Lipase modification of milk fat: Impact on changes of triacylglycerol distribution and melting profiles

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ABSTRACT

Milk fat plays significant role in functional, nutritional, physical and chemical properties in dairy and dairy products. Dairy products also contain nutritional resources and essential fatty acids. Consumer demand is increasing due to nutritional and pleasant flavor containing in dairy and dairy products. Modification of milk fat by lipases for the aim of increasing pleasant flavor to be added in bakery products and other dairy and dairy products in decades is highly demanded. Triacylglycerols (TAGs) from hydrolyzed milk fat by Lipozyme-435 and Novozyme-435 were analyzed by using ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry to assess their redistribution. Percentage TAGs (CN 28–34) and TAGs (CN 36–42) with at least two short chain fatty acids and with short and medium chain fatty acids were respectively decreased in both Lipozyme-435 and Novozyme-435 treated AMF. As consequences, TAGs (CN 44–54) with at least two long chain fatty acids were observed to increase in both Lipozyme-435 and Novozyme-435 treated AMF. The melting and crystallization melting profiles of both Lipozyme-435 and Novozyme-435 treated AMF were also modified compared to AMF. In addition, solid fat content behave plasticity at certain temperature range of SFC values.

Keywords: Hydrolyzed milk fat; Lipase; Triacylglycerol; Melting and crystallization profiles; Solid fat content; Mass/mass spectrometry
INTRODUCTION

Worldwide, milk fat is the third main lipid source for human nutrition (Aguedo et al., 2008) and imparts excellent flavor and superior mouth-feel to milk products (Aguedo et al., 2008; Reddy, 2010). Milk fat is an important ingredient in bakery and confectionery industry technology. To increase the usage of milk fat as food ingredient, several modifying milk fat technology have been suggested such as acyl exchange of the fatty acids on the triacylglycerol (TAGs) backbone (Grall and Hartel, 1997). Milk fat being an important fat in dairy industry (Arul et al., 1988), has a heterogeneous nature which contains complex fatty acid composition results in a mixture of TAGs with different chemical and physical properties (Büyükbeşe et al., 2014; Destaillats et al., 2006). Those physical and chemical properties are influenced by the chain length of the fatty acid residues, their degree of un-saturation, and their binding position on the glycerol backbone (Balcão et al., 1998; Schenkel et al., 2013). Having different TAGs with different fatty acids in chain length and degree of un-saturation, milk fat is widely melting from about -40 ºC to 40 ºC (Arul et al., 1988; Fatouh et al., 2005; Queirós et al., 2016; Ramel and Marangoni, 2016). Modification to provide a variety of functionalities in different food applications can be achieved due to a diversity of fatty acids contained in milk fat (Prado et al., 2012). Therefore, the study on TAGs identification, structure and distributions is important with regard to nutritional functions, quality control and technological characteristics (Zhou et al., 2013).

Milk fat is the most complex naturally occurring fats due to the wide variety of almost 400 different fatty acids (Bugeat et al., 2015) with non random distribution which results in thousands TAGs (Robinson and MacGibbon, 1998). Modification of milk fat
composition to improve its nutritional and functional properties is of considerable interest for expanding its use in the food industry (Lopez et al., 2006). Some of such approaches are hydrolyzed milk fat fraction enriched in conjugated linoleic acid and trans-vaccenic acid (Martínez-Monteagudo et al., 2014), enrichment of anhydrous milk fat in polyunsaturated fatty acid residues from linseed and rapeseed oils through enzymatic interesterification (Aguedo et al., 2008) and unsaturated fatty acid enriched vs. control milk triacylglycerols (Bugeat et al., 2015). Controlled hydrolysis of milk fat to maximize the production of short-chain fatty acids (C4:0–C8:0) for the aim of enhancing flavor development in dairy products is another approach to expand milk fat utilization which involves hydrolyzing the TAGs to free fatty acids and glycerol via enzymatic hydrolysis (Martínez-Monteagudo et al., 2014).

On hydrolyzing milk fat by lipase to impart its flavor in dairy and dairy products, a little emphasis has been made to characterize their TAGs left after hydrolysis. During hydrolysis of milk fat by lipase, lipase plays a big role to cleave the bond on TAGs molecule whereby fatty acids are located. Depending on which lipase is employed during hydrolysis, the expected final product is a milk fat with improved and pleasant flavor. To obtain this, the lipase with a characteristics of cleaving the bond at sn_1,3 positions is highly accepted due to their ability to cleave the bond at sn_1 and sn_3 positions in a TAG molecule (Nyyssölä et al., 2015). However, the lipase that cleave only at sn_1,3 positions to our knowledge is still a challenge, this end up with certain cleavage of other fatty acids with long chain fatty acids which have no much impact on flavor development. Long-chain saturated acids, such as stearic acid, are primarily located at sn_1 position and also unsaturated fatty acids, such as oleic and linoleic acids are at sn_3
position (Ruiz-Gutierrez and Barron, 1995). The targeted fatty acids especially with short chain fatty acids are highly located at sn_1,3 positions of a TAG molecule (Nyyssölä et al., 2015). Those short chain fatty acids such as butyric, caproic and octanoic acids are responsible for strong flavor on improved milk fat flavor (Omar et al., 2016).

In order to obtain desired and pleasant flavor during hydrolysis, controlled hydrolysis is essential. The controlled hydrolysis can be achieved by employing specific lipase and at the same time controlling the hydrolysis period. In this case it implies that not all TAGs are cleaved during the hydrolysis which is targeting the improved flavor as the end product. The left TAGs still contribute and play important roles to the final product and therefore has to be studied to understand the altered fat (Neff et al., 2001). The study carried out by Neff and Byrdwell (1995) revealed that the oxidative stability of soy bean oil was partly depended on the TAGs composition and their structure.

During hydrolysis of milk fat, the melting and crystallization profiles are modified due to the fact that certain fatty acids on TAGs molecules are cleaved especially those contain short chain fatty acids. The modifications are expected to improve the chemical and physical properties when added to other products at dairy and dairy products at processing industry. Many properties such as texture, mouth-feel, and rheology that are important for product quality are affected by crystallisation of milk fat (Wiking et al., 2009). The high melting fractions can be used as shortening in puff pastry to impart a desirable butter flavor, fat bloom inhibitors in chocolate, cocoa butter replacement in confectionary products, while low melting fractions can be added in biscuits, short breads and cold spreadable butter (Lopez et al., 2006). The rheological properties of fats are
generally functions of the solid fat content (SFC) and other factors such as size, shape,
number of the crystals and clusters (Wiking et al., 2009).

Little work has been done in characterizing the impact of TAGs and melting profiles on
hydrolyzed milk fat. Therefore, the aim of the present study was to identify and quantify
the TAGs after controlled milk fat hydrolysis in 24 hours by using Lipozyme-435 and
Novozyme-435 and hence study their melting profiles which in turn affect the final dairy
and dairy products.

MATERIALS AND METHODS

Anhydrous milk fat (AMF) with moisture content of 0.14% was a gift from Kerry Oil &
Grains Industries Shanghai Company. The immobilized lipases (Lipozyme-435 (≥ 20000
U/g) from Rhizomucor miehei and Novozyme-435 (≥ 5000 U/g) from Candida antarctica
were gifts from Novozyme (Shandong) Innovation & Business Center, China. The OPO
(1,3-dioleoyl-2-palmitoylglycerol) and OOO (tri-olein) (≥95%) TAGs standards were
obtained from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA). n-Hexane,
acetonitrile and iso-propanol were of HPLC grade. All other solvents were of the
analytical or HPLC grade.

Fatty acid analysis

Fatty acids were determined by converting the free fatty acids released to their methyl
esters (FAME). In briefly the sample was prepared out as follows: A 50 mg of milk fat
was added in 2 mL of n-hexane before 0.5 mL 2 N KOH of methanol solution was added.
The mixture was vigorously shaken with vortex in 2 minutes. To remove excess water a
sodium thiosulfate was also added. Then the mixture was centrifuged at 10000 rpm for 10
minutes. A supernatant was passed through 0.45 μm membrane filter and then a 0.5 μL
sample was injected into the gas chromatograph (Shimadzu GC-2010, Tokyo Japan) equipped with an FID detector, with a capillary column, PEG-20M 30M i.d. 0.32 mm x 1.0 μm. The initial column temperature was set at 100 °C for 3 minutes before ramped to 180 °C for 8 minutes at a rate of 10 °C/min and then maintained at this temperature for 1 minute. The second ramp was reached to 240 °C for 20 minutes at a rate of 3 °C/min and this temperature was maintained for 9 minutes. The injector and detector temperature was set at 250 °C. The pressure of the carrier gas (N₂) was maintained at 110.0 kPa at the rate of 3 mL/min and H₂ gas was at a flow rate of 47 mL/min. The air flow rate was set at 400 mL/min and the split ratio was set at 1:12. The fatty acids were identified by a comparison of retention times to the 40 fatty acids standards.

Performance of milk fat hydrolysis reactions

Hydrolysis of AMF with Lipozyme-435 and Novozyme-435 were carried out at 55 °C in a 125 mL Erlenmeyer flask as described in detail in our previous study (Omar et al., 2016).

Ultra Pressure Liquid Chromatography Analysis

The TAGs in hydrolyzed milk fat were identified and quantified by a Waters Acquity Ultra Pressure Liquid Chromatography (UPLC) system coupled to Waters Qu-ToF Premier mass spectrometer using a ACQUITY UPLC BEH C18 analytical column (i.d. 2.1×50 mm, 1.9 μm). A 10 mM ammonium acetate was introduced to the mobile phase to serve as an electrolyte. The efficient separation of TAGs was carried out by two mobile phases, (A) acetonitrile/isopropyl alcohol (1:9, v/v) and (B) 40% acetonitrile at 300 μL/min. Initially, 70% of mobile phase A was used and kept for 1 min, then allowed to reach 87% in 30 min before held for 1 min, and then was returned to the initial 70% in 1
min and equilibrated for 4 min. The temperature of a sample chamber was set at 20 °C while keeping the column temperature at 45 °C. A 1.0 μL was set as dead volume and each sample was analyzed in triplicate.

Mass Spectrometry Conditions

The exactive mass spectrometer operated in the positive ion mode was performed on a Waters Qu-ToF Premier mass spectrometer with a mass range of 200 to 1500 m/z in 1 s duration. High-purity nitrogen was used as nebulizer and drying gas was set at a constant flow rate of 1.5 ml/min. The source parameters were: 400 °C as desolvation temperature; 100 °C as source temperature; 3.5 V capillary voltages and; 20 V sampling cone voltage. MS/MS analysis was set at collision energy of 35 V. The sodium format was used previously to calibrate the instrument and the lock mass spray for precise mass determination was set by leucine enkephalin in the positive ion mode. All the data were acquired and processed by Mass Lynx V4.1 software.

Melting profiles conditions

Melting and crystallization profiles of AMF, Lipozyme-435 and Novozyme-435 treated AMF were determined by using differential scanning calorimetry (DSC Q2000 V24.9 Build 121, TA Instruments, New Castle, DE, USA). In order for the analysis to take place, the system was purged with nitrogen gas at 20 m/Lmin during the analysis, and also nitrogen was used to serve as a refrigerant to cool the system. Indium, eicosane, and dodecane standards were used during calibration. The samples in the ranges (5–8 mg) were hermetically sealed in an aluminum pan, heated to 80 °C, and held for 5 min to destroy completely the previous crystal structure, before the samples allowed to cool at -40 °C and maintaining for 5 minutes. This allowed the melting profiles to be obtained by
heating the samples to 80 °C at a rate of 10 °C/min. The melting and crystallization profiles were recorded from -40 °C to 80 °C. An empty aluminum pan was used as a reference and each sample was done in duplicate.

**Solid fat content analysis**

The SFC of AMF, Lipozyme-435 and Novozyme-435 treated AMF were performed by using AM4000MQC (Oxford, Oxfordshire, UK) low-resolution nuclear magnetic resonance (NMR). Approximately, 2.5 ml of melted sample was placed in NMR tubes before kept at 80 °C for 30 min, and then tempered at 0 °C for 90 min, and finally 30 minutes at each 5 °C intervals measuring temperature. The SFC was determined from 0 °C – 40 °C temperature range. All samples were carried out in triplicate.

**Statistical Analysis**

SPSS statistical software (v. 19.0, IBM SPSS, Chicago, IL, USA) was used in data analysis. All analyses were carried out in triplicate, and were reported as means ± standard deviations. The box-plots were treated by Origin 8.5 (Origin Lab, Northampton, MA, USA).

**RESULT AND DISCUSSION**

**Fatty acids composition**

Eighteen fatty acids were identified by GC analysis from the ranges of 0.25 to 33.93%. Those were butanoic acid (1.96%), caproic acid (1.58%), caprylic acid (1.04%), capric acid (2.67%), lauric acid (5.04%), myristic acid (13.31%), pentadecanoic acid (1.07%), palmitic acid (34.74%), palmitoleic acid (1.69%), margaric acid (0.40%), stearic acid (10.42%), oleic acid (22.43%), linoleic acid (1.21%), linolenic acid (0.51%), linolenic acid (0.61%), stearidonic acid (1.00%), arachidic acid (0.13%) and gadoleic acid
Lauric, myristic, palmitic, stearic and oleic acids were the most abundant fatty acids.

**Triacylglycerol identification and composition**

A Qu-ToF MS with ESI is a rapid and unambiguous tool for TAG profiles composition and TAGs are detected mostly as sodiated molecules [M+Na]+. The OPO and OOO standards with sodiated [M+Na]+ adducts of 881 and 907 were respectively used. All the TAGs in AMF, Lipozyme-435 and Novozyme-435 treated AMF were observed as sodiated [M+Na]+ and ammoniated [M+NH4]+ adducts.

Table 1 shows the identified TAGs and their relative percent peak area for Lipozyme-435 and Novozyme-435 treated with AMF. In viewing Table 1 there is clear evident that the two lipases hydrolyzed the milk fat which resulted in to new re-distribution of TAGs. The percentage TAGs (carbon number (CN) 28–34) which had short chain fatty acids especially the one contain at least two short chain fatty acids and percentage TAGs (CN 36–42) with some of short and medium chain fatty acids generally decreased after 24 hours of hydrolysis in both Lipozyme-435 and Novozyme-435 treated with AMF. Fig. 1a and Fig. 1b show chromatograms and TAGs identified in Lipozyme-435 and Novozyme-435 treated with AMF respectively. The increase in TAGs (CN 44–54) percentage with long-chain fatty acids especially the one containing at least two long chain fatty acids were also observed in both Lipozyme-435 and Novozyme-435 treated with AMF. These observations suggest that the degree of hydrolysis was dependent on the number of carbon atoms of the TAGs; with hydrolysis preferentially affecting TAGs containing esterified short-chain fatty acids, as a consequence of the loss of short chain of...
fatty acids on TAGs, there is an increase in the relative percent of long-chain fatty acids on TAGs (Fontecha et al., 2006).

The major TAGs found in Lipozyme-435 treated AMF were PoPoCo, PoPoCy, CyOL, CaOL, PLaO, LaOO, PPP/PMO, OOPo, OOO and OOS/SSL. These major TAGs are responsible for the physical and functional properties of the product (Tzompa-Sosa et al., 2016) due to their high influence compared to other little amount of TAGs contained. Fig. 2 (a,b,c,d) shows MS/MS spectra of four highest major TAGs obtained by Lipozyme-435 treated AMF. Those TAGs were CyOL (11.06±0.14%), CaOL (10.69±0.32%), OOPo (15.23±0.32%) and OOO (10.32±0.10%). During identification of TAGs the parent and daughter ions are used to come up with the right TAG. For instance, the daughter ions of CyOL (with parent ion m/z 767.58) were [Cy-L]+ (m/z 463.32), [Cy-O]+ (m/z 465.40) and [O-L]+ (m/z 601.51). The fragments formations were due to the neutral loss of caprylic, linoleic and oleic acids along with ammonia.

Table 1 also shows the major TAGs found in Novozyme-435 treated AMF and those were PoPoCo, PoPoCy, CoPO, CyOL, OOCy, OOCa, LaOO, PPP/PMO, OOM/PPL, OOO and OOS/SSL. Fig. 3 (a,b,c,d) shows the MS/MS spectra of four highest major TAGs found in Novozyme-435 treated AMF. The highest TAGs were OOCy (11.20±0.32%), OOCa (9.41±0.20%), OOM/PPL (16.54±0.44%) and OOO (13.57±0.26%). The parent ion of (OOO) m/z 907.71 was used with daughter ion of [O-O]+ (m/z 603.48) to identify this tri-olein. The high amount of OOM/PPL (16.54±0.44%) produced during Novozyme-435 treated AMF may be among the contributing factor which led to less oxidative stability in our previous study (Omar et al., 2016) as compared to Lipase-435 treated AMF (OOPo). This is due to the fact that TAG contained
with fatty acid in which there is one or more double bonds less stable compared to the
one contained by TAG with one or no double bonds. The lipid contains high amount of
linoleic acid may be un-stable due to the possibility of oxidizing compound at the double
bond position. In addition, the study conducted by Neff and Byrdwell (1995) showed that
the oxidative stability of SBO partly depends on the TAG composition and their
structure.

**Melting and crystallization profiles**

The study analyzed and investigated thoroughly AMF, Lipase-435 and Novozyme-435
treated AMF melting and crystallization profiles obtained. Fig. 4 (a,b) shows melting and
crystallization profiles of AMF, Lipase-435 and Novozyme-435 treated AMF. AMF
revealed melting range from -38.36 °C to 36.61 °C (Fig. 4a). This was contributed by
broad (-38.36 °C– -2.80 °C), sharp shoulder (-2.80 °C–9.67 °C), very long sharp shoulder
(9.67 °C–20.29 °C) and finally broad (20.29 °C–36.61 °C) melting curves. Milk fat
contains different TAGs which consisting short, medium and long chain fatty acids.
These fatty acids are the main contributors of the milk fat being with broad melting
ranges (Fatouh et al., 2005; Ramel and Marangoni, 2016). However, there were changes
in melting profiles when Lipase-435 and Novozyme-435 treated AMF were used (Fig.
4a). In Lipozyme-435 treated AMF a broad (-38.62 °C– -16.20 °C) was experienced, then
followed with very long broad (-3.60 °C–23.47 °C), thereafter followed by (23.47 °C–
31.43 °C), before reached final (31.43 °C–37.80 °C) melting temperature ranges. On the
study conducted by Tunick and Malin (1997) found that the fresh cow milk mozzarella
samples had a low temperature melting region from about -30 °C to 11°C, with a short
peak shoulder between 7 °C and 8 °C, thereafter followed by medium temperature
melting region with a tall peak at about 17 °C before preceded to a high melting region
from about 21°C to 38 °C.

For Novozyme-435 treated AMF a broad (-38.89 °C– -11.56 °C) was observed, followed
with very broad (-11.56 °C–22.67 °C), thereafter by (22.67 °C–33.82 °C), before reached
final with short broad (31.43 °C–40.45 °C) melting temperature curves. The sharp
shoulder (-2.80 °C–9.67 °C) in AMF was brought far away when Lipozyme-435 and
Novozyme-435 treated AMF were used (-38.62 °C– -16.20 °C) and (-38.89 °C– -11.56
°C) respectively. This implies that both Lipozyme-435 and Novozyme-435 treated AMF
contained high amount of TAGs which made up with short and medium chain fatty acids,
but also with saturated fatty acids. The short and medium chain fatty acids, with saturated
fatty acids are responsible in lowering the melting point of a lipid. In general, the melting
point of the fat decreases with decreasing chain length and increasing degree of un-
saturation of the fatty acids in milk fat (Smiddy et al., 2012). In viewing the very long
sharp shoulder (9.67 °C–20.29 °C) of the AMF, a modification of a long broad melting
temperature was also observed by both Lipozyme-435 and Novozyme-435 treated AMF.
This long broad range of temperature may consist with TAGs with some medium and
long chain fatty acids. Finally, the broad (20.29 °C–36.61 °C) melting profile was
observed in AMF while in both Lipozyme-435 and Novozyme-435 treated AMF were
(31.43 °C– 37.80 °C) and (31.43 °C– 40.45 °C) respectively. Surprisingly, the final
melting region of the Novozyme-435 treated AMF was brought to higher temperature
(40.45 °C) than the AMF and Lipozyme-435 treated AMF. This can be explained by the
fact that Novozyme-435 treated AMF contained high percent relative area of TAGs with
long chain fatty acids which may be a reason to extend the melting curve to higher
temperature. Since kinds and quantities of individual fatty acids located at the TAG glycerol moiety affect the food formulation and product functional properties, such as melting profile range (Neff and Byrdwell, 1995). Rao et al. (2001) studied the thermal profile of coconut oil triglycerides with a stearic acid level of 2% and the one with modified lipids containing varying levels of stearic acid and found that increase in the level of stearic acid substitution in coconut oil triglycerides, there was also a gradual corresponding shift in the peak at higher melting temperatures.

Fig. 4b shows crystallization curves of AMF, Lipozyme-435 and Novozyme-435 treated AMF. In observing AMF, a broad range of (-34.96 °C–13.86 °C) and short arm (13.86 °C–15.53 °C) were observed. However, for Lipozyme-435 and Novozyme-435 treated AMF three melting regions were observed in each. A broad range of (-36.02°C–0.97°C), then short shoulder (7.04 °C–16.44 °C) and finally with very long sharp shoulder (16.44°C–25.84°C) melting temperature were observed in Lipozyme-435 treated AMF. While for Novozyme-435 treated AMF, broad range of (-35.30 °C–0.92 °C), thereafter short shoulder (7.82 °C–19.90 °C) and finally with very long sharp shoulder (19.90 °C–29.71 °C) melting temperature were also observed. The gradual increase in crystallization temperature towards higher temperature is associated with an increase in long chain of saturated (Rao et al., 2001) and un-saturated fatty acids. The crystallization temperature region in both Lipozyme-435 and Novozyme-435 treated AMF were characterized by shifting to approximately similar temperature regions which was evident that the TAGs contained short, medium and long chain fatty acids and hence crystallize at different temperature regions (Martínez-Monteagudo et al., 2014). Long chain and saturated fatty acids are concentrated at high crystallization temperature peak region, including C16:0,
C18:0 and C20:0, medium-chain saturated (C10:0, C12:0 and C14:0) and long chain unsaturated fatty acids (C18:1 t11, C18:1 ω-9) are concentrated in the middle melting point fraction while the peak at lower crystallization temperature region consisted exclusively of short chain fatty acids such as C6:0 and C8:0 [17]. This is also supported by Haddad et al. (2011) who found in their study that the TAG composition is responsible for the melting and crystallization behavior and the rheological properties of a milk fat.

**Solid fat content**

The consistency of AMF, Lipozyme-435 and Novozyme-435 treated AMF were tested by SFC using NMR set at the range of 0 °C to 35 °C as shown in Fig. 5. The SFC is an important parameter used in monitoring milk fat and milk fat products at different temperatures to evaluate their suitability when added in different food products in the food industry.

AMF, Lipozyme-435 and Novozyme-435 treated AMF showed the decrease on SFC on each testing temperature. Figure 4 shows the SFC values of AMF from 0 °C to 15 °C for each testing temperature being a little bit higher compared to Lipozyme-435 and Novozyme-435 treated AMF. The phenomenon may be explained that AMF contained many TAGs with short chain fatty acids compared to Lipozyme-435 and Novozyme-435 treated AMF. With Novozyme-435 treated AMF slightly increase in SFC values at that testing temperature was observed than in Lipozyme-435 treated AMF. However, at the testing temperature range of 15 °C to 25 °C, the SFC values of AMF and Novozyme-435 treated AMF were approximately the same, though for Lipozyme-435 treated AMF was a little bit higher than AMF even at 30 °C. Another interesting point to note was when Lipozyme-435 and Novozyme-435 treated AMF at testing temperature ranges of 15 °C to
35 °C, Novozyme-435 treated AMF was slightly lower in SFC values than Lipozyme-435 treated AMF. This may be explained by the fact that Novozyme-435 treated AMF had higher relative percent area of OOO (13.57±0.26%) TAG compared to OOO (10.32±0.10 %) TAG of Lipozyme-435 treated AMF. In addition, Novozyme-435 treated AMF had higher relative percent area of OOM/PPL (16.54±0.44 %) TAG compared to OOPo (15.23±0.32 %) TAG of Lipozyme-435 treated AMF. The fatty acids contained in TAG molecule play an important role in changing the characteristic of a lipid contained. The un-saturated fatty acid with one or more double bond tends to be liquid at room temperature while saturated fatty acids are solid at the same temperature. Since Novozyme-435 treated AMF was comprised with high percent of OOO and OOM/PPL TAGs may be the reason behind being with the lower SFC values compared to Lipozyme-435 treated AMF. Additionally, many TAGs with short chain fatty acids were found in Lipozyme-435 treated AMF than in Novozyme-435 treated AMF (Table 1). In general, both Lipase-435 and Novozyme-435 treated AMF behave plasticity in the temperature range of SFC values and this may allow their use in many dairy and dairy products. According to Rao et al. [28] the temperature range in which the fats are plastic (retain their consistency) is obtained by measuring the SFC in the range of 15–35%.

CONCLUSIONS

The TAGs composition of both Lipozyme-435 and Novozyme-435 treated AMF were significantly changed after 24 hours of controlled hydrolysis. The percentage of TAGs (CN 28–40) with some short and medium chain fatty acids were decreased as consequence percentage of TAGs (CN 44–54) with long chain fatty acids increased after 24 hours of controlled hydrolysis. The decrease in amount of TAGs with short chain fatty acid...
acids and those with medium chain fatty acids can be related with an increase in amount of the TAGs with long chain fatty acids which may suggest the selectivity of TAGs with short chain fatty acids for the lipase used during hydrolysis. Both melting and crystallization profiles of Lipase-435 and Novozyme-435 treated AMF revealed the changes compared to AMF; and furthermore behaved plasticity at certain temperature range of SFC values which may aid for further dairy and dairy products processing.

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Author contributions


REFERENCES


**Figure Captions**

Fig. 1 Chromatograms and TAGs identified from Lipozyme-435 and Novozyme-435 were treated with AMF in 24 hours.

Fig. 2 Some of major triacylglycerol mass spectra obtained when Lipozyme-435 was treated with AMF in 24 hours.

*AMF (anhydrous milk fat)*

Fig. 3 Some of major triacylglycerol mass spectra obtained when Novozyme-435 was treated with AMF in 24 hours.

*AMF (anhydrous milk fat)*

Fig. 4 Melting and crystallization profiles obtained by AMF and when Lipozyme-435 and Novozyme-435 was treated with AMF in 24 hours.

*Note:* (a) represents melting profiles while (b) represents crystallization profiles
**Fig. 5** Solid fat content obtained by AMF and when Lipozyme-435 and Novozyme-435 was treated with AMF in 24 hours.

**Table 1** Triacylglycerol obtained when AMF was treated with Lipozyme-435 and Novozyme-435 for 24 hours

<table>
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<tr>
<th>RT</th>
<th>[M+NH4]^+</th>
<th>[M+Na]^+</th>
<th>CN</th>
<th>TAG identified</th>
<th>DAG Fragment Ions Obtained [M-RCOO+H]^+</th>
<th>% TAG Obtained</th>
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<td>661</td>
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<td>30</td>
<td>BuCaP/CoCaM</td>
<td>175 [Bu-P]^+ 411 [Bu-La]^+ 383 [Ca-La]^+ 383 [Cy-La]^+ 439 [La-La]^+</td>
<td>0.17±0.04</td>
</tr>
<tr>
<td>2.76</td>
<td>600</td>
<td>605</td>
<td>32</td>
<td>CaCaLa/LaCy</td>
<td>383 [Ca-Ca]^+ 411 [Ca-La]^+ 383 [Cy-La]^+ 439 [La-La]^+</td>
<td>nd</td>
</tr>
<tr>
<td>2.82</td>
<td>624</td>
<td>629</td>
<td>34</td>
<td>CyCyL</td>
<td>327 [Cy-Cy]^+ 463 [Cy-L]^+</td>
<td>0.60±0.09</td>
</tr>
<tr>
<td>3.69</td>
<td>626</td>
<td>631</td>
<td>34</td>
<td>CyCyO</td>
<td>327 [Cy-Cy]^+ 465 [Cy-O]^+</td>
<td>2.32±0.06</td>
</tr>
<tr>
<td>3.82</td>
<td>628</td>
<td>633</td>
<td>34</td>
<td>CyCaS/LaLaCa</td>
<td>355 [Cy-Ca]^+ 467 [Cy-S]^+ 495 [S-Ca]^+ 411 [Ca-La]^+ 439 [La-La]^+</td>
<td>nd</td>
</tr>
<tr>
<td>4.81</td>
<td>680</td>
<td>685</td>
<td>38</td>
<td>PoPoCo</td>
<td>409 [Po-Co]^+ 547 [Po-Po]^+</td>
<td>4.06±0.07</td>
</tr>
<tr>
<td>6.24</td>
<td>708</td>
<td>713</td>
<td>40</td>
<td>PoPoCy</td>
<td>437 [Po-Cy]^+ 547 [Po-Po]^+</td>
<td>8.21±0.13</td>
</tr>
<tr>
<td>11.87</td>
<td>792</td>
<td>797</td>
<td>46</td>
<td>OOCa</td>
<td>493 [O-Ca]^+ 603 [O-O]^+</td>
<td>nd</td>
</tr>
<tr>
<td>12.79</td>
<td>792</td>
<td>797</td>
<td>46</td>
<td>PoPoM</td>
<td>521 [M-Po]^+ 547 [Po-Po]^+</td>
<td>0.72±0.09</td>
</tr>
<tr>
<td>14.10</td>
<td>820</td>
<td>825</td>
<td>48</td>
<td>LaOO</td>
<td>521 [La-O]^+ 603 [O-O]^+</td>
<td>8.62±0.11</td>
</tr>
<tr>
<td>16.34</td>
<td>874</td>
<td>879</td>
<td>52</td>
<td>OOPo</td>
<td>575 [O-Po]^+ 603 [O-O]^+</td>
<td>15.23±0.32</td>
</tr>
<tr>
<td>18.69</td>
<td>902</td>
<td>907</td>
<td>54</td>
<td>OOO</td>
<td>603 [O-O]^+</td>
<td>10.32±0.10</td>
</tr>
<tr>
<td>19.77</td>
<td>896</td>
<td>901</td>
<td>54</td>
<td>LLL</td>
<td>599 [L-L]^+</td>
<td>1.22±0.09</td>
</tr>
<tr>
<td>19.87</td>
<td>852</td>
<td>857</td>
<td>50</td>
<td>PPS</td>
<td>551 [P-P]^+ 579 [P-S]^+</td>
<td>nd</td>
</tr>
<tr>
<td>22.04</td>
<td>880</td>
<td>885</td>
<td>52</td>
<td>SPS</td>
<td>579 [S-P]^+ 605 607 [S-S]^+</td>
<td>0.79±0.25</td>
</tr>
<tr>
<td>23.01</td>
<td>906</td>
<td>911</td>
<td>54</td>
<td>SOS</td>
<td>605 [O-S]^+ 607 [S-S]^+</td>
<td>2.87±0.11</td>
</tr>
<tr>
<td>23.91</td>
<td>908</td>
<td>913</td>
<td>54</td>
<td>ASP</td>
<td>579 [S-P]^+ 607 [A-P]^+ 635 [S-A]^+</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td>25.07</td>
<td>934</td>
<td>939</td>
<td>56</td>
<td>AAPo</td>
<td>605 [A-Po]^+ 663 [A-A]^+</td>
<td>0.39±0.09</td>
</tr>
</tbody>
</table>
aNote: $T_R$ (retention time), CN (carbon number), TAG (triacylglycerol), DAG (diacylglycerol), nd (not detected), Bu (butanoic acid), Co (caproic acid), Cy (caprylic acid), Ca (capric acid), La (lauric acid), M (myristic acid), P (palmitic acid), Po (palmitoleic acid), S (stearic acid), O (oleic acid), L (linoleic acid), A (arachidic acid).
Details of manuscript submission

Our manuscript entitled **Lipase modification of milk fat: Impact on changes of triacylglycerol distribution and melting profiles** is submitted for the purpose to seek publication to your journal so as to extend the scientific knowledge in this area. The interest in identification and quantification of milk fat triacylglycerols has recently increased not only on milk fat triacylglycerols which influence in further milk fat processing but also on dairy and dairy processing milk fat products. However, identification is not an easy job of these triacylglycerols due to the fact that milk fat contains more than 400 fatty acids which results in thousands of triacylglycerols. Many scientific reports present used long period in separation of the triacylglycerols contained in milk fat, which consume time, high cost of analysis and high solvent consumption. This leads to un-economic balance but also un-easy job especially when someone is dealing with many samples. In our study we used UPLC/Qu-ToF ESI MS/MS to analyze hydrolyzed milk fat by lipases. Therefore, the study will be able to increase the existing knowledge in this area and also help in further studies of triacylglycerols in milk fat and dairy products. The study also focused on melting and crystallization profiles which also impart further processing of dairy and dairy products.

Authors’ declaration

1. We declare that this manuscript is from our original finding and has not been previously published, is not currently submitted for publication to any other journal, and will not be submitted elsewhere before a decision is made by this journal and the provided data are original finding of our research. All authors concur with the submission. The manuscript does not neither contain experiments
using animals nor does contain human studies. All authors concur with the
submission.

Authors declared no conflict of interest.

We declare that the manuscript is strictly prepared according to the Journal format as
provided in the instructions to authors.

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Fig. 1

(a)

(b)
Fig. 5

The graph illustrates the effect of temperature on the (\%) SFC of AMF, Lipozyme-435 treated AMF, and Novozyme-435 treated AMF. The percentage of SFC decreases as the temperature increases from 0°C to 35°C.