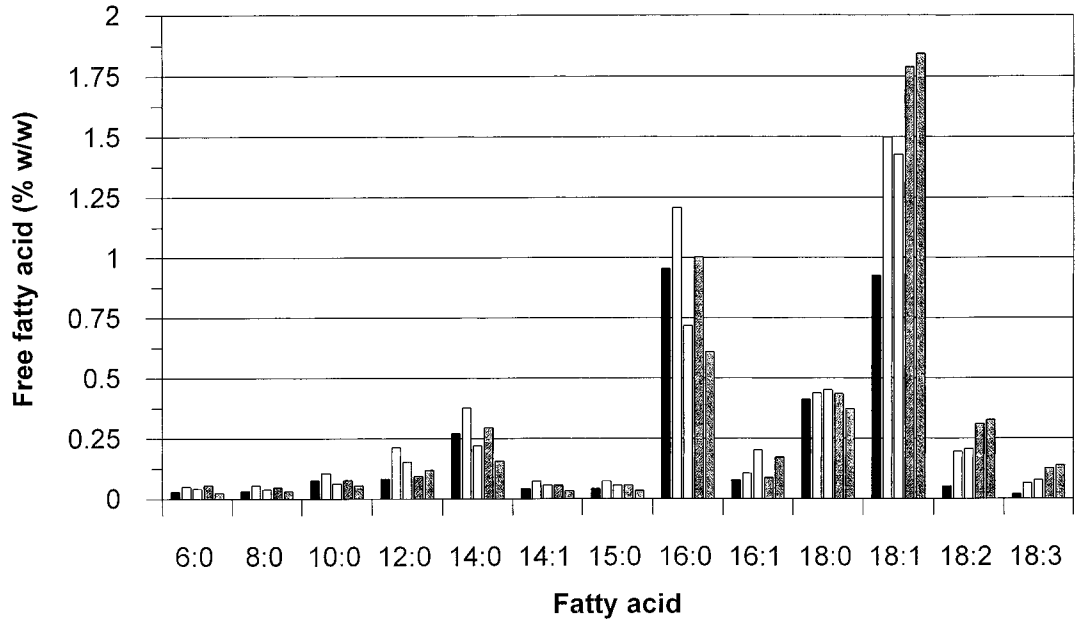

Figure 6. Fatty acids (% w/w; bars in each grouping represent, from left to right, 100, 90, 80, 70 and 60% w/w) released in each blend following 96 h of enzymatic interesterification.

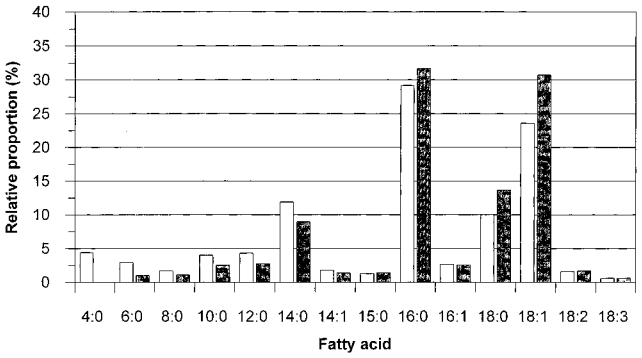

Figure 7. Comparison of native fatty acid composition (% w/w; white bars) and free fatty acids released (% w/w; gray bars) following 96 h of enzymatic interesterification for butter fat.

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