MILK FAT TRIACYLGLYCEROLS:

Their variability, relations with fatty acids, DGAT1, β polymorphs and melting fractions

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Abstract

Milk fat (MF) triacylglycerol composition varies within a population of dairy cows. The variability of MF triacylglycerols and their structure was partially explained by the fatty acid (FA) composition of the MF, and by *DGAT1* K232A polymorphism. The FA C16:0 and C18:1cis-9 play a major role in understanding the changes seen in triacylglycerol profile and structure because they are the most abundant FAs in MF and are negatively correlated. MFs with low ratio C16:0/C18:1cis-9 were decreased in triacylglycerols with 34 and 36 carbons and were increased in triacylglycerols with 52 and 54 carbons. These changes in MF composition greatly affected the crystallization behavior of MF by changing the types of polymorphs formed during its crystallization. MF with low ratio C16:0/C18:1cis-9 formed stable and metastable polymorphs (β and β', respectively), whereas MF with high ratio C16:0/C18:1cis-9 formed exclusively metastable polymorphs (β') when the fat was crystallized at 20°C. The changes in MF composition also affected the melting behavior of MF by changing the melting point of the MF fractions.

List of Abbreviations

CN- Carbon number

DGAT1- Diacylglycerol-acyltransferase isoform 1

DHB- 2,5-dihydroxybenzoic acid

FA- Fatty acid

GC-FID- Gas chromatography with flame ionization detector

GPAT- Glycerol-3-phospate acyltransferase

mtGPAT- Mitochondrial glycerol-3-phospate acyltransferase

HMF- High melting fraction

LMF- Low melting fraction

MALDI-TOF MS- matrix-assisted laser desorption/ ionization- time of flight

MMF- Middle melting fraction

MF- Milk fat

MAG- Monoacylglycerol

pNMR- Pulse nuclear magnetic resonance

SFA- Saturated fatty acid

TAG- Triacylglycerol

TLC- Preparative thin layer chromatography

UFA- Unsaturated fatty acid

WAXD- Wide-angle x-ray diffraction

Table of Contents

Chapter I

Introduction

Milk fat composition

Milk fat (MF) is one of the major components of milk. Milk contains 3.0 to 6.0% of fat, but typically MF content is in the range between 3.5 and 4.7% (Walstra *et al.*, 2005). Due to its unique taste and nutritional value, MF is used as an ingredient in many food products. The main type of lipids in MF are triacylglycerols (TAG) (98% approx.), which are composed of 3 fatty acids (FA) attached to a glycerol backbone. More than 400 FA have been identified in MF resulting in a wide range of TAG (Jensen, 2002). The pool of FA in the mammary gland has two sources: dietary FAs that are transported through the blood from the rumen to the mammary gland and FAs produced *de novo* in the mammary gland from acetic and propionic acid (Dils, 1986). Blood derived and *de novo* synthesized FAs can be further transformed in the mammary gland by the action of elongases and desaturases (Jump, 2009, Guillou *et al.*, 2010). Elongases add two carbons to FAs and desaturases introduce double bonds to FAs. One of the most important enzymes is Δ9-desaturase, which adds a cis-9 double bond to saturated FA (Schennink *et al.*, 2008). The FA in the mammary gland enter the glycerol-3-phosphate pathway having TAG as end products. The glycerol-3-phosphate pathway is catalysed by four enzymes, namely glycerol-phosphate acyltransferase, acylglycerol-phosphate acyltransferase, phosphatidic acid phosphohydrolase and diacylglycerol acyltransferase (DGAT) (Coleman and Lee, 2004) (Figure 1).

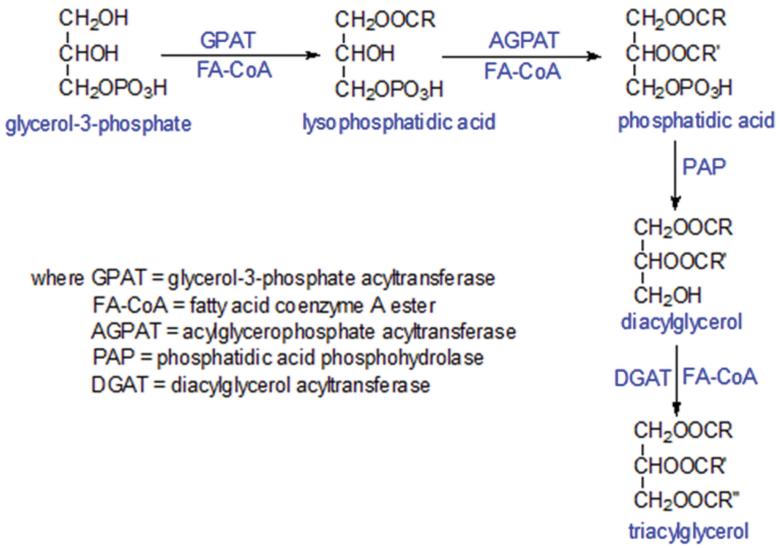

Figure 1. Synthesis of triacylglycerols via the glycrol-3-phosphate pathway (taken from Christie, 2014).

Milk fat FA composition can be modified by nutrition and management, by genetics or by industrial processes (Walker *et al.*, 2004). MF rich in unsaturated C18 FA is obtained by diets rich in fresh grasses or with dietary oil supplementation (DePeters *et al.*, 2001, Capuano *et al.*, 2014). In the Netherlands, diets rich in fresh grasses are typical for summer diets. MF with a saturated FA profile is obtained when the cows are fed with diets rich in concentrates and silage, which in the Netherlands is typical for winter diets. Figure 2 shows the variation of saturated FA in MF during a year in the Netherlands (Heck *et al.*, 2009). Moreover, MF composition can also be genetically modified. For instance, an increase in saturated FA (C16:0) has been associated to the K allele of *DGAT1* K232A polymorphism. As previously mentioned, *DGAT1* is an enzyme involved in TAG synthesis.

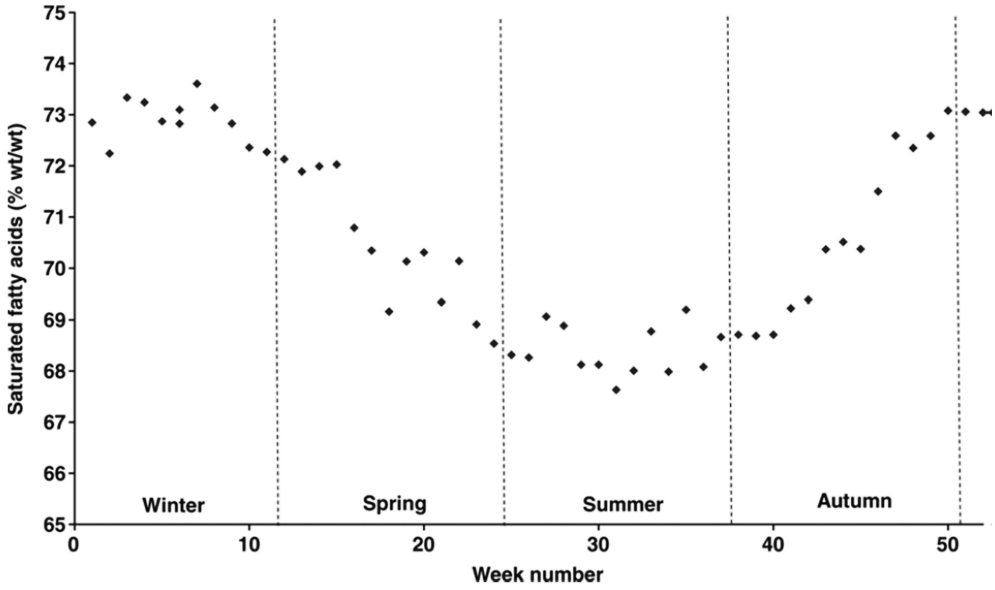

Figure 2. Weekly variation in the concentration of saturated fatty acids (g/100 g of fatty acids) in Dutch bovine raw milk in 2005 (taken from Heck et al, 2009).

Milk fat composition has been traditionally characterized on FA profile. MF FA profile is analyzed using FA methyl esters (FAMEs) produced after cleavage of the FA from TAG and subsequent methylation. Identification and quantification of the FAMEs is carried out by GC-FID. MF FA profile has been extensively studied and average values can be found elsewhere (Jensen, 2002, Heck *et al.*, 2009). Knowing the FA profile of a fat may be sufficient to characterize the nutritional value, it is, however, not enough to understand the physical-chemical properties of a fat because these are also determined by the TAG profiles. Therefore, in this study MF was characterized on both FA and TAG composition.

Triacylglycerols

Triacylglycerols can be described by the total number of carbons (CN), which is the sum of the carbon atoms of the three FA within the TAG; by the number of double bonds within the TAG and by the positioning of the FA within the glycerol backbone (Figure 3). So far, about 405 TAG species have been identified in MF, ranging from 22 to 54 CN and with up to 4 double bonds (Gresti *et al.*, 1993). However, if the positioning of FAs are taken into account an even larger number of TAG species exist. This large number of TAG species in MF makes the thermal behavior and functional properties of MF complicated.

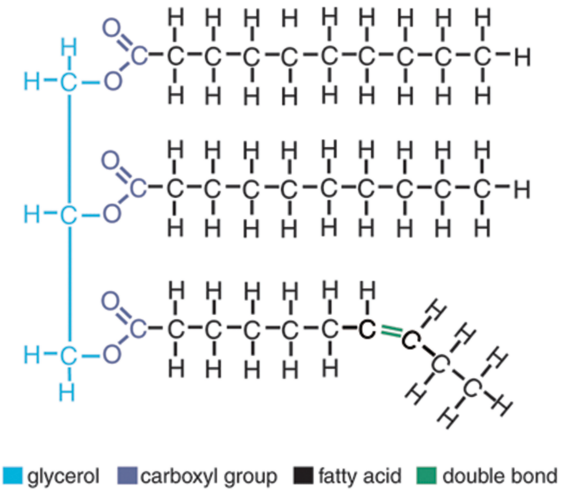

Figure 3. Triacylglycerol structure with 30 number of carbons in the fatty acids and one double bond (CN30:1) (source: http://creationwiki.org/pool/images/7/74/Triglyceride.gif).

Profiling of MF TAG is carried out by GC-FID and it is also used to identify adulteration (CommissionRegulation (EC)No.273/2008). This technique allows the separation of 16 TAG according to their CN but information on unsaturation level is not obtained (Figure 4A). Recent developments in lipid analysis have allowed fast separation and identification of TAG species according to CN and unsaturation level. This characterization is done using matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) (Figure 4B). Using this technique it is possible to identify more than 80 different TAG species in MF (Picariello et al., 2007). However, information on the positioning of the FA within the TAG is not obtained. In chapters 2 to 5 of this thesis both techniques, GC-FID and MALDI-TOF, were used to describe MF TAG composition.

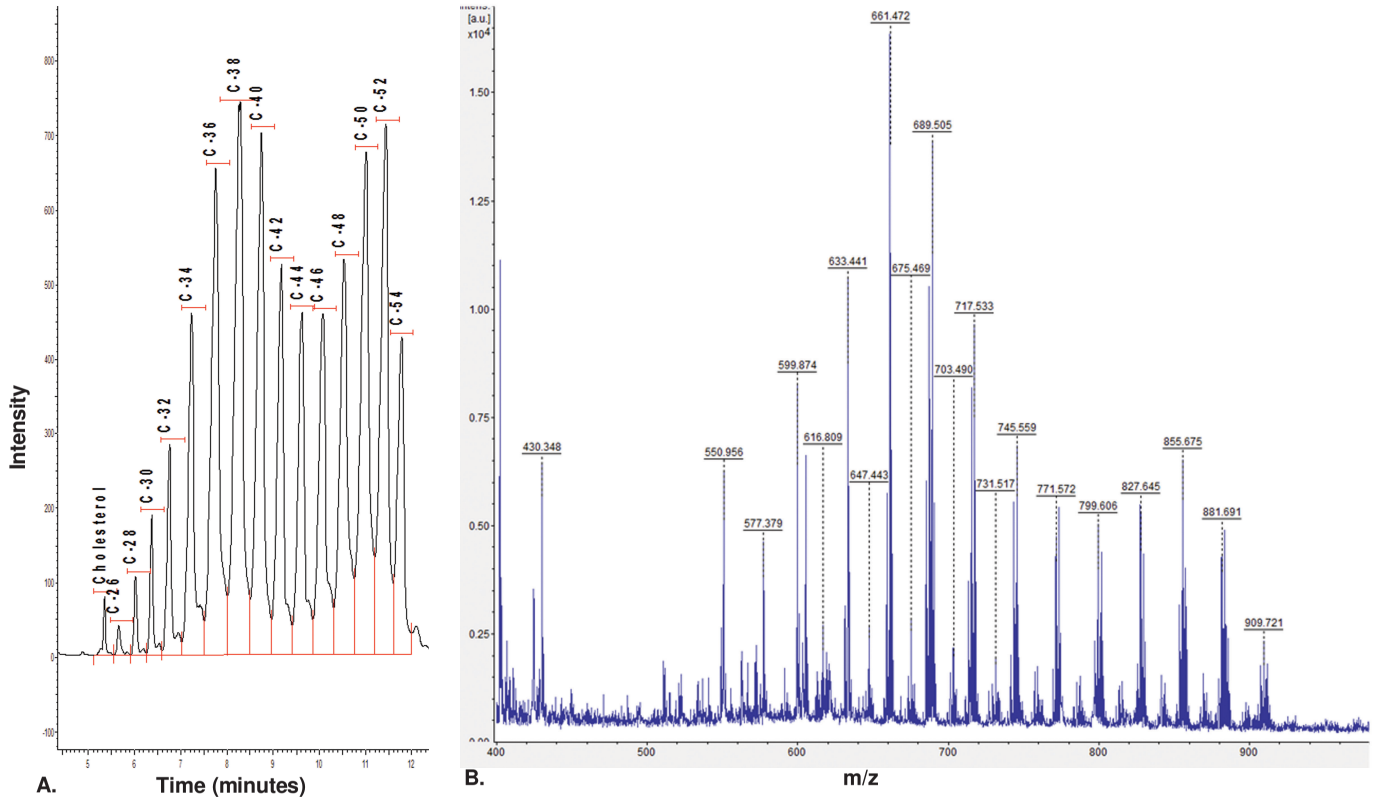

Figure 4. Milk fat triacylglycerol profile. A. Profile obtained from GC-FID analysis; B. Profile obtained from matrix assisted laser desorption ionization time-of-flight (MALDI-TOF).

Fatty acids within bovine MF TAG are not randomly distributed. The positions of each FA within the TAG are defined by a stereospecific numbering, namely sn-1, sn-2, sn-3 (Figure 3). This system is recommended by IUPAC. When the TAG molecule is shown in a Fisher projection, the carbon in the middle of the glycerol molecule is numbered as sn-2, the carbon above this is sn-1 whereas that underneath becomes sn-3 (Christie, 2011). The distribution of FA within TAG has been studied using either a regiospecific or a stereospecific approach. In the regiospecific approach, the FA placed at sn-1,3 and sn-2 are determined, whereas with the stereospecific approach, the FA at the sn-1, sn-2 and sn-3 positions are determined. Analysis of positioning of FAs within the TAG involves partial deacylation of the TAG by enzymatic or chemical means and further analysis of the FA composition from the acyl moieties. In MF TAG, short-chain FAs and C18:0 are predominantly located at sn-1,3, whereas C14:0 is predominantly located at sn-2. Furthermore, C16:0 and C18:1cis-9 are abundant either at sn-1,3 and sn-2 (Angers *et al.*, 1998, Blasi *et al.*, 2008).

DGATI K232A polymorphism

Diacylglycerol acyltransferase catalyses the last step of TAG synthesis in the mammary gland (Coleman and Lee, 2004). A polymorphism in the gene encoding for *DGAT1* has been identified in cattle, namely, *DGAT1* K232A polymorphism (Cases *et al.*, 1998). The *DGAT1* allele that encodes lysine at position 232 (232K) is associated with decreased milk yield (kg), increase in fat content (%), higher concentration of saturated fat and C16:0 and lower concentrations of C14:0, unsaturated C18 and CLA (Grisart *et al.*, 2002, Schennink *et al.*, 2007). Since this enzyme is involved in TAG synthesis, we hypothesized that *DGAT1* K232A polymorphism will influence either the quantity of TAG species produced in the mammary gland or the TAG structure. In the following chapters we will discuss the effect of *DGAT1* K232A polymorphism on MF TAG profile and on the structure of TAG.

Thermal behaviour of milk fat

The most essential physical properties of MF are crystallization and melting behavior because these properties affect the functional and sensory attributes of MF-containing products (*i.e.* firmness and spreadability of butter, structure of whipped cream, glossiness and mouth feel of chocolate and smoothness of ice-cream) (Narine and Marangoni, 1999a, Campos *et al.*, 2002b, Wiking *et al.*, 2009a, Marangoni *et al.*, 2011). Since TAG are the most abundant lipids in MF, they provide the relevant physical characteristics to the fat such as crystallization and melting point, polymorphism, solid fat content, etc. Studying the thermal behavior of MF with different FA and TAG composition may help to better control functional and technological aspects of MF.

Crystallization behaviour

Crystallization starts with nucleation, which is the formation of tiny embryonic crystals called nuclei. The formed nuclei grow and form crystals. TAG are the fundamental unit of crystals and can pack in different crystal arrangements that differ in stability (Sato, 2001). According to the Ostwald rule of stages, unstable crystals will be formed before metastable and stable crystals. Formation of these crystals is affected by cooling rate, crystallization temperature and amount of liquid fat. Several studies have published on these factors (Grotenhuis *et al.*, 1999, Lopez *et al.*, 2001a, Lopez *et al.*, 2001c, Lopez *et al.*, 2005b). In this thesis we describe the effect of FA and TAG compositions on the crystallization of MF at 20°C based on the types of polymorphs formed.

Melting behaviour

The melting point of each TAG depends on its FA composition and on the positioning of the FA within the TAG. If we take into account that in MF more than 400 FA have been identified and, theoretically, 10^5 different TAG species exist (Walstra *et al.*, 1992), then we may expect that MF has an extremely wide melting range. In reality, the melting temperature of MF ranges from -40 to 40^0C. TAG with a melting point higher than 40°C do exist in MF but they normally dissolve in the lower melting TAG fraction (Walstra *et al.*, 1992), mainly because these high melting TAG are present in low amounts.

The melting behavior of a fat depends on its composition and on the crystallization history. Crystallization history is described as cooling rate, final crystallization temperature and storage time. These factors determine the types of crystals formed and their composition. For instance, fast cooling favors the formation of unstable crystals (α crystals) and promotes co-crystallization. This results in a broader melting range and a lower melting maxima (Breitschuh and Windhab, 1996). MF composition also affects melting behavior by determining the melting range of the fat. For instance, MF with a high concentration of unsaturated C18 FA or short chain FA has a lower melting point than the average MF (Buldo *et al.*, 2013). To our knowledge, no study has reported about the effect of TAG composition on MF melting behavior. Therefore, in chapter 5 we describe the results and conclusions of the study on MF melting behavior with variable TAG composition.

Aim of our study and outline of this thesis

The objectives of the present study were to describe the variability of MF TAG profile and structure within a population of Dutch Holstein-Friesian cows. To understand the relation of TAG with MF productive traits, days in milk (DIM) and *DGAT1* K232A polymorphism. And lastly, to study the effect of TAG and FA profile on the thermal behavior of MF.

Chapter 2 "Milk fat triacylglycerols and their relations with milk fatty acid composition, *DGAT1* K232A polymorphism and milk production traits", describes the variability of TAG on MF from individual cows. We investigated the effect of FA composition, *DGAT1* K232A polymorphism and milk production traits (fat content, morning milk yield) and DIM on MF TAG profile in the Dutch Holstein-Friesian dairy cattle population. In this study large differences in MF TAG profile were seen among cows implicating relevant differences in MF physical and functional properties. The implications of these differences in composition are described in Chapter 4 and 5.

Chapter 3 "Influence of C16:0 and long-chain FA on normal variation of bovine MF TAG **structure"** reports the TAG structure of individual cows with variate FA profiles. Our aim was to determine if differences in MF TAG structure exist among individual cows and to determine how MF FA profile and *DGAT1* K232A polymorphism are related to the structure of MF TAG.

In **Chapter 4 "Formation of β polymorphs in milk fats with large differences in triacylglycerol profiles"** we describe the effect of MF TAG composition on the formation of MF polymorphs that are thermodynamically stable at 20°C. We aimed to better understand the relation between MF TAG composition and crystals thermodynamically stable at 20°C, such as β' and β.

In **Chapter 5 "Melting points of milk fat fractions: the importance of crystallization history and milk fat composition"** we describe the relation between MF composition and melting fractions. The aim of this study was to investigate the effect of crystallization history and FA and TAG composition on melting behavior of MF. Two types of crystallization processes were used, namely isothermal and non-isothermal crystallization.

Chapter 6 contains information on the experimental design, sample selection and data analysis of the studies. Moreover, we discuss the importance of using MF samples from individual cows and the relevance of the study of TAGs. Furthermore, we describe the results obtained in this thesis in a broader context and conclude with main observations and recommendations.

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Chapter 2

Milk fat triacylglycerols and their relations with milk fatty acid composition, *DGAT1* K232A polymorphism and milk production traits

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Abstract

Milk fat (MF) triacylglycerols (TAGs) determine the physical and functional properties of butter and products rich in MF. To predict these properties, it is necessary to understand the variability of FA, TAGs, their associations and their effect on milk productive traits, DIM and genes related to fat synthesis. Therefore, the aim of this research was to study the variability of TAG using MF from individual cows and to investigate the effect of FA composition, *DGAT1* K232A polymorphism, DIM and milk production traits (fat content and morning milk yield) on MF TAG profile in the Dutch Holstein-Friesian dairy cattle population.

Large differences in MF TAG profiles were seen among cows. We showed that the variability of TAGs is highest for low and high molecular weight TAG (TAG with carbon number 26-30 and 52-54, respectively) and lowest for TAG with carbon number (CN) 38, which was the most abundant TAG. Saturation index (SFA/UFA) and the ratio C16:0/C18:1cis-9 showed significant effects on TAG CN34, 36, 52 and 54. TAG CN34 and 36 increased as the saturation index and ratio C16:0/C18:1cis-9 increased, while the opposite was seen for TAG CN52 and 54. Moreover, the *DGAT1* K232A polymorphism significantly affected TAG CN38. We showed that the relative concentration of TAG with CN38 was higher in cows with *DGAT1* KK genotype. Production traits (fat content and morning milk yield) and DIM had no significant effect on TAG profile. This is a relevant observation since considerable increases of milk yield and fat content has been seen in the Netherlands over the last 60 years. The large differences shown between individual cows in MF TAG profile imply differences in physical properties of MF.

Keywords: Triacylglycerol, milk fat, *DGAT1*, milk productive trait, milk synthesis.

Introduction

Milk fat **(MF)** triacylglycerols **(TAGs)** determine the physical and functional properties of butter and products rich in MF (Parodi, 1979, Hawke and Taylor, 1995). However, predicting the functional properties of these products is difficult because these properties are influenced not only by FA composition, but also by the positioning of FA within the glycerol backbone and by the concentration of individual TAG species. Moreover, it is known that the concentration of FAs, the positioning of FAs within the TAG and the concentration of TAG species change among seasons and feeding regimes (Gresti *et al.*, 1993, Capuano *et al.*, 2014, Tzompa-Sosa *et al.*, 2014). These characteristics of TAG are largely regulated by the availability of FA present in the system and by the enzymes involved in lipid synthesis, which respond to genetic factors (Bionaz and Loor, 2008, Smiddy *et al.*, 2012, Tzompa-Sosa *et al.*, 2014).

Milk fat TAG is synthesized via the glycerol-3-phosphate pathway. Its reactions are catalysed by four enzymes, namely glycerol-phosphate acyltransferase, acylglycerol-phosphate acyltransferase, phosphatidic acid phosphohydrolase and diacylglycerol acyltransferase **(DGAT)** (Coleman and Lee, 2004). A change in the amino acid sequence of an enzyme could result in a change of its function such as the one reported for *DGAT1* K232A polymorphism. The *DGAT1* K allele has been associated with decreased milk yield (kg) and increased in fat content (%), higher concentration of saturated fat and C16:0 and a lower concentration of C14:0, unsaturated C18 and CLA (Schennink *et al.*, 2007). Moreover, *DGAT1* K232A polymorphism has been associated with a change in the positioning of the FA within the TAG (Tzompa-Sosa *et al.*, 2014).

Bovine MF is composed of TAG with carbon number **(CN)** between 22 and 54 (Gresti *et al.*, 1993, RuizSala *et al.*, 1996). Most studies agree that the concentrations of individual TAGs vary among seasons and feeding regimes (DePeters *et al.*, 2001, Capuano *et al.*, 2014). For instance, MF of cows in pasture is rich in unsaturated C18 FA, has a decreased relative concentration of TAG CN34, 36, 42, 44 and 46 and has an increased relative concentration of TAG CN24, 26, 40, 50, 52 and 54, as compared with cows in a winter diet with no fresh grass (Capuano *et al.*, 2014). A similar effect has been reported for cows with dietary oil supplementation (DePeters *et al.*, 2001). Overall, studies agree that diets rich in unsaturated C18 FA, such as fresh grass or canola supplementation, increase the relative concentrations of TAG CN52 and 54 and decrease the relative concentration of TAG CN34, 42 and 44.

The effect of feeding on MF TAG profile in pooled samples (DePeters *et al.*, 2001, Capuano *et al.*, 2014) and the effect of *DGAT1* K232A polymorphisms on the FA profile have been previously studied (Schennink *et al.*, 2007), however, no

information about variability of MF TAGs in a dairy cow population using individual samples, nor the effect of *DGAT1* K232A polymorphism on TAG profile have been reported. Understanding the variability of MF TAG in a cow population and the role of FA composition, *DGAT1* K232A polymorphism and milk productive traits on the variation of MF TAG profiles can help to find opportunities to modify physical properties of MF. The advantage of using MF from individual cows over bulk milk is that information on FA composition, productive traits and the genetic background of each individual cow is available and can be related to changes in TAG profile. The aim of this research was to study the variability of TAG on MF from individual cows, as well as to investigate the effect of FA composition, *DGAT1* K232A polymorphism and milk production traits (fat content, morning milk yield) and DIM on MF TAG profile in the Dutch Holstein-Friesian dairy cattle population.

Materials and Methods

Sample selection of individual milk fats from a population of Dutch Holstein-Friesian cows

The MF samples and FA compositions used in this study were part of the Dutch Milk Genomics Initiative where morning milk samples were collected from 1918 first lactation Holstein-Friesian cows on 398 commercial farms in the Netherlands (Schennink *et al.*, 2007, Heck *et al.*, 2012). The FA composition of this population has been previously reported and discussed (Stoop *et al.*, 2008, Heck *et al.*, 2012). Milk samples were collected during the months February and March, which corresponds to the winter season in the Netherlands. The typical winter diet in the Netherlands consist mainly of concentrate, corn and grass silage (Heck *et al.*, 2009).

In the current study we assured large variation in fat content, and consequently in FA composition, by randomly selecting a similar number of MF samples from cows with a fat content from 3.0 to 3.9%, from 4.0 to 4.9% and from 5.0 to 6.0% (Figure 1). Furthermore, within each group *DGAT1* genotype AA and KK are represented. Genotyping of the *DGAT1* K232A polymorphism was performed as part of a previous study (Schennink *et al.*, 2007). In total, 54 cows were selected, 26 with *DGAT1* AA genotype and 28 with *DGAT1* KK genotype (Figure 1). The 54 selected MF samples belonged to different commercial herds (46 herds in total) and therefore the samples provide the best possible representation of management and feeding regimes.

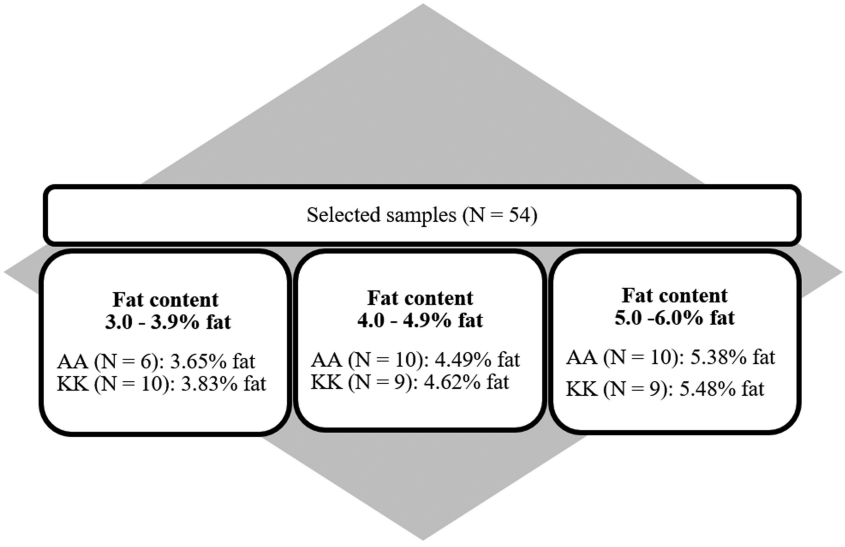

Figure 1. Sample selection of individual milk fats from a population of Dutch Holstein-Friesian cows.

Analysis of TAG Profile

Isolation of MF was performed by the Dutch Milk Controlling Institute Qlip Laboratories (Zutphen, the Netherlands) as described by Tzompa-Sosa *et al.*, (2014a). Prior to TAG analysis, we performed a qualitative determination of lipid classes to our MF samples by silica gel thin later chromatography (TLC) according to Tzompa-Sosa et al (2014a). The TLC plates showed that MF had only TAG and no traces of other lipid compounds were found. Therefore, no further purification was performed.

The MF TAG profile was analysed by the Dutch Milk Controlling Institute Qlip Laboratories (Zutphen, the Netherlands) in accordance with the reference method for analysis and quality evaluation of milk and milk products from the European Commission Regulation (CommissionRegulation(EC)No.273/2008). With this method TAG are separated according to the total number of carbon atoms in the aliphatic chain of the TAG (Christie and Han, 2010). Samples were analysed in duplicate. The concentration of each TAG was determined as a percentage of total TAG content (g/100 g of FA). And the absolute amount of each TAG was calculated using the formula $absolute\,amount\,of\,TAGi = \frac{FY * TAGi}{100}$; where TAG_i is a TAG in g/100 g of FA (from 24 to 54 CN) and FY is fat yield in grams per morning milking.

Statistical Analysis

The effect of FA, milk production traits (fat content, morning milk yield) and DIM on TAG profile was analysed using the model

$$y_{ij} = \mu + \beta\, x_{ij} + e_{ij} \quad (1)$$

The effect of *DGAT1* K232A polymorphism on TAG profile was analysed using the model

$$y_{ij} = \mu + DGAT1_i + e_{ij} \quad (2)$$

where y_{ij} are observations for individual carbon number traits (CN24-54); μ is the overall mean, β is the regression coefficient; x_{ij} is the covariable describing the effect of FA, fat content, morning milk yield or DIM; *DGAT1* is a fixed effect of *DGAT1* genotype; and e_{ij} is the residual, which includes differences on feeding and management.

To account for multiple testing, the Bonferroni correction was used to calculate the appropriate significance level. In total 16 individual carbon number traits (CN24-54) were analysed and therefore $\left(p = \frac{\alpha}{n} = \frac{0.05}{16}\right)$ $p < 0.003$ was considered significant. Data analysis were performed using IBM SPSS statistics software (Version 21; Armonk, NY).

Results and Discussion

Variability of milk fat triacylglycerols

Morning milk yield was on average 12.09 ± 3.2 kg with a fat content of 4.63 ± 0.7 % (Table 1) and the cows in this study were on average 175.81 ± 40.8 DIM. The fat content in our study was similar to the average fat content reported for winter milk in the Netherlands in 2005 (4.6% of fat) (Heck *et al.*, 2009) and to the reported yearly average fat content for the Netherlands in 2010 (4.4% of fat) (Bijl *et al.*, 2013). The MF TAG profile is shown in Table 1 in relative concentrations (g/100 g of FA) and in absolute amounts (g/morning milking). The absolute amount is relevant from a physiological perspective because it indicates the amount (in g) of TAG produced in the mammary gland. TAG CN36, 38, 48 and 50 showed the highest values both in relative concentrations and in absolute amounts. Similar values have been reported in other studies where bulk milk was analyzed (Smiddy *et al.*, 2012, Capuano *et al.*, 2014). The coefficient of variation (C.V.) for relative concentrations was highest (>15%) for low (CN26-30) and high molecular weight TAG (CN52-54) and lowest (< 5%) for TAG CN38, which was the most abundant TAG. When TAG profile was expressed in absolute amounts, the C.V. was high (>20%) for all TAG, which reflects the large variability in fat yield among cows.

Table 1. Milk productive traits and triacylglycerol composition in relative concentration (g/100g of FA) and in absolute amount (g/morning milking) of milk fat from individual cows in a winter diet (N=54)

	Mean (S.D.)		C.V. (%)	Mean (S.D.)		C.V. (%)
Fat content	4.63	(0.71)	15			
Morning milk yield	12.09	(3.16)	26			
DIM	175.81	(40.75)	23			
Triacylglycerols	Relative concentration (g/100g of FA) (N= 54)			Absolute amount (g/ morning milking) (N = 47)[1]		
CN24 + cholesterol	0.25	(0.02)	8	1.4	(0.32)	23
CN26	0.24	(0.05)	21	1.3	(0.45)	35
CN28	0.61	(0.12)	20	3.4	(1.13)	33
CN30	1.26	(0.21)	17	7.0	(2.16)	31
CN32	2.8	(0.37)	13	15.6	(4.36)	28
CN34	6.63	(0.55)	8	36.8	(8.88)	24
CN36	11.63	(0.8)	7	64.2	(14.49)	23
CN38	12.2	(0.59)	5	68.1	(13.67)	20
CN40	9.21	(0.48)	5	50.6	(11.79)	23
CN42	7.53	(0.47)	6	41.8	(9.54)	23
CN44	7.63	(0.56)	7	42.4	(9.45)	22
CN46	8.6	(0.56)	7	47.7	(10.15)	21
CN48	10.09	(0.58)	6	55.4	(11.17)	20
CN50	10.99	(1.01)	9	59.7	(12.31)	21
CN52	7.56	(1.13)	15	40.5	(9.81)	24
CN54	2.79	(0.76)	27	14.6	(4.56)	31

[1] Information on morning milk yield was missing for 7 samples. No absolute TAG amount was calculated for this samples.

Relation between FA and milk fat triacylglycerol profile

The associations of FAs (mol %) in relation to TAG profile measured in relative concentrations (g/100g of FA) (Table 2) and absolute amounts (g/ morning milking) (see supplementary information) was quantified. These effects were higher on relative concentrations than in absolute amounts. It appears that FA with common synthesis pathways have similar effects on TAGs. We showed that an increase in *de novo* FA C6:0 to C14:0 increase TAG CN26-34, CN42-46 as measured in relative concentrations and absolute amounts. In contrast, an increase in FA C6:0 to C14:0 decreased TAG CN 50-52 in relative concentrations. FA C4:0 showed a different effect than the other short chain FA probably, because this FA is synthesized *de novo* from elongation of C2:0 and can also be obtained from blood derived FA from rumen fermentation. Moreover, an increase in FA C14:1cis-9 and C16:1cis-9, increased TAG CN 44 and 46 in relative concentration. An increase in long chain saturated FA C16:0 and C18:0 decrease low molecular weight TAG and increase high molecular weight TAG but this effect is only seen when TAG is measured in relative concentrations.

Non-linear relationships between FA and TAG were also tested by extending model 1 with a quadratic component. For C4:0 and C6:0 we found a significant quadratic relation with CN38 ($p < 0.001$ and $p = 0.003$, respectively). This means that the concentration of FA C4:0 and C6:0 on TAG CN38 increases until reaching the maximum possible concentration of these FA on TAG CN38, which is about 12.8 g/100 g of FA. After reaching the maximum concentration of TAG CN38, FA C4:0 and C6:0 continued to be esterified at TAG CN38, but the concentration of TAG CN38 will not be increased.

We also studied the association between the saturation index (SFA/UFA) and the ratio C16:0/C18:1cis-9 with the TAG profile. The saturation index summarizes the effect of all saturated and unsaturated FA. C16:0 and C18:1cis-9 are the two most abundant FA, they are negatively correlated and have an opposite effect on physical properties of MF such as solid fat content. Therefore the relation between the ratio C16:0/C18:1cis-9 and the TAG profile was studied. Both the saturation index and C16:0/C18:1cis-9 ratio, showed a significant linear relation with TAG CN34, 36, 52 and 54 (Figure 2). We showed that the difference on TAG CN34, 36, 52 and 54 among individual cows can be large (Figure 2). For instance a difference of 4.8% in TAG CN36 was seen between the maximum and the minimum concentration in MF. The relation between C16:0/C18:1cis-9 ratio and TAG profile found in our study for a dairy cow population are in agreement with other studies investigating the TAG composition in diets rich in unsaturated C18 FA (DePeters *et al.*, 2001, Capuano *et al.*, 2014). These studies showed that a diet rich in unsaturated C18 FA increase TAG CN40, 50, 52 and 54 and decrease TAG CN32, 34, and 36. Furthermore, an

Table 2. Linear effects of *DGAT1* K232A polymorphism, milk productive traits, DIM and FA on the relative concentrations of TAG (g/100 g of FA). Effects in bold are considered significant at a level P < 0.003[1] (N =54).

Triacylglycerol	*DGAT1* K232A [2]	Morning milk Yield	Fat content	DIM	C4:0	C6:0	C8:0	C10:0	C12:0	C12:1 cis-9	C14:0	C14:1 cis-9	C15:0	C16:0	C16:1cis-9	C18:0	C18:1cis-9	Total C18:1trans	Saturation Index	Ratio 16:0/C18:1cis-9
CN24[3]						**0.032*** **	**0.06*** **	**0.02** **												
CN26		0.01*			0.02*	**0.14*** **	**0.22*** **	**0.07*** **	**0.04*** **	0.49**				**-0.01*** **		-0.01*		0.05*		
CN28		0.013*			0.05*	**0.33*** **	**0.56*** **	**0.18*** **	**0.10*** **	1.19**	0.05*			**-0.02*** **		-0.03*				
CN30		0.02*				**0.53*** **	**1.00*** **	**0.34*** **	**0.20*** **	**2.33** **	**0.11*** **			-0.03**		**-0.08*** **				
CN32		0.036*				**0.79*** **	**1.58*** **	**0.57*** **	**0.41*** **	**4.66*** **	**0.26*** **			-0.04*		**-0.19*** **	-0.08*			
CN34						**1.12*** **	**1.75*** **	**0.61*** **	**0.49*** **	4.60*	**0.35*** **		-1.06*			**-0.35*** **	**-0.22*** **		**0.67*** **	**0.63** **
CN36			0.31*		**0.51*** **	**1.56*** **							-1.51*	**0.20*** **		**-0.30** **	**-0.41*** **	-0.95*	**1.40*** **	**1.78*** **
CN38	-0.41**				**0.59*** **	**1.65*** **	0.96*		-0.27*		**-0.35*** **						-0.15**		0.48*	0.57*
CN40		0.05*			0.26**	**1.35*** **	**2.04*** **	**0.53*** **						**-0.12*** **				0.64**		-0.52**
CN42					**-0.27** **		**1.94*** **	**0.93*** **	**0.54*** **	**5.36** **	**0.39*** **					**-0.21*** **	**-0.15*** **		0.39*	
CN44					**-0.49*** **		1.02*	**0.71*** **	**0.65*** **	**8.60*** **	**0.49*** **	0.82**			0.58*	**-0.31*** **	-0.15**			
CN46					**-0.52*** **	**-1.13*** **			**0.45*** **	**8.15*** **	**0.35*** **	**1.04*** **			**0.96*** **	**-0.28*** **		-0.61*		
CN48					**-0.47*** **	**-1.87*** **	**-2.41*** **	**-0.60*** **					1.29*	**0.09** **	0.67*			-0.60*		
CN50		-0.10*				**-2.51*** **	**-4.67*** **	**-1.52*** **	**-1.02*** **	**-13.74*** **	**-0.52** **	-1.33*		0.12*		**0.40*** **	0.24*			
CN52						**-1.76** **	**-3.65*** **	**-1.40*** **	**-1.11*** **	**-13.46*** **	**-0.70*** **					**0.76*** **	**0.56*** **	1.17*	**-1.57*** **	**-1.69*** **
CN54									**-0.50*** **		**-0.37*** **			**-0.19*** **		**0.47*** **	**0.43*** **	**1.42*** **	**-1.37*** **	**-1.67*** **

[1] Bonferroni correction was used to calculate the appropriate significance (P = α/N = 0.05/16 = 0.003)

[2] *DGAT1* KK genotype is set to zero

[3] Including cholesterol content

increased C16:0/C18:1cis-9 ratio implies an increase in C16:0 and a decrease in C18:1cis-9, which could increase MF melting point above the cow's temperature. The cow might overcome this problem by increasing the concentration of low molecular weight TAG (CN34 and 36) and decreasing the amount of high molecular weight TAG (CN52 and 54).

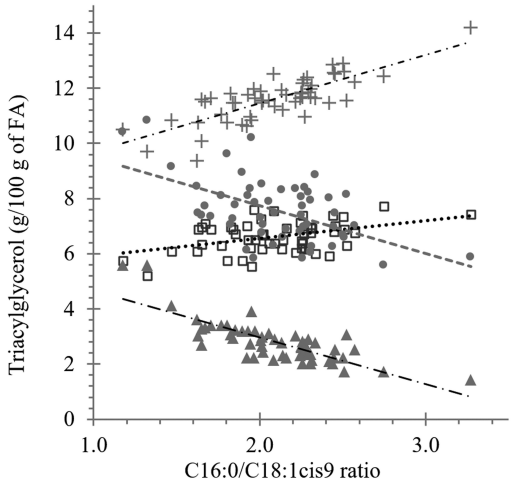

Figure 2. Change of concentration of milk fat triacylglycerols with a change in C16:0/C18:1cis-9 ratio (N = 54). CN34 ($p < 0.001$); CN36 ($p < 0.001$); CN52 ($p < 0.001$); CN54 ($p < 0.001$); □ CN34; + CN36; ● CN52; ▲ CN54.

The changes in TAG profile seen in this study can have an impact on the mechanisms of MF crystallization. Crystallization starts with nucleation, which is the formation of tiny embryonic crystals called nuclei. Nucleation is related to the concentration of TAGs, since an appreciable number of TAGs (supersaturation) should be present to form a nuclei that can further grow into a crystal. This means that nucleation will occur faster in MF with high concentrations of TAGs able to nucleate at the given temperature. Further studies on crystallization behaviour of MF are needed to elucidate the concrete effects of differences in MF TAG and FA profile.

Effect of *DGATI* K232A polymorphism and productive traits on MF TAG profile

The effect of *DGAT1* K232A polymorphism on TAG profile was tested in relative concentration (g/100g of FA) (Table 2) and absolute amounts (g/ morning milking) (See supplementary information). We have shown that *DGAT1* K232A polymorphism had a significant effect on the TAG CN38, which is the most abundant TAG. Cows with *DGAT1* KK genotype are increased in this TAG (Figure 3). In the present study, samples were selected in such a way that *DGAT1* AA and KK have similar fat content. Therefore effects of the *DGAT1* K232A polymorphism in the current study

represent effects adjusted for differences in fat content. A random sample from the population would show larger differences between *DGAT1* KK and AA genotypes for fat content and fat composition and corresponding effects on TAG.

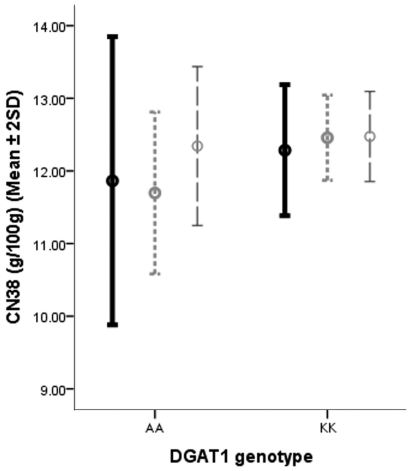

Figure 3. Relative concentration (g/100 g of fat) of TAG CN38 grouped per fat content and *DGAT1* genotype. fat content 3-3.9%; fat content 4-4.9%; fat content 5-6%.

The K allele of *DGAT1* is associated with increased levels of TAG CN38. The average difference of TAG CN38 in absolute amount between genotypes (AA and KK) was 9.49 g/morning milking and in relative amounts the average difference was of 0.41 g/100g of FA. This difference in TAG CN38 alone might not be enough to promote a change in the physical properties of MF. However it shows that cows with *DGAT1* KK genotype produced higher amount of TAG CN38 suggesting that changes are happening in MF TAG synthesis. *DGAT1* K allele has been associated with an increase in C16:0 FA (Schennink *et al.*, 2007). It is known that TAG species 6:0-16:0-16:0, 4:0-16:0-18:0 and 4:0-16:0-18:0 are the main TAG subspecies in TAG CN38 (Gresti *et al.*, 1993). We suggest that part of the excess of C16:0 in cows with *DGAT1* KK genotype is being esterified in TAG CN38 together with short chain FA. In this way, the TAG can decrease its melting point. This mechanism could help the cow to regulate the melting point of MF and overcome the increase in melting point produced by an overall increase in C16:0. A detailed analysis on subspecies of TAG CN38 can help to further understand the alterations in MF TAG synthesis caused by *DGAT1* K232A polymorphism.

Previously we discussed the quadratic effect of FA C4:0 and C6:0 on TAG CN38. We further investigated the interaction between *DGAT1* K232A polymorphism and C4:0 and C6:0 on TAG CN38. We showed that these interactions were significant ($p < 0.001$) (Figure 4). Figure 4 shows the relation between TAG CN38 and FA C4:0

and C6:0. It is shown that both genotypes of *DGAT1* follow the same trend. However, cows with *DGAT1* KK genotype are increased in TAG CN38 and tend to contain a higher concentration of FA C4:0 and C6:0 as compared with *DGAT1* AA genotype. This confirms our previous suggestion that species of TAG CN38 from cows with *DGAT1* KK are increased in short chain FA and most likely esterified with FA C16:0.

In the present study, DIM and productive traits, namely fat content and morning milk yield, had no significant effects on TAG profile (Table 2) indicating that these traits do not affect MF TAG synthesis. This is a relevant observation since a considerable increase of milk yield and fat content has been seen in the Netherlands over the last 60 years (Bijl *et al.*, 2013).

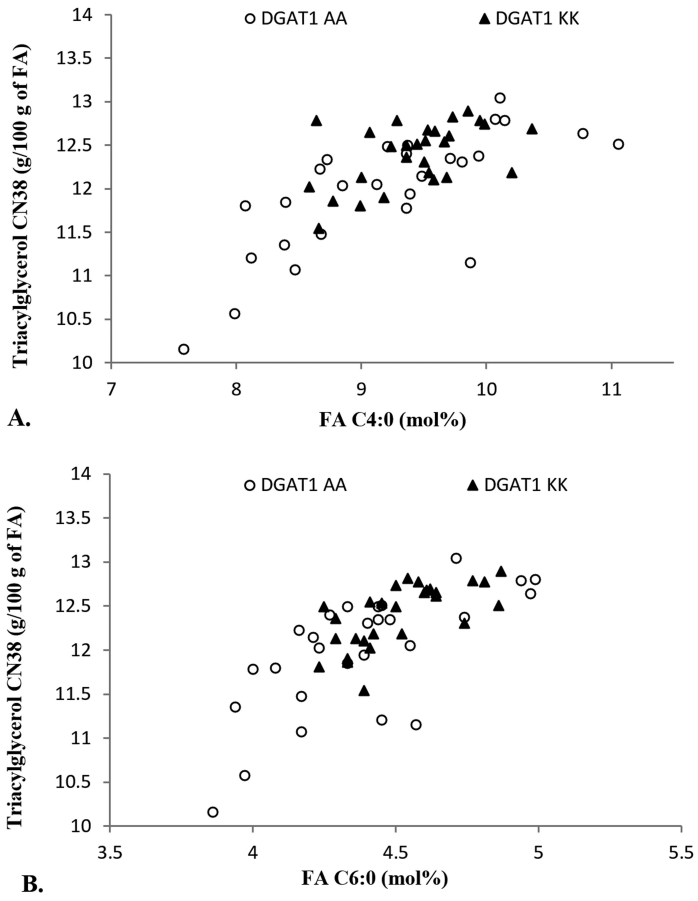

Figure 4. Quadratic effect between milk fat TAG CN38 and FA C4:0 (A) and C6:0 (B).

Conclusions

The present study provides insight into the variability of MF TAG profile of individual cows. We showed that saturation index (SFA/UFA) and the ratio C16:0/C18:1cis-9 significantly affect TAG CN34, 36, 52 and 54. An increase in the saturation index and in the ratio C16:0/C18:1cis-9 increased the relative concentration of TAG CN34 and 36 and decreased TAG CN52 and 54. *DGAT1* K232A polymorphism significantly affected TAG CN38. Cows with *DGAT1* KK genotype were increased in TAG CN38, measured both in relative concentration and in absolute amounts. We suggest that part of the excess of C16:0 in cows with *DGAT1* KK genotype caused by K allele is being esterified in TAG CN38 together with short chain FA C4:0 and C6:0. In this way, MF will have a decreased melting point diminishing the effect of a higher C16:0 concentration in MF. The productive traits, including DIM, fat content and milk yield, had no significant effects with TAG profile. Overall, large differences in MF TAG profile were seen among cows. These differences in MF TAG profile imply differences in its physical properties.

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Table S1. Linear effects of *DGAT1* K232A polymorphism, milk productive traits, DIM and FA on the abolute amounts of TAG (g/morning milking). Effects in bold are considered significant at a level P < 0.003[1,2] (N = 47).

Triacylglycerol	*DGAT1* K232A[3]	Fat content	DIM	C4:0	C6:0	C8:0	C10:0	C12:0	C12:1 CIS9	C14:0	C14:1 CIS9	C15:0	C16:0	C16:1CIS9	C18:0	C18:1CIS9	Total C18:1trans	Saturation Index	Ratio 16:0/ C18:1cis9
CN24[4]	-0.16*				0.44*	0.734**	0.21*	0.16*					-0.05*						
CN26	-0.24*				**0.93***	**1.60***	**0.47***	**0.33***	3.82*				**-0.10***				0.60*		
CN28					**2.26***	**4.09***	**1.27***	**0.86***	9.31*				**-0.24***				1.41*		
CN30					**3.91***	**7.47***	**2.42***	**1.74***	18.21*	0.87*			**-0.43****		-0.62*				
CN32					6.54**	**12.89***	**4.31***	**3.69***	37.61*	1.97*			-0.78**		-1.38*				
CN34					12.00 *	19.66**	6.01*	**5.78****											
CN36	-6.82*			7.22*	19.49*														
CN38	**-9.49****			7.15*	20.23*												11.86*		
CN40	-6.44**				15.33*	24.81**		5.35*					-1.96**						
CN42						21.82**	8.26**	**6.64***		3.64*			-1.45**						
CN44						16.30*	6.87*	**7.18***	78.59*	4.13*			-1.40*						
CN46								**6.42****					-1.36*						
CN48	-4.24																		
CN50	-5.26*																		
CN52	-5.43*		-0.07*										-1.26*		3.36*	2.79*		-7.43*	-11.37**
CN54	-3.28**												**-1.13***		1.67**	1.82***	7.88**	**-5.84***	**-8.4***

[1] Bonferroni correction was used to calculate the appropriate significance (p = α/N = 0.05/16 = 0.003)
[2] Information on morning milk yield was missing for 7 samples. No absolute TAG amount was calculated for this samples.
[3] *DGAT1* KK genotype is set to zero
[4] Including cholesterol content

Chapter 3

Influence of C16:0 and long-chain FA on normal variation of bovine MF TAG structure

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Abstract

Fatty acids (FA) are non-randomly distributed within milk fat (MF) triacylglycerols (TAG). Moreover, the structure of MF TAG differs with feeding regimens. So far, nothing is known about the variation of MF TAG structure among individual cows. A deep understanding of the normal variation of TAG structures and the relationships between MF FA profile and its TAG structure could help to better control functional and compositional differences between MFs from various sources and to increase the knowledge on MF synthesis. The focus of the present study was to determine the regiospecific TAG structure of individual samples of winter MF from Dutch Holstein-Friesian cows with a wide variation of FA profiles and with 2 diacylglycerol acyltransferase 1 (*DGAT1*) genotypes: *DGAT1* K232A genotype AA and *DGAT1* K232A genotype KK. From an initial set of 1,918 individual MF samples, 24 were selected. The selected samples had a wide range of FA composition and had either *DGAT1* K232A genotype AA or KK. The structure analysis was done with a regiospecific approach. This analysis is based on the acyl degradation of TAG by a Grignard reagent and further isolation of sn-2 monoacylglycerols by thin layer chromatography. An intra- and interpositional approach was used to study the structural variation. With the intrapositional approach, the amount of an FA at the secondary (sn-2) and primary (sn-1,3) positions was related to its total amount in the TAG. With the interpositional approach, the proportion of C8:0, C10:0, C14:1 cis-9, C16:1 cis-9, and C18:1cis-9 at sn-2 was positively correlated with the amount of C16:0 in the triacylglycerol; in contrast, saturated C14:0, C16:0, and long-chain saturated FA (C14:0–C18:0) were negatively correlated. These observations suggest that the amount of long-chain saturated FA in TAG influences the positioning of other FA in the TAG. With an interpositional approach, the *DGAT1* polymorphism had a significant effect on the proportional positioning of C16:0 at sn-2. These results provide a new direction to controlling functional and compositional differences between MFs.

Keywords: *DGAT1*, triacylglycerol structure, regiospecific analysis, glycerol-3-phosphate acyltransferase

Introduction

Bovine milk fat (MF) is mainly composed (98%) of triacylglycerols (TAG), which are formed of three FA esterified to a glycerol backbone (Jensen, 2002). The most abundant FA in bovine MF are C16:0 (on average 32.6 wt%), C18:1cis-9 (on average 18.0 wt%), C14:0 (on average 11.6 wt%) and C18:0 (on average 8.7 wt%) (Heck *et al.*, 2012). The abundance of these FA is related to the cow´s diet and to their genetic characteristics (Palmquist *et al.*, 1993, Schennink *et al.*, 2007). A diet rich in fresh grasses, for instance, will increase the amount of unsaturated fatty acids (UFA) such as C18:1, omega-3 and omega-6 FA in MF; in contrast, a diet based on maize-silage or rich in hay will increase the amount of saturated fatty acids (SFA) in MF (Palmquist, 2006, van Valenberg *et al.*, 2013). Genetic differences also play a role in the FA abundance. For instance, the *DGAT1* K232A polymorphism has been associated with an increase in C16:0 and a decrease in C14:0, unsaturated C18:1 and conjugated linoleic acid (Schennink *et al.*, 2007). Moreover, C16:0 and C18:1cis-9 are negatively correlated and its concentration is affected by the fat content of MF (Stoop *et al.*, 2008).

Fatty acids within bovine MF TAG are non-randomly distributed (Parodi, 1979b). A regiospecific approach can be used to study the distribution of FA in the TAG, which allows a differentiation of FA esterified in the primary (sn-1,3) and secondary position (sn-2). In MF TAG, short chain FA and C18:0 are predominantly located at sn-1,3, while C14:0 is predominantly located at sn-2. Furthermore, C16:0 and C18:1cis-9 are abundant at sn-1,3 and sn-2 (Angers *et al.*, 1998, Blasi *et al.*, 2008). The distribution of FA within the TAG can be affected by the nutrition of dairy cows (Morrison and Hawke, 1977, Parodi, 1979b, Christie and Clapperton, 1982), which can vary with the season. However, no differences in distribution of FA on TAGs in MF has been reported between seasons (Parodi, 1979b) which can be a consequence of pulling the milk from different farms. Hence, in pooled milk the seasonality is lost due to a variety of breeds, feeding regimes and lactation stages. A modification in the TAG structure suggests that the distribution of FA could be triggered by differences in blood derived FA or by changes in the activity of enzymes related to fat synthesis that respond to availability of FA for TAG synthesis.

The positioning of the FA in the TAG affects the metabolic fate of ingested FA. Saturated FA at the secondary position are absorbed more efficiently than saturated FA at the primary positions (Kallio *et al.*, 2006) because FA in the secondary position will be absorbed as 2-monoacylglycerol (2-MAG) and this leads to better emulsification properties during digestion (Mattson *et al.*, 1979). The positioning of FA at the secondary position becomes relevant for new born babies. It has been reported that infants fed with formulas in which C16:0 is mainly in the primary position

present a lower absorption of this FA than when C16:0 is placed at the secondary position (Tomarell *et al*., 1968, Lopez-Lopez *et al*., 2001).

A deep understanding of the normal variation of TAG structures and the relation between MF FA profile and its TAG structure could help to better control functional and compositional differences between MFs from various sources. In this study, we determined the TAG structure of individual samples of winter MF from 24 Dutch Holstein Friesian cows, 13 with the *DGAT1* K232A genotype AA and 11 with the *DGAT1* K232A genotype KK. Our aim was to determine if differences in MF TAG structure exist among individual cows and to determine if MF FA profile is related to the structure of MF TAG.

Materials and Methods

Sample Selection of Individual Milk Fat

The samples used in this study were part of the Dutch Milk Genomics Initiative where morning MF samples were collected from 1918 first lactation Holstein-Friesian cows on 398 commercial herds in the Netherlands (Schennink *et al.*, 2007, Heck *et al.*, 2012). We studied MF from the months February and March, which corresponds to winter season in the Netherlands. The typical winter diet in the Netherlands consists of 31% concentrate, 44% grass silage, and 25% corn silage (Heck *et al.*, 2009). We assured a wide range of FA composition by randomly selecting MF samples from individual cows with a fat content within a range of 2.85-5.76% and with either *DGAT1* K232A genotype AA or KK. Genotyping of the *DGAT1* K232A polymorphism was performed with a TaqMan allelic discrimination assay (Applied Biosystems Inc., Foster city, CA) and was performed as part of a previous study (Schennink *et al.* (2007). .. In total, 24 cows were selected, 13 with *DGAT1* K232A genotype AA and 11 with *DGAT1* K232A genotype KK. To avoid confounding with herd effects in our study, all the 24 selected MF samples belonged to different commercial herds.

Fat Extraction of Milk Fat

To extract the fat fraction from milk, we warmed 100 mL of milk in a water bath at 28°C. Then 5 mL of HCl (4M) was added to the milk and shaken for 45 min until fat was clearly separated. Next, the fat phase was washed with cold water. Finally, the fat phase was poured into a centrifuge tube and warmed in an oven at 70°C for 60 min and then centrifuged at 3500 rpm for 10 minutes. A clear fat phase was present at the top of the centrifuge tube. This fat was used for regiospecific structure analysis.

Regiospecific Structure Analysis of Milk Fat

The regiospecific distribution of TAG was determined by a chemical degradation with a Grignard reagent and further isolation of 2-MAG by preparative thin layer chromatography (TLC) (Christie and Moore, 1969). 2-MAG obtained after Grignard degradation have been proven useful for structure analysis of natural TAG (Becker *et al.*, 1993, Angers *et al.*, 1998, Turon *et al.*, 2003). These acyl moieties allow direct determination of the composition of the native TAG. In this study, ethyl magnesium bromide was used to degrade TAG from MF. The degradation degree of TAG was of 62%. After degradation, sn-2 MAG were isolated by preparative TLC and its FA composition was determined by capillary gas chromatography with flame ionization detector (GC-FID). The FA composition of sn-1,3 was calculated from the

composition of sn-2 MAG and TAG. The TAG degradation was done as follows, 50 mg of anhydrous MF was weighted in an oven dried 10 mL vial hermetically closed with a Teflon cap. Then, 2 mL of dry diethyl ether was added to dissolve the fat. The fat was allowed to react for 30 s with 200 µL 3M ethyl magnesium bromide in dry ether (Aldrich, The Netherlands, CAS nr 925-90-6) while being stirred; the reaction was stopped with 50 µL of glacial acetic acid followed by 1.5 mL of 10% boric acid solution. The acyl moieties were extracted 3 times with 2mL diethyl ether saturated with boric acid and subsequently washed 3 times with 2% sodium bicarbonate, water and brine (sodium chloride saturated aqueous solution). The water phase from the mixture was removed using a separator funnel. Then, the solvent phase was dried under nitrogen. To separate the acyl moieties, the residue was dissolved in 100 µL of diethyl ether and applied on a silica gel plate (20x20 cm, 500 microns thickness) (Analtech Inc., Newark, DE) previously sprayed with 5% boric acid in methanol. The plates were developed using chloroform/acetone (85:15, v/v) 1% acetic acid solution (Turon *et al.*, 2002). Two references for MAGs were used: 2-oleoylglycerol and 1-oleoyl-rac-glycerol (Sigma-Aldrich, The Netherlands). Iodine vapour was used to stain the acyl moieties. In the TLC plate, sn-2 MAGs (Rf = 0.37) ran ahead of sn-1,3 MAG (Rf = 0.29). The band corresponding to sn-2 MAG was scraped from the silica plates and transferred to 10 mL test tubes vials. The glycerides were extracted from the silica gel two times with diethyl ether aided by sonication (10 min). Precipitation of the silica was achieved by centrifuging 10 min at 14,000 rpm. The glycerides (4-5 mg) corresponding to sn-2 fraction were dissolved in diethyl ether, were transferred to a 900 µL vial and the solvent was evaporated under a stream of nitrogen.

The FA composition of the isolated sn-2 MAG and of the unreacted fat was analysed at the Dutch Milk Controlling Institute Qlip Laboratories (Zutphen, The Netherlands) in accordance with the international standard ISO 15885:2002/IDF 184:2002. For both samples, approximately 5 mg was methylated and analysed for FA composition with GC-FID with a WCOT fused silica column (CP WAX 52 CB, 30 m x 0,25 ID, DF = 0,25 µm, Agilent Technologies). This column allows the separation of the 12 most abundant FA.

Calculations for intra- and interpositional approach

Intrapositional approach. With this approach the amount of a FA reported is relative to all FA at that position. Therefore, the sum of the proportions of all FA present at each position will sum 100%; with this approach, the total amount of a FA in the TAG is not considered.

The FA profile of sn-2 MAG, recovered after Grignard degradation, and the FA profile of the unreacted fat were obtained in g/100g and were transformed to mol%. Both profiles were used to calculate the FA profile at sn-1,3 by using the formula:

$$\text{position}1,3_{FA\ 1} = \frac{3 * TAG_{FA\ 1} - \text{position}2_{FA\ 1}}{2}$$ (Christie, 2003),

where position 2 is the amount of a FA at sn-2 and TAG the amount of the same FA in the unreacted TAG.

Interpositional approach. With this approach the distribution of one FA over the three positions is determined. The FA profiles obtained in the intrapositional approach were used to calculate the proportion of a FA placed at sn-2 and sn-1,3. The formulas used were:

$$\%\ sn2_{FA\ 1} = \frac{\text{position}2_{FA1}}{\text{position}2_{FA\ 1} + (\text{position}1,3_{FA\ 1} * 2)} * 100$$

$$\%sn1,3_{FA\ 1} = \frac{\text{position}1,3_{FA\ 1} * 2}{\text{position}2_{FA\ 1} + (\text{position}1,3_{FA\ 1} * 2)} * 100\ ;$$

where position 2 was the amount of a FA at sn-2 (in mol%) and position 1,3 was the amount of the same FA at sn-1,3 (in mol%).

Statistical Analysis

Analysis were performed using IBM SPSS statistics software (version 21; IBM Corp., Armonk, NY). Concentrations and proportions of FA in the TAG and at sn-2 and sn-1,3 were subjected to bivariate Person correlations. The effect of *DGAT1*, fat content, FA in the TAG and their interactions on FA at sn-2 were estimated using the model:

$$yij = \mu + DGAT1i + \beta 1\ Fij + \beta i2\ (DGAT1i * Fij) + eij$$

where y_{ij} is the dependent variable, μ is the overall mean, *DGAT1* is a fixed effect of *DGAT1* genotype 'i' (AA or KK), β_1 is the regression coefficient relative to F_{ij}; F_{ij} is the covariable describing the effect of the concentration of an FA in the TAG or fat content, β_{i2} are the regression coefficients relative to the concentration of an FA in the TAG or fat content within the *DGAT1* genotypes; ($DGAT1_i \times F_{ij}$) describes the interaction between estimated effects of *DGAT1* K232A (AA or KK genotypes) and the concentration of an FA in in the TAG or fat content; and e_{ij} is the residual effects, which are assumed to be normally distributed. A significance level of $P < 0.05$ was used throughout the study.

Results

Fatty acids in MF TAG are non-randomly distributed (Parodi, 1979b). The distribution of FA within the TAG molecule has been previously reported with an intra- and interpositional approach (Blasi *et al.*, 2008). While the intrapositional approach helps to identify the most abundant FA at one position, the interpositional approach helps to understand the distribution of each FA over the positions in the TAG. In this study, we report and discuss our results based on both approaches (Table 1).

Intrapositional Approach

In this study we found that the most abundant FA at sn-2 are C14:0, C16:0 and C18:1cis-9; and the most abundant FA at sn-1,3 are C4:0, C16:0 and C18:1cis-9 (Table 1). Short chain FA such as C4:0-C8:0 are almost absent at sn-2. C14:0, C16:0, C18:0 and C18:1cis-9 showed the highest variability in its distribution over sn-2 and sn-1,3; these FA are also the most abundant FA in the TAG. In this study, we found significant positive correlations between the total amount of a FA in the TAG and its distribution over sn-1,3 and sn-2 (Table 2). Short, middle and long chain FAs, for instance, have a positive correlation between its total amount in the TAG and the amount present at sn-1,3. The total amount of C12:0 in the TAG is positively correlated with its amount at sn-2. Moreover, significant negative correlations were found between the total amount of a FA in the TAG and the amount of other FA at sn-2 and sn-1,3 (Table 2). For instance, the total amount of C16:0 in the TAG is negatively correlated to the amount of C6:0, C8:0, C10:0 and C18:1cis-9 at sn-1,3 and to the amount of C14:0 and C18:0 at sn-2.

Table 1. Total fatty acid composition and regiospecific distribution of fatty acids from milk fat triacylglycerols with an intra- and interpositional approach. Means ± standard deviation, (n=24). Literature data from (1) Turon, F. *et al.* 2002 and (2) Blasi, F. *et al.* 2008, (3) Morrison and Hawke; sn-1,3 data from Blasi, F. *et al.* 2008 were calculated by author.

Compound	Triacylglycerol (mol%)				Intrapositional (mol%)				Interpositional (% mol)		
Position	α,β,α'				sn-2[4] β		sn-1,3[5] α,α'		sn-2 %[6] β		sn-1,3 %[7] α,α'
	Min.	Max	Average ±S.D.	Literature[1/2]	Average ±S.D.	Literature[1/2]	Average ±S.D.	Literature[1/2]	Average ±S.D.	Literature[2/3]	Average ±S.D.
Fat content	2.9	5.8	4.32 ± 0.82								
Saturation index[8]	2.4	3.9	3.31 ± 0.43								
C4:0	8.3	10.9	9.40 ± 0.78	7.6/6	-	-/0.4	14.10 ± 1.17	11.4/8.8	-	2.2/0	100
C6:0	3.9	5.2	4.45 ± 0.32	4.2/2.9	0.2 ± 0.06	0.4/0.9	6.58 ± 0.51	6.1/3.9	1.48 ± 4.08	10.1/12	98.52 ± 4.08
C8:0	2	2.6	2.15 ± 0.17	2.1/1.7	0.47 ± 0.75	1.9/0.2	2.99 ± 0.43	2.2/2.5	7.16 ± 11.51	4.7/52	92.84 ± 11.52
C10:0	3.3	4.5	3.88 ± 0.32	4.5/3.4	2.59 ± 1.88	6.9/1.4	4.52 ± 1.02	3.3/4.4	22.33 ± 16.14	12.4/51	77.67 ± 16.14
C12:0	3.1	7.2	4.61 ± 0.85	4.9/3.9	4.93 ± 1.77	7.6/4.8	4.46 ± 1.17	3.6/3.5	35.87 ± 11.86	39.1/60	64.13 ± 11.87
C14:0	9.7	13.3	11.31 ± 0.90	-	16.73 ± 3.66	20.3/22.8	8.59 ± 1.97	9.7/8.3	49.39 ± 10.6	53.5/63	50.61 ± 10.59
C14:1 cis-9	0.9	1.9	1.34 ± 0.23	-	0.34 ± 0.5	-	1.85 ± 0.38	-	8.25 ± 11.76		91.75 ± 11.76
C15:0	0.8	1.5	1.11 ± 0.18	-	0.9 ± 0.58	-	1.21 ± 0.35	-	27.24 ± 17.09		72.76 ± 17.09
C16:0	22.3	32.5	27.90 ± 3.14	32.4/31.6	40.73 ± 4.45	42.9/44.1	21.48 ± 4.94	27.1/25.4	49.22 ± 7.48	42.8/43	50.78 ± 7.48
C16:1 cis-9	0.8	1.9	1.24 ± 0.26	-/1.8	0.49 ± 0.58	-/2.5	1.62 ± 0.48	-1.5	13.37 ± 15.35	42.2/-	86.63 ± 15.32
C18:0	4	10.1	7.41 ± 1.15	9.7/6.6	11.47 ± 3.45	6.2/5.3	5.36 ± 2.15	1.5/7.3	50.24 ± 19.53	24.2/18	49.76 ± 19.53
C18:1cis-9	17.6	23.3	17.05 ± 2.81	13.3[9]/19.2	15.11 ± 2.89	9.6/15.7[9]	16.34 ± 4.26	15.1/21[9]	33.54 ± 7.25	24.8/24[9]	66.46 ± 7.26

[4]Isolated fraction obtained after Grignard degradation of milk fat triglycerides

[5]Calculated from the FA profile from sn-2 and TAG using the formula $\text{position } 1,3_{FA\,1}=\frac{3*TAG_{FA\,1}-\text{position } 2_{FA\,1}}{2}$

[6]Calculated from the FA profile of sn-2 and sn-1,3 in mol% using the formula $\%\ sn2_{FA\,1}=\frac{\text{position } 2_{FA\,1}}{\text{position } 2_{FA\,1}+(\text{position } 1,3_{FA\,1}*2)}*100$

[7]Calculated from the FA profile of sn-2 and sn-1,3 in mol% using the formula $\%\ sn1,3_{FA\,1}=\frac{\text{position } 1,3_{FA\,1}*2}{\text{position } 2_{FA\,1}+(\text{position } 1,3_{FA\,1}*2)}*100$

[8] $Saturation\ index=\frac{Total\ saturated\ FA}{Total\ unsaturated\ FA}$

[9]Reported as C18:1 total

Table 2. Significant correlations between amount (mol%) of a fatty acid in the triglyceride and the amount (mol%) at sn-2 and sn-1,3.

	Triacylglycerol (mol%)												
	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1 cis-9	C15:0	C16:0	C16:1 cis-9	C18:0	C18:1cis-9	LCSFA[1]
sn-2 (mol%)													
C4:0													
C6:0												-0.37†	
C8:0									0.44*				0.46*
C10:0									0.41*				0.48*
C12:0					0.46*	0.44*						0.43*	
C14:0			0.47*	0.44*					-0.38†				-0.42*
C14:1 cis-9									0.39†				0.43*
C15:0													
C16:0										0.36†			
C16:1 cis-9									0.47*				0.52**
C18:0	0.41†	0.38†						-0.43*	-0.38†				
C18:1cis-9							-0.46*				0.49*		
LCSFA													
sn-1(3) (mol%)													
C4:0	1**	0.84**	0.41**			-0.34†		-0.40†		-0.40†			
C6:0	0.78**	0.83**	0.68**						-0.37†				-0.43*
C8:0			0.50**	0.37†					-0.54**				-0.57**
C10:0				0.40†					-0.42*				-0.48*
C12:0				0.37†	0.74**	0.54**					-0.37†		
C14:0		-0.36†	-0.51*			0.44*			0.36†	-0.38†			0.49*
C14:1 cis-9							0.77**				-0.44*		
C15:0								0.58*		0.60*	-0.43†		0.55**
C16:0								0.34†	0.9**		-0.36†	-0.36†	0.89**
C16:1 cis-9								0.46*		0.80**			
C18:0					-0.38†						0.63**		
C18:1cis-9									-0.68**				-0.74**
LCSFA[1]													0.87**

**Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

†Correlation is significant at the 0.10 level (2-tailed).

[1] Long chain saturated fatty acids from C14:0 to C18:0

Interpositional Approach

The interpretation of the variability of TAG structures within a cow population is easier with an interpositional approach because it places all the FA under the same scale (0-100%). With the interpositional approach we found that the preferential positioning of FA in the TAG varies according to the FA carbon length and its unsaturation level. Middle and long chain SFA such as C12:0, C14:0, C16:0, C18:0 prefer the sn-2 position (Figure 1). Meanwhile, short chain FA and UFA, such as C4:0-C8:0 and C14:1, C16:1 and C18:1, prefer the sn-1,3 position. The interpositional approach did not show significant correlations between the total amount of specific FA in the TAG and their amount at sn-2 or sn-1,3, except for C16:0. The proportion of most FA at sn-2 significantly correlated with the amount of C16:0 and long chain SFA (C14-C18) in the TAG (Table 3).

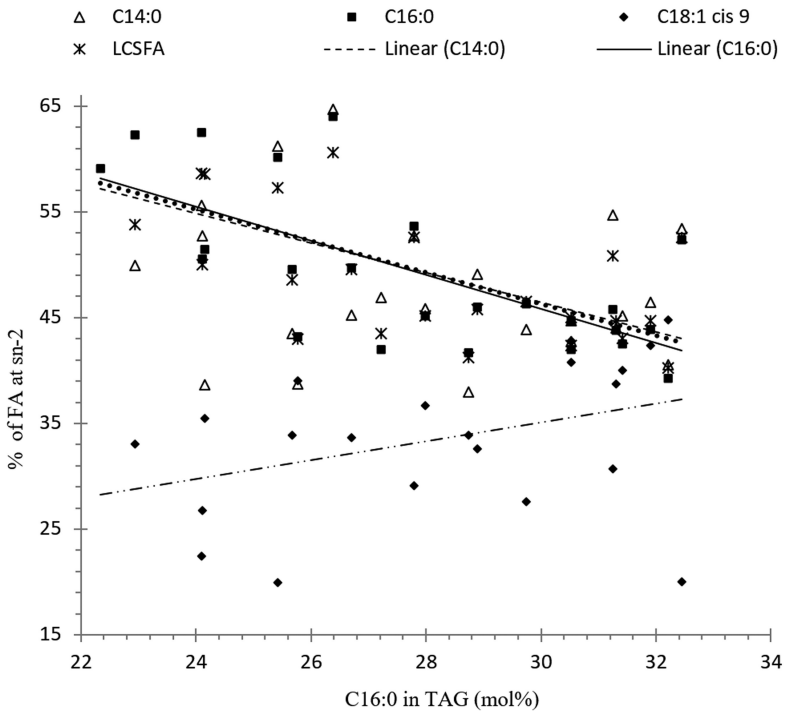

Figure 1. Percentage of C14:0, C16:0 and C18:1cis-9 esterified at sn-2 and its relation to the total amount of C16:0 in the TAG (mol%). % C14:0 at sn-2 = 88.327-1.396*C16:0 in TAG;R^2 = 0.17; p=0.04
% C16:0 at sn-2 = 94.134-1.61* C16:0 in TAG ;R^2 = 0.45; p<0.0001
% C18:1cis-9 at sn-2 = 8.367+0.891* C16:0 in TAG;R^2 = 0.13; p = 0.1
% long chain saturated fatty acids at sn-2 = 110.02-2.198*C16:0 in TAG; R^2=0.37; p = 0.002

Table 3. Significant correlations between the amount (mol%) of fatty acids in the triglyceride and the proportion of them at sn-2.

	Triacylglycerol (mol%)												
	C-4:0	C-6:0	C-8:0	C-10:0	C-12:0	C-14:0	C-14:1 cis-9	C-15:0	C-16:0	C-16:1 cis-9	C-18:0	C18:1cis-9	LCSFA
% sn-2													
C4:0													
C6:0													
C8:0									0.44*				0.47*
C10:0									0.40†				0.47*
C12:0													
C14:0			0.524**	0.34†					-0.41*				-0.50*
C14:1 cis-9									0.47*				0.50*
C15:0													
C16:0									-0.68**				-0.71**
C16:1 cis-9									0.47*				0.54**
C18:0													
C18:1cis-9							-0.40†		0.38†				0.49*
LCSFA									-0.66**				-0.75**

**Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

†Correlation is significant at the 0.10 level (2-tailed).

[1] Long chain saturated fatty acids from C14:0 to C18:0

Effect of *DGAT1* polymorphism and CI6:0 on TAG structure

The interaction of *DGAT1* polymorphism and C16:0 in the TAG had a significant effect on C16:0 at sn-2. This effect was significant when measured in mol % basis (p=0.05) as well as when measured as a proportion (p=0.04) (Figure 2). No further effect was seen for fat content or for the interaction of *DGAT1* polymorphism with another FA.

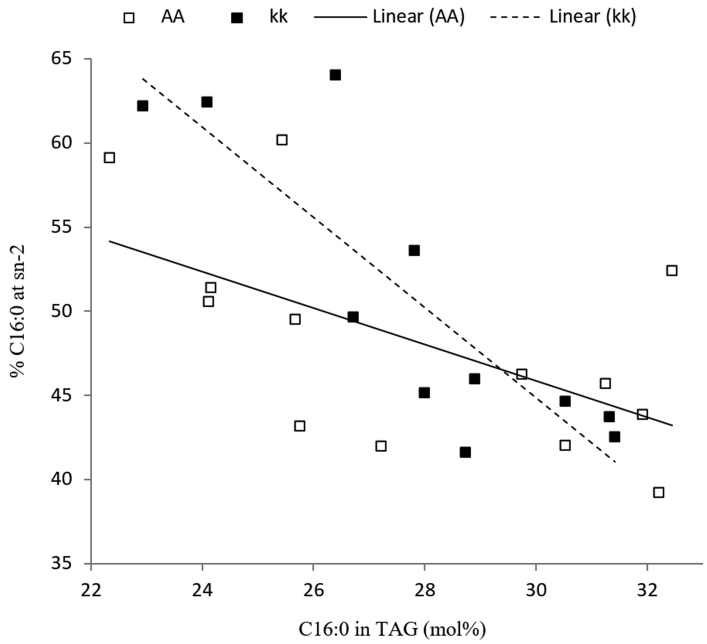

Figure 2. Effect of the interaction of C16:0 at the secondary position (sn-2) with the amount of C16:0 in the triacylglycerol (TAG: P = 0.04). *DGAT1* = diacylglycerol acyltransferase.

Discussion

Intrapositional approach

Our results are in accordance with previous studies (Parodi, 1979b, Turon *et al.*, 2002, Blasi *et al.*, 2008) except for C18:0. In our study the amount of C18:0 at sn-2 was similar to the values reported by Christie and Clapperton (1982), however, almost a double amount was found by Blasi *et al.,* (2008), Parodi (1979) and Turon *et al.* (2002). The differences found in C18:0 are not likely to be associated to the method used to analyse structure because Christie and Clapperton (1982) used an enzymatic approach while Turon *et al.* (2002) used a chemical approach, such as the one used in this study.

Moreover, we found large variability among individual cows, which made it interesting to test for correlations. These correlations were indeed found between short, middle and long chain SFA (table 2). These FA have a positive correlation between its total amount in the TAG and the amount present at sn-1,3. Our observations on correlations between total amount in the TAG and at sn-2 and sn-1,3 are similar to results previously reported by (Parodi, 1983). These correlations indicate the complexity of the interactions between FA during MF synthesis. The relations between the total amount of a FA in the TAG and at sn-2 or sn-1,3 are an important aspect that should be considered in the interpretation and comparison of different studies on MF TAG structure.

Interpositional Approach

In this study most FA at sn-2 significantly correlated to the amount of C16:0 and long chain SFA (C14-C18) in the TAG (table 3). These correlations were unexpected because there are no reports of influence of C16:0 over positioning of other FA in the TAG. These correlations give an indication that C16:0 and other long chain SFA play a role in the positioning of several FA in the TAG molecule.

Previous studies had reported the TAG structure with an interpositional approach (Morrison and Hawke, 1977, Blasi *et al.*, 2008). The interpositional approach was used in these studies to compare MF strucutres from different species or bovine MF TAG structures from cows with different diets. The propotions of a FA at sn-2 show a large variation among studies and might look contradictory between them. However, as shown in this study, the proportions of a FA at sn-2 will dependend on the original amount of C16:0 and long chain SFA (C14-C18) in the TAG. This remark should always be considered when interpreting the results.

Role of CI6:0 in milk fat triacylglycerol structure

The most abundant FA in MF is C16:0; this FA varies greatly between diets (Jensen, 2002). Our results show that the proportion of other FA at sn-2 is correlated to the total amount of C16:0 in the TAG. This arrangement may be related to FA preference of enzymes involved in MF TAG synthesis. So far, only glycerol-3-phospate acyltransferase **(GPAT)**, which esterifies the FA at sn-1, was shown to have a preference for SFA at the expenses of UFA (Monroy *et al.*, 1973, Bremer *et al.*, 1976, Coleman and Lee, 2004). GPAT has two isoforms (mitochondrial and microsomal) with the same FA preference but to different degrees. Until now, only mtGPAT has been annotated and characterized (Bionaz and Loor, 2008). The order of FA preference of mtGPAT is C16:0> C18:0≈C14:0>C12; UFA are less used by mtGPAT (Monroy *et al.*, 1973). The activity of mtGPAT is eight to ten times higher with C16:0-CoA than with C14:0, C18:0 or C18:1-CoA (Kinsella and Gross, 1973). Moreover, in heart and adipose tissues, GPATs are mainly mediated through the availability of FA and competing substrates (Aas and Daae, 1971). We suggest that an increase in availability of C16:0 and other long chain SFA for lipid synthesis in mammary epithelial cells will increase the activity of both isoforms of GPAT in mammary gland. This increase in activity will then increase the proportion of C16:0 and other long chain SFA acylated at sn-1 at the expenses of sn-2. The decrease of long chain SFA at sn-2 would be counterbalanced by other FA. This hypothesis explains our findings, where we saw an increase in the proportion of C16:0 and other long chain SFA at sn-1,3 as C16:0 increased in the TAG. In this study, the decrease of C16:0 and other long chain SFA at sn-2 was counterbalanced by an increase of, among others, C18:1.

Additional support of an the increase in C16:0 available for TAG synthesis can be obtained by quantifying sphyngomyelin in MF because C16:0 CoA is a common precursor of both lipids. Therefore, if C16:0 increases in the initial pool of FA available for fat synthesis, sphyngomyelin content will also increase. (Argov-Argaman *et al.*, 2013) found that an increase in sphyngomyelin content is related to an increase in MF content, which in turn is related to an increase in C16:0 in the TAG. Moreover, the rate of increase of sphyngomyelin in MF was related to *DGAT1* genotype (Argov-Argaman *et al.*, 2013). This observation supports our hypothesis, since an increase in MF content implies an increase in C16:0 in the TAG (Stoop *et al.*, 2008), and we showed that an increase in C16:0 in the TAG will influence TAG structure.

C16:0 could increase its availability for MF synthesis in mammary cells via *de novo* synthesis or via blood derived absorption into mammary epithelial cells. *De novo* C16:0 is synthesized in the cytosol of the mammary epithelial cells after a series of biochemical steps that elongate C2:0-CoA and C4:0-CoA (Bionaz and Loor, 2008). A

genetic predisposition could lead to a more efficient elongation leading to an excess of C16:0 compared to the shorter SFA, with C16:0 becoming dominant. The mechanism of stopping the elongation of C2:0 and C4:0 is not known but it could be linked to enzyme activity. The significant effect found in this study for *DGAT1* K232A genotype KK on the proportion of C16:0 placed at sn-2 suggests that genetics could have an effect on TAG structure. It is plausible that the *DGAT1* K232A genotype KK increases the availability of C16:0 for lipid synthesis by affecting other enzymes or cell organelles related to lipid synthesis. Blood derived C16:0 and other long chain SFA could increase their availability for MF synthesis with the diet. Blood derived FA enters the mammary epithelial cell via flip-flop and protein-mediated mechanisms (Bionaz and Loor, 2008). Once inside the cell, blood derived and *de novo* produced C16:0 long chain SFA are activated by attaching an Acyl-CoA. C16:0 and long chain FA with CoA are transported by FABP3 to mitochondria or endoplasmic reticulum (ER) where they serve directly as substrate for TAG synthesis (Bionaz and Loor, 2008). The increase of C16:0 and other long chain SFA available for MF synthesis may then result in an increased mitochondrial and microsomal GPAT activity, leading to a change in TAG structure as discussed previously. Studies on GPAT activity of mammary epithelial cells are necessary to confirm this hypothesis. Moreover, the observed effect of *DGAT1* on TAG structure will need to be confirmed on a larger number of animals.

Regiospecific structural analysis of TAG with chemical or enzymatic degradation of TAG has been used to determine the average FA composition of sn-2 and sn-1,3 of vegetable and marine oils as well as human and ruminant MF (Angers *et al.*, 1998, Lopez-Lopez *et al.*, 2002, Turon *et al.*, 2002). The advantages and limitations of both approaches have been widely discuss by others (Yurkowsk.M and Brockerh.H, 1966, Becker *et al.*, 1993, Turon *et al.*, 2002). Recently, new techniques to determine the regioisomerism of individual molecular species of TAG have been described (Kubo *et al.*, 2013, Linderborg *et al.*, 2014). However, these techniques still need to be optimized and validated to be used on bovine MF.

Implications

In the Netherlands, MF content has increased by 20% since the 1950's (Bijl *et al.*, 2013). This increase is the result of breeding and feeding strategies taken in the Netherlands during the last century (Heck *et al.*, 2009). This increase in fat content has led to higher amounts of SFA and especially of C16:0 (Stoop *et al.*, 2008). Breeding specialists should thus be aware that the increase in SFA impacts MF TAG structure because higher amounts of C16:0 in MF implies lower proportions of long chain SFA, especially of 16:0 and C14:0, at sn-2 and higher proportions of C18:1cis-9 at the same position. An increase in long chain SFA has an effect in the thermal properties of MF. Since higher proportions of C16:0 at sn-2 are desired for

infant nutrition (Lopez-Lopez *et al.*, 2001), bovine MF with low concentrations of C16:0 in its overall profile could better fit infant nutrition requirements. It is likely that certain MF fractions will give a profile richer in C16:0 at sn-2.

Conclusions

Individual differences in MF TAG structures were found within a dairy cattle population. To understand the changes in TAG structure among cows, two approaches were used in the present study. With an intrapositional approach the change of a FA at sn-2 and sn-1,3 was related to its total content in the TAG. With an interpositional approach the concentration of C16:0 in the TAG was negatively correlated to the proportion of C16:0, and C14:0 and was positively correlated to the proportion of C18:1 at sn-2. We suggest mtGPAT indirectly controls the amount of FA that are esterified at the sn-2 position by selectively esterifying C16:0 at sn-1. As a consequence, the positioning of C14:0 and C18:1cis-9 at sn-2 is less dependent on its own amount in the TAG but is related to the presence of 16:0 at sn-1. *DGAT1* K232A genotype AA showed a significant effect on the proportion of C16:0 placed at sn-2; *DGAT1* K232A genotype KK showed a higher change rate in the proportional positioning of C16:0 at sn-2. Since *DGAT1* esterifies a FA at sn-3, we cannot draw any conclusions about the FA preference of this enzyme. Further studies could investigate on the behaviour of C16:0 at sn-1 and the effect of *DGAT1* polymorphism on sn-3 with a stereospecific analysis of MF TAG. High amounts of C16:0 at sn-2 are desirable for infant nutrition because C16:0 in human milk is mainly placed at sn-2. Therefore, if MF is used as an ingredient for infant formulas, a MF with low amounts of C16:0 in the TAG will fit better this requirement.

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Chapter 4

Formation of β polymorphs in milk fats with large differences in triacylglycerol profiles

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Abstract

In this study, we characterized the polymorphism of milk fat (MF) with varying TAG compositions during isothermal crystallization at 20ºC. TAG composition of MF from seven individual cows was determined using GC-FID and MALDI-TOF MS and MF polymorphism was studied using X-ray diffraction. Results showed that TAG profile determines the polymorphic behavior of MF. Saturated TAG with carbon number 34-38 promoted the formation of α polymorphs, while unsaturated TAG with 52-54 promoted the formation of the β polymorphs. Furthermore, MFs with unsaturated fatty acid profiles were increased in unsaturated TAG with 52-54 carbons. The presence of MF crystals in the β polymorph has been controversial over the years as most authors mainly find MF in α and β′ form. In our work, we showed that the β polymorph is formed in MF based on its TAG composition.

Keywords: milk fat crystallization, triacylglycerol, lipid polymorphism, β polymorphism, X-ray diffraction.

Introduction

Milk fat (MF) is composed of triacylglycerols (TAG) with a wide range of fatty acids (FA) with different chain lengths and sn-positional distribution. These characteristics contribute to its complex thermal behaviour. Average TAG compositions of MF have been presented over the years (Jensen *et al.*, 1991), however, significant differences in TAG profiles of MF can be found when comparing milk from individual cows (Tzompa-Sosa *et al.*, 2014, Tzompa-Sosa *et al.*, 2016). These differences in TAG profile could be responsible for the differences observed in the polymorphism and the thermal behaviour of MF.

During crystallization, TAG can pack into a number of characteristic structural crystal polymorphs (Marangoni *et al.*, 2012). The formation of these crystal forms is affected by several factors such as cooling rate, crystallization temperature, amount of liquid fat and TAG composition. More than one crystal form can coexist in MF as was shown before (ten Grotenhuis *et al.*, 1999, Lopez *et al.*, 2001, Lopez *et al.*, 2005, Cisneros *et al.*, 2006). The polymorphic forms found in MF are α, β' and β, listed in increasing degree of packing density and melting point (Deman, 1992, Lopez *et al.*, 2001, Sato, 2001). These polymorphic forms are characterized by d-spacings of 4.15Å (α), 4.2 and 3.8Å (β'), and 4.6Å (β), using wide-angle x-ray diffraction (WAXD).

When MF is subjected to fast cooling rates (i.e., >0.1 °C/min), the α polymorph is formed followed by the formation of β' and β (Mazzanti *et al.*, 2004, Lopez *et al.*, 2005, Fredrick *et al.*, 2011, Marangoni *et al.*, 2012). Whereas at slower cooling rates (i.e., ≤0.1 °C/min), initial nucleation of TAG into the β' form is favoured (Martini *et al.*, 2001, Marangoni *et al.*, 2012) and no β polymorphs are formed (Mazzanti *et al.*, 2004). The differences on polymorphic formation is related to the activation energy of formation of each polymorph and on the formation temperature. Polymorphs with a low activation energy of formation, such as α, are loosely packed, nucleate faster and have lower crystallization temperatures than polymorphs with a higher activation energy of formation, such as β' and β (MacNaughtan *et al.*, 2006). With slower cooling rates the time span to the formation of the first polymorph is longer as compared with high cooling rates. Therefore, if enough time is given, polymorphs with high activation energy and longer nucleation time will be formed. For this reason, the cooling rate determines the type of polymorph.

Crystallization temperature is another important factor that affects the formation of polymorphs in MF. At given crystallization temperatures, both liquid and solid fat phases exist due to the complexity of the MF TAG composition (Timms, 1980). This is important in polymorphic transformation as a substantial amount of liquid fat is needed for the rotation and rearrangement of TAG into more stable forms (Cisneros *et al.*, 2006). At low temperatures therefore, transition of less stable to

more stable polymorphs (e.g. α to β') is delayed or completely hindered whereas at higher temperatures polymorphic transformation to more stable polymorphs is enhanced (ten Grotenhuis *et al.*, 1999, Cisneros *et al.*, 2006, Bugeat *et al.*, 2011). At crystallization temperatures higher than or equal to 20°C, α polymorphs completely dissolve in the remaining molten phase however, β' and β polymorphs remain because they can coexist with the melt. At temperatures between -8 and 20°C, co-existence of the α and β' polymorphs occurs whereas at temperatures below -8°C only the α polymorph exists (ten Grotenhuis *et al.*, 1999, Lopez *et al.*, 2001, Mazzanti *et al.*, 2004).

Polymorphic forms are influenced by TAG composition, however little information about the relation between the variation of MF TAG profile and polymorphic behaviour has been reported. The concentration of TAG species is very important since it determines the capability of polymorphic formation during a crystallization process. The interest in studying the thermal and structural behaviour of stable polymorphs in MF with various TAG profiles arose from the contradicting observations in the formation of β polymorphism in MF (Deman, 1961, Woodrow and deMan, 1968, Timms, 1979, ten Grotenhuis *et al.*, 1999, Mazzanti *et al.*, 2004). The presence of β polymorphs in MF was associated to slow cooling rates by De man (1961), whereas Mazzanti, et. al (2004) concluded that β polymorph was related to high cooling rates, while others concluded that cooling rate was not a determinant factor for its formation (Woodrow and deMan, 1968). Traces of β polymorphs have been found at 15°C after 16-24 h of isothermal crystallization (Timms, 1979). In other studies using different cooling rates and crystallization temperatures, no β polymorphs were seen (ten Grotenhuis *et al.*, 1999). These differences have been attributed to variation in MF composition (ten Grotenhuis *et al.*, 1999). It has been suggested that TAG forming β polymorphs have strict TAG structural requirements. They are formed when a certain degree of TAG symmetry is achieved, that is, when the FA present in the TAG does not differ in more than two carbons (Dsouza *et al.*, 1990, Deman, 1992, Sato, 2001, Campos *et al.*, 2002). Our study aimed to better understand the relation between MF TAG profile and crystals thermodynamically stable at 20°C, such as β' and β. β' polymorphs with a fine needle like structure are favorable in MF-containing food products as they provide thermal stability (i.e., higher melting point) and are associated with desirable sensory attributes and functional properties, such as a smoothness and better air incorporation than β polymorphs (Wiederma. Lh, 1968, Narine and Marangoni, 1999, Ghotra *et al.*, 2002). Polymorphs of MF from individual cows with large differences in their TAG profile were studied at isothermal conditions at 20°C after rapid cooling (20°C/min) by X-ray diffraction (XRD) and solid fat content (SFC) was studied by pulse NMR (pNMR). Identification of individual TAG species as well as relative intensities was performed by matrix-assisted laser desorption/ ionization- time of flight (MALDI-TOF MS).

Materials and Methods

Sample selection

Milk fat from individual cows as well as information on FA composition was available from the Dutch Milk Genomics Initiative (Stoop *et al*., 2008, Heck *et al*., 2012). Information on TAG profile by GC was available from an earlier study by us (Tzompa-Sosa *et al*., 2016). TAG profiling by GC allows a quantitative determination of main 16 TAG in MF. Using the information on FA and TAG composition we calculated the saturation index (SFA/USFA) and the ratio of 34-38/52-54 TAG of the samples in our original pool of 54 cows. These TAG ratios were selected because a previous study showed that these TAG had an effect on saturation index and on the ratio C16:0 to C18:1cis9 (TAG with CN 34, 36, 52 and 54) or to *DGAT1* K232A polymorphism (TAG with CN38)(Tzompa-Sosa *et al*., 2016). Moreover, these TAG showed high variability in MF. From our original pool of 54 cows, we selected seven samples of MF from individual cows with contrasting 34-38/52-54 TAG ratio. The selected samples were: Two MF with high 34-38/52-54 TAG ratio; 3 MF with average 34-38/52-54 TAG ratio; and two MF with low 34-38/52-54 TAG ratio.

Identification of individual TAG species by MALDI-TOF MS

Milk fat TAG were profiled based on carbon number (CN) and unsaturation level using MALDI-TOF MS (UltrafleXtreme, Bruker Corporation). The method of analysis was based on (Picariello *et al*., 2007) with some minor modifications. MF was dissolved in chloroform (10 mg of MF/ml of solvent) and stored in a glass tube with Teflon cap. The sample solution was mixed with 1 M NaCl at a ratio of 1:1 (v/v) and centrifuged for 3 minutes at 946 g (Eppendorf centrifuge 5430R). The upper aqueous layer was discarded and the lower organic layer was used for MALDI-TOF MS analysis. The matrix for MALDI-TOF MS analysis was 2,5-dihydroxybenzoic acid (DHB) dissolved in methanol containing 0.1% trifluoroacetic acid. A 0.1 mM NaCl solution was used as cationization agent. MALDI-TOF MS equipment was calibrated using maltodextrin, which is used as a reference for molecules with molecular weights between 400 and 3500 Da. Each MF samples was analysed in triplicate. Per repetition, 1000 laser shoots were acquired. The relative intensity of each identified TAG was calculated using the sum intensities of all TAG normalized to 100%. The relative intensities of TAG were the mean of the three repetitions.

XRD analysis

XRD patterns were recorded using Philips PAN analytical X'Pert XRD System. Monochromated CuKα radiation with a wavelength, λ equal to 1.5418Å was used to obtain diffraction patterns in the 2Θ range 15 to 30°, thus covering the wide

angle region. One XRD analysis per MF type was performed. MF samples were first melted to 70°C to dissolve all crystals. After 10 minutes at 70°C, the MF (~100µL) was transferred into in a sample holder previously stabilized at 70°C. The sample holder with the sample was then immediately positioned inside the XRD which was cooled to 20°C using a water bath. The sample was let to isothermally crystallize at 20°C for 120 min. XRD scans were recorded every 5 minutes during the entire crystallization time. The wide angles recorded were converted to d-spacings 3.0 to 6.0Å which correspond to the d-spacings of the different crystal forms commonly observed during MF crystallization. D-spacings were calculated using Bragg's law, given in equation 1.

$$n\lambda = 2d\sin\Theta \qquad \text{(equation 1)}$$

Intensities, in counts per second, were plotted against d-spacings to identify MF crystals that were formed during isothermal crystallization. Data collection and identification of the peaks corresponding to MF crystals was done using the software OPUS 6.5. The presence of each polymorph was also visually assessed.

Solid fat content

The solid fat content (SFC) of MF during isothermal crystallization at 20°C for 120 minutes was measured using a Bruker Minispec pNMR. About 5 mL of MF was first melted in a water bath at 70°C for 10-15 minutes and then 2 mL of melted fat was poured into a pNMR glass tube. The tube with the sample was placed again in a water bath at 70ºC for 15 minutes to melt crystals previously formed. The tube was then placed in the pNMR at 20°C for 120 minutes. SFC was measured each minute for 120 minutes. The SFC after 120 min was considered the maximum value during the measuring time. The analysis was performed in duplicate.

Statistical analysis

Milk fat samples with different TAG profiles were clustered according to the relative intensities of TAG species with 34-38 and 52-54 CN identified by MALDI-TOF MS. A hierarchical clustering with Euclidean distance was performed. To visually summarize the information, the cluster analysis was shown as a heat map. The relative abundance of each TAG is represented in the heat map with different colours. The TAG with the highest relative abundance (5.5% of total TAG) was presented in red whereas the TAG with the lowest relative abundance (0.24% of total TAG) was presented in dark blue. The clustering analysis as well as the heat map was performed using MeV software (version 4.9.0).

Results and Discussion

Milk fat was isothermally crystallized at 20°C for 120 min. During crystallization we observed three types of polymorphs in MF, namely, α (4.15 Å), β' (3.8 and 4.2 Å) and β (4.6 Å) (Figure 1). Results of the order of appearance of the polymorphs (induction time), intensity and their stability during crystallization are shown and are discussed in terms of TAG composition either using the ratio 34-38/52-54 TAG or the individual species of TAG identified by MALDI-TOF MS. The FA composition of these two groups of TAG differs. TAG with CN 34-38 are mainly composed of two long chain FAs and one short chain FA whereas TAG with CN52-54 are composed of three long chain FAs.

The ratio 34-38/52-54 TAG was also selected because previous studies showed and effect of these groups of TAG on saturation index and on the ratio C16:0 to C18:1cis9 (Tzompa-Sosa *et al.*, 2016). Furthermore, TAG with CN 38 was added to the ratio because in this same study this TAG was associated to a genetic effect ($p = 0.005$) and had a moderate correlation with saturation index ($r = 0.34$; $p = 0.01$).

In our study α polymorph, observed at d- spacing = 4.15 Å, was found in some the MF samples. This polymorph was formed within the first 5 minutes of isothermal crystallization. In some samples the intensity of α polymorph became less pronounced after 20 ± 5 min (AR2 and LR2), whereas in others samples this peak apparently remained (HR1 and HR2). The absence of α polymorph in some samples (AR1, AR3 and LR1) can be related to either a slow formation speed or to the impossibility of these samples to form this polymorph due to their TAG composition. The formation of α polymorphs was found to be related to the ratio 34-38/52-54 TAG since the samples with high ratio 34-38/52-54 TAG showed a clear α peak as compared to MF with low and average 34-38/52-54 TAG ratio (Figure 1). To study the importance of TAG species on the formation of α polymorph, we studied the relative abundance of the TAG species 34-38 and 52-54 and showed it as a heat map (Figure 2). MF with no apparent peak for α polymorph grouped in one cluster. This MF samples had a decreased abundance of TAG 34:0, 35:0 and 38:0 and an increased abundance of TAG 39:0 and most unsaturated TAG with 37 to 39 and 52-54 CN. TAG 34:0 and 35:0 are some of the most abundant TAG species in MF and are typically formed by two long chain saturated FA and one short chain saturated FA (Gresti *et al.*, 1993, Picariello *et al.*, 2007). The separation into one cluster of MF samples where α polymorph was absent indicated that the abundance of the TAG play an important role in the initial formation of α polymorph at 20°C. It also supports the suggestion that α polymorphs are formed from low and middle melting TAG (ten Grotenhuis *et al.*, 1999), such as TAG 35:0, 34:0 and 38:0. Various TAG might ultimately nucleate and grow as α polymorphs when the melt is shock-cooled to 20°C, but for most TAG

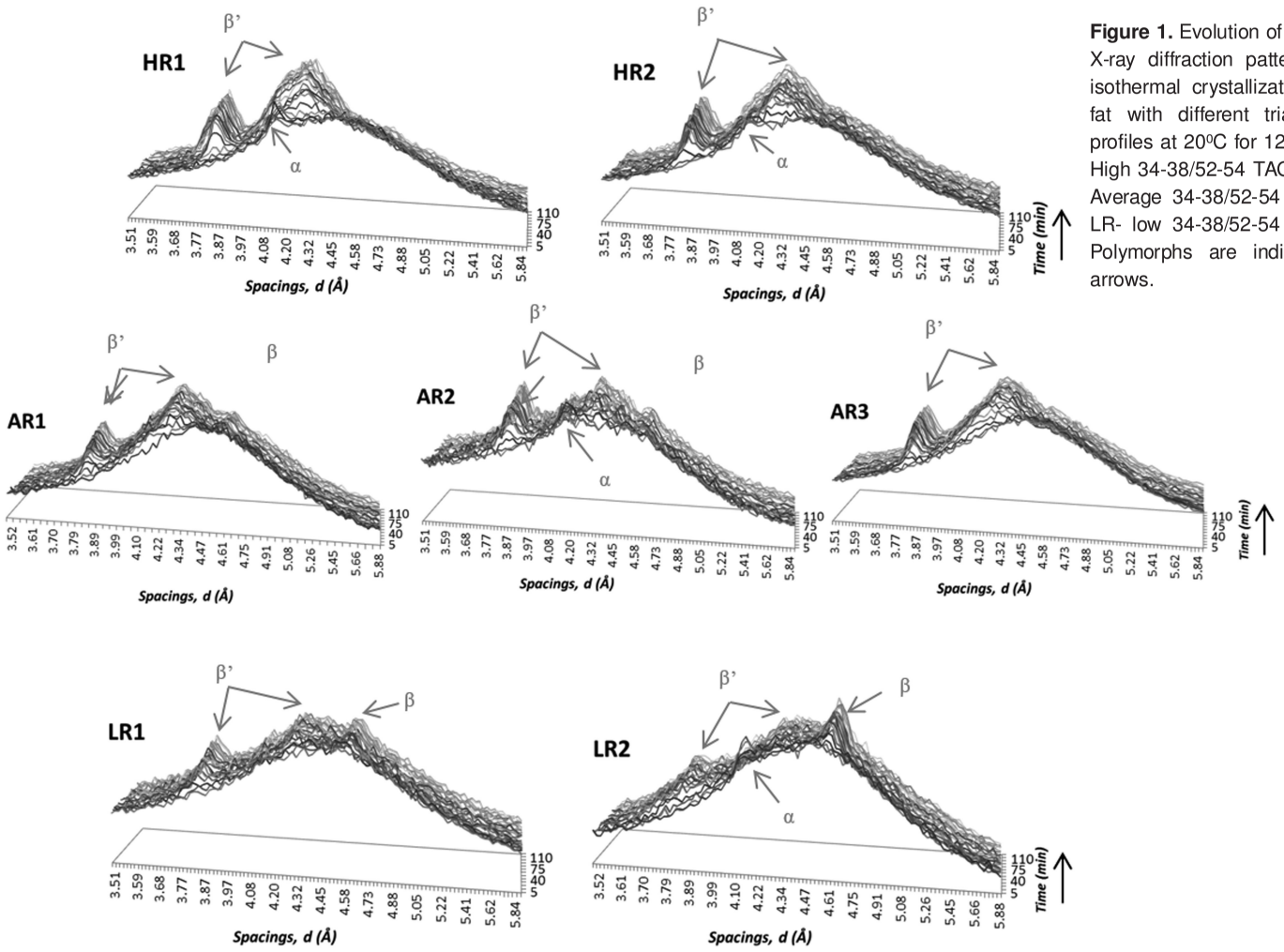

Figure 1. Evolution of wide angle X-ray diffraction patterns during isothermal crystallization of milk fat with different triacylglycerol profiles at 20^{0}C for 120 min. HR- High 34-38/52-54 TAG ratio; AR- Average 34-38/52-54 TAG ratio; LR- low 34-38/52-54 TAG ratio. Polymorphs are indicated with arrows.

separately it might be kinetically very slow because of their low concentration. Only TAG 34:0, 35:0 and 38:0 are sufficiently abundant to lead to fast α polymorph nucleation at 20°C. During growth they can probably incorporate other species into the growing α polymorphs since this polymorph has some freedom to fitting different molecules in one crystal lattice (Walstra, 1987).

Rapid cooling of the melt to 20°C was applied in our study. With this cooling process we were able to record α polymorphs even at the clear point of α, which is 20°C (ten Grotenhuis, van Aken *et al.* 1999). By flash-cooling of the melt to 20°C, α polymorphs nucleate and grow at high supersaturation (or undercooling) from the melt, hence crystallization will be fast and likely a broad range of TAG species will co-crystallize. Hence crystallization will not be very selective for TAG species. We suggest that TAG 34:0, 35:0 and 38:0 promote the nucleation of α polymorphs. Once the nuclei is formed, other TAG species could incorporate into this polymorph. Furthermore, the nucleation of TAG into α polymorph is favored because arrangement of TAG into α form requires a lower activation free energy and a more loose structure than β'and β (ten Grotenhuis *et al.*, 1999, van Aken *et al.*, 1999, Wiking *et al.*, 2009). As confirmed by our study, α polymorph formed by flash cooling is metastable for many TAG species, leading to a gradual recrystallization to more stable polymorphic crystals over time.

The second polymorphic form observed was β', observed at d-spacing = 3.8 Å and 4.2 Å. The intensity of peaks related to the β' polymorph in relation to other peaks within each XRD pattern differed among MF samples. The intensity of this polymorph was related to the ratio 34-38/52-54 TAG. MF with high 34-38/52-54 TAG ratio (≥ 4.4), as in sample HR1 and HR2, showed relatively high intensities of peaks related to β' polymorphism whereas MF with a low 34-38/52-54 TAG ratio (≤ 1.6), as in sample LR1 and LR2, showed relatively low intensities of peaks related to the β' polymorph. The clustering analysis of the relative abundance of individual TAG species with 34-38 and 52-54 carbons does not gives information about the key TAG involved in the formation of β' as most MF TAG can pack as β' polymorph. However, the apparent relation between the 34-38/52-54 TAG ratio and the intensity of β' polymorph shows that the abundance of TAG with CN 34-38 is of importance for the formation of β' polymorphs. The most abundant TAG species with CN 34-38 are saturated (Figure 2). These TAG are also some of the most abundant TAG in MF (Picariello *et al.*, 2007). It is likely that an increase abundance of saturated TAG with CN 34-38 promoted the formation of β' polymorph. These samples could also have at the same time, low melting point for their corresponding α polymorph, thus too high solubility at 20°C. Therefore, saturated TG with CN34-38 could be forming α polymorphs from the melt but they later recrystallize into β'polymorphs

as they cannot compete with β' formation. TAG with CN 34-36 have been positively associated with saturation index(Tzompa-Sosa *et al.*, 2016). Consequently, MF with high saturation index will be increased in TAG with CN 34-36 causing an increase in β' polymorphism upon crystallization at 20°C. The β' polymorphs provide smoothness and better air incorporation than β polymorphs and are desired in spreads, margarine, and shortenings for puff pastry as β' crystals are small and needle-like (Wiederma.Lh, 1968, Narine and Marangoni, 1999, Ghotra *et al.*, 2002).

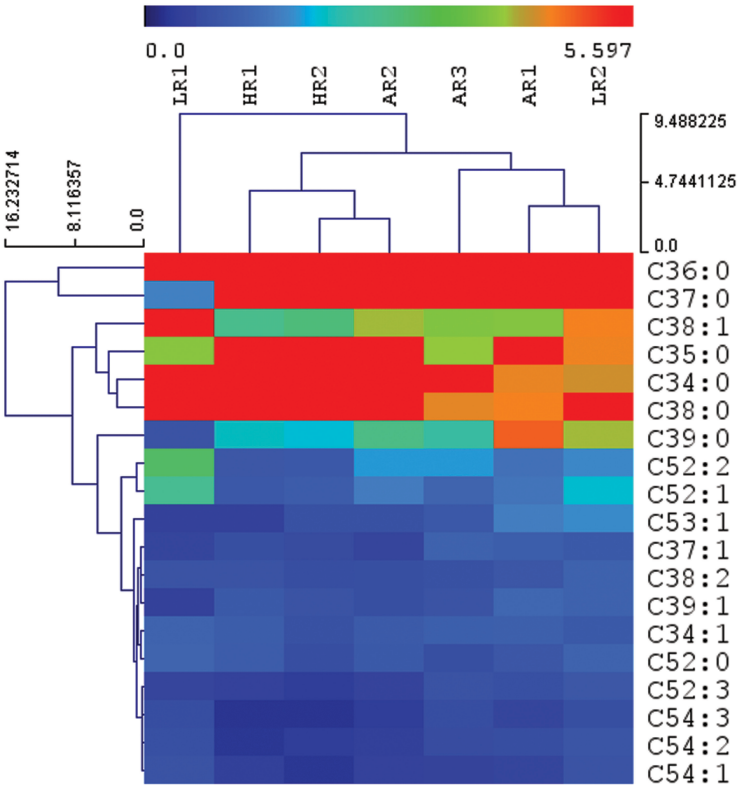

Figure 2. Relative abundance of milk fat triacylglycerols with carbon number from 34 to 38 separated according to carbon number and level of saturation by matrix assisted laser desorption/ionization- time of flight (MALDI-TOF MS). Samples and triacylglycerols are clustered using Hierarchical Clustering using Euclidean distance. The colours determine the relative abundance of a TAG ranging from 5.59% (red) to 0 % (dark blue) of total TAG. LR- low ratio; AR- average ratio; HR- high ratio.

A third polymorphism was observed at d-spacing = 4.6 Å, which is characteristic of β polymorph. Two different types of TAG are responsible for the formation of β' and β polymorphs since appearance of the peak related to β polymorph occurred at the same time as the β' polymorph (Table 1). Once again, the intensity of the XRD peak for β polymorph was related to the ratio 34-38/52-54 TAG. MF with low and average 34-38/52-54 TAG ratio (≤2.16) showed peaks for β polymorphs (Figure 1, samples AR1, AR2, LR1 and LR2). Moreover, the lower the 34-38/52-54 TAG ratio,

the higher the relative intensity of β polymorphs in relation to other peaks within each XRD pattern up to the point in which the β polymorphs became predominant (sample LR2). The identified TAG species with CN 52-54 are mainly unsaturated with up to three double bonds (Figure 2). This suggests that a high concentration of unsaturated TAG with CN 52-54, which are mainly long chain FA, promotes the formation of β polymorphism. Previous studies showed that, the concentration of TAG with CN 52-54 is negatively correlated with saturation index ($r = -0.57$ and $r = -0.75$, respectively; $p<0.001$)(Tzompa-Sosa *et al.*, 2016). Therefore, MF with low saturation index will tend to have an increased concentration of CN 52-54 and this will promote the formation of β polymorphism when it is crystallized at 20°C.

The importance of the concentration of unsaturated TAG with CN52-54 on the formation of β crystals was confirmed in one sample having a ratio 34-38/52-54 TAG = 2.13 (sample AR3). This sample had an average 34-38/52-54 TAG ratio similar to sample AR1 and AR2, but it was also low in TAG with CN 52-54 (12.05 g/100 g of TAG) and did not show β polymorphism. This suggests that for the formation of β polymorphs in MF at 20°C, a minimum amount of TAG with CN 52-54 is necessary. In the case of samples showing β polymorph (AR1, AR2, LR1 and LR2), the concentration of TAG 54 plus 54 was above 13 g/ 100 g of fat (Table 1). Formation of β polymorph requires strict TAG structure, namely FA that do not differ in two carbons (Dsouza *et al.*, 1990, Deman, 1992, Sato, 2001, Campos *et al.*, 2002). In MF, the TAG that might cover this structural requirement are TAG with CN52-54, because they are formed mainly by FA with 16 and 18 carbons (Gresti, et al, 1993). A detailed FA composition of the TAG species with CN 52-54 can confirm if the FA within these TAG does not differ on more than two carbons, which has been suggested as a structural requirement for β polymorph.

Other requirements for the formation of β polymorph include the presence of a considerable amount of MF in liquid state (Timms, 1980, Cisneros *et al.*, 2006). The liquid fraction present in the system influence the rotation and rearrangements of TAG into more stable polymorphs, such as β' and β (Cisneros *et al.*, 2006). The liquid fraction is especially relevant when a small amount of liquid fat is present in the fat system because the mobility of TAG is impeded, as shown in high melting fraction of MF with SFC above 90% at 5°C (Cisneros *et al.*, 2006). Therefore, in our study we measured the amount of SFC after 120 min of isothermal crystallization at 20°C (Table 1). The range of SFC seen among our samples was large (SFC between 16 and 35.5%), which was related to the large differences in TAG composition among the fat samples. Furthermore, SFC was positively correlated to saturation index ($r = 0.85$, $p < 0.05$) and to the relative concentration of TAG with CN 34 and 36 ($r = 0.72, 0.78$; $p = 0.07$ and 0.04, respectively). In our study, SFC was always below 35.5% providing enough liquid fraction for the polymorphic transformations to take place. Thus, the liquid fraction might not play a role in the formation of stable polymorphs in MF at 20°C.

Table 1. Triacylglycerol profile by GC (g/100g), saturation level, polymorphic and thermal characteristics of anhydrous milk fats with different triacylglycerol profiles. HR- high ratio 34-38/52-54 TAG; AR- average ratio 34-38/52-54 TAG; LR- low ratio 34-38/52-54 TAG.

	HR1	HR2	AR1	AR2	AR3	LR1	LR2
Triacylglycerols							
CN24	0.26	0.26	0.24	0.25	0.26	0.24	0.23
CN26	0.24	0.27	0.21	0.14	0.25	0.20	0.17
CN28	0.61	0.73	0.52	0.39	0.66	0.52	0.38
CN30	1.30	1.55	1.07	0.88	1.35	1.05	0.72
CN32	2.96	3.40	2.46	2.18	2.87	2.26	1.62
CN34	7.45	7.73	6.09	5.76	6.09	5.23	4.84
CN36	14.18	12.42	10.83	10.74	9.37	9.71	10.27
CN38	13.04	12.05	11.78	11.35	10.16	11.90	12.57
CN40	8.94	9.30	9.07	8.42	8.82	9.90	8.84
CN42	7.80	8.42	6.78	6.96	7.88	7.08	5.34
CN44	7.61	8.40	6.95	7.36	8.30	6.63	5.14
CN46	8.18	8.89	8.27	8.73	9.40	7.55	6.76
CN48	9.51	9.75	10.52	10.88	11.02	9.42	10.27
CN50	10.61	9.51	11.94	12.95	11.52	11.88	14.44
CN52	5.88	5.59	9.15	9.61	8.43	10.82	12.94
CN54	1.43	1.73	4.12	3.40	3.62	5.61	5.48
Ratio 34-38/52-54 TAG	4.74	4.40	2.16	2.14	2.13	1.63	1.50
Saturation level[1]	3.83	3.37	2.13	2.50	2.34	2.18	1.97
Solid fat content (%)[2]	35.50	27.60	21.40	21.04	20.53	27.20	16.08
Induction time of polymorphisms (min)by XRD							
α polymorph[3]	0	0	absent	0	absent	absent	0
β' polymorph	≈ 20	≈ 15	≈ 20	≈ 20	≈ 15	≈ 20	≈ 25*
β polymorph	absent	absent	≈ 10*	≈ 15*	absent	≈ 10	≈ 20

[1] Saturation Level = Total saturated fatty acids / total unsaturated fatty acids

[2] Solid Fat content at 20°C after 120 min of isothermal crystallization.

[3] Induction and disappearance time

* Traces

Conclusion

We showed that the concentration of TAG influence the type of polymorphs present in MF when it is rapidly cooled and isothermally crystallized at 20°C. The concentration of TAG is relevant to the formation of a nuclei and growth of a stable polymorph at a given temperature because the melt should be supersaturated. Supersaturation is favored when an increased amount of TAG are able to structure in a specific polymorph. Under conditions of supersaturation, the number of nuclei formed in a given time lapse (nucleation rate) is dependent on the degree of supersaturation. Any nucleus will grow out at a rate dependent on the degree of supersaturation. As shown in this work, the conditions of supersaturation vary among MF samples and TAG profiles.

The formation and intensity of α and β' polymorphs were related to the abundance of TAG with CN 34-38. An increased in the abundance of TAG with CN 34-38 was related to an increased in the intensity of peaks related to the β' polymorph within each XRD. Moreover, α polymorphs were not present in MF samples with decreased abundance of TAG 35:0, 34:0 and 38:0 and increased abundance of TAG 39:0 and most unsaturated TAG with 37-39 and 52-54 CN.

In our study we confirmed that the presence of β polymorphs is dependent on MF TAG composition, as previously suggested (ten Grotenhuis *et al.*, 1999, Wiking *et al.*, 2009). Moreover, we showed that fast cooling does not prohibit the formation of β polymorphs (20°C/min). Therefore, cooling rate is not a critical factor in the formation of β polymorphs. We suggest that the concentration of unsaturated TAG with CN 52 to 54 and the presence of a substantial amount of liquid fat are equally important for the formation of β polymorph. Therefore, if the concentration of unsaturated TAG with CN 52-54 is low, no β polymorph will be formed. In this way we explain the presence of β polymorph in MF and solve the controversy surrounding this polymorph.

Studies regarding MF polymorphism are of interest because the macroscopic physical properties of MF will greatly vary depending on the type, number and interaction of the various polymorphs formed. This is the first time that formation of polymorphs and TAG composition are combined in order to explain the experimental results of MF crystallization.

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Chapter 5

Melting point of milk fat fractions: the importance of crystallization history and milk fat composition

Introduction

Milk fat (MF), an important component of milk, is used in the food industry for its desirable organoleptic properties. The physical properties of MF greatly influence the products rich in fat. The physical properties of MF are described by crystallization and melting behavior. Crystallization of MF has been thoroughly studied and comprehensive reviews can be found elsewhere (Mortensen, 1982, Walstra, 1987).

Cooling rate, final temperature and storage time determine crystallization history and ultimately affect melting behavior of MF (Walstra, 1987, Lopez and Ollivon, 2009, Larsen *et al.*, 2014). Cooling rate determines the types of crystals formed during crystallization, whereas the final temperature influences the type of TAG to be crystallized and storage time allows polymorphic transformations in MF (Walstra, 1987). When MF is rapidly cooled at rates > 0.1 °C/min with a few minutes of storage at the final temperature (≤ 10 min), it crystallizes mainly into the α polymorph, which is the least stable crystal (Lopez *et al.*, 2005). However, when MF is slowly cooled at rates < 0.1 °C/min, α, β'and β polymorphs are formed (Lopez *et al.*, 2001). The melting behavior of MF is determined by the types of metastable and stable crystals formed during crystallization and, if present, the re-crystallization of unstable crystals occur during melting.

Non-isothermal and isothermal crystallization are used to study crystallization history and melting behavior of fats. During non-isothermal crystallization, the fat is cooled at a determined rate until the final temperature is reached. This final temperature is kept for a few minutes (<10 min) and subsequently the fat is heated. During isothermal crystallization, the fat is cooled until the desired final temperature is reached. Then, the fat is stored at the final temperature for a prolonged time (usually some hours). After storage, the fat is heated until reaching its melting point. If enough storage time is given (between 20 and 35 min), polymorphic transformations will take place (ten Grotenhuis *et al.*, 1999).

The melting behavior of MF varies between non-isothermally and isothermally crystallized MF. When MF has been non-isothermally crystallized to temperatures about -60 °C with a few minutes of storage, it shows three endothermic peaks during melting. This melting behavior has been interpreted as melting of three fractions (low, middle, and high) that crystallize separately and behave as independent solid phases (Timms, 1980, Lopez *et al.*, 2005, Lopez and Ollivon, 2009). However, when MF has been isothermally crystallized at 20, 17, 14 and 10 °C, the three typical melting fractions of MF are not seen any more (ten Grotenhuis *et al.*, 1999). The differences in melting behavior after non-isothermal and isothermal crystallization are explained by the presence of different types of crystals in the fat; metastable and

stable crystals are present in isothermally crystallized fat, whereas the least stable crystals are mainly present in non-isothermally crystallized fats.

Melting behavior has been less studied compared to crystallization behavior. However it is well known that melting behavior is affected by crystallization history and TAG composition (Taylor *et al.*, 1978, Walstra, 1987). The effect of variate TAG composition on polymorphism of MF has been previously studied (chapter 4, this thesis). In the present study we describe the effect of variate MF composition and crystallization history on the melting behavior of MF. We used MF with variate FA and TAG composition. MF TAG was profiled on carbon number (CN) and unsaturation level using MALDI-TOF. The effect of crystallization history on MF melting behavior was studied using two types of crystallization conditions, namely non-isothermal and isothermal crystallization.

Materials and Methods

Samples of Individual Milk Fat

The MF samples in this study were provided by the Dutch Milk Genomics Initiative. Information on FA composition was available from previous studies in this Initiative (Stoop *et al*., 2008, Heck *et al*., 2012). For our study we selected six MF samples from individual cows with variate FA composition.

Identification of individual TAG species by MALDI-TOF

Milk fat TAG were profiled on carbon number (CN) and unsaturation level using MALDI-TOF. The method of analysis was based on Picariello *et al.*, (2007) with some minor modifications. MF was dissolved in chloroform (10 mg of MF/ml of solvent) and stored in a glass tube with Teflon cap. The sample solution was mixed with 1 M NaCl at a ratio of 1:1 (v/v) and centrifuged for 3 minutes at 946 g (Eppendorf centrifuge 5430R). The upper aqueous layer was discarded and the lower organic layer was used for MALDI-TOF analysis. The matrix for MALDI-TOF analysis was 2,5-dihydroxybenzoic acid (DHB) dissolved in methanol containing 0.1% trifluoroacetic acid. A 0.1 mM NaCl solution was used as cationization agent. MALDI-TOF equipment was calibrated using maltodextrin, which is used as a reference for molecules with molecular weights between 400 and 3500 Da. Each sample was measure in triplicate. We acquired 1000 laser shoots per repetition. The intensities of TAG were the mean of the three repetitions. The relative intensity of each identified TAG was calculated using the sum intensities of all TAG normalized to 100%. The results of TAG profiling were shown as heat maps using MeV software (version 4.9.0).

Thermal analysis

Thermal analysis was performed using a TA Q1000 differential scanning calorimeter (DSC) (New Castle, DE) calibrated with indium. MF (15 ± 1 mg) was loaded in stain steel pans of 50 µL hermetically sealed with a cap of the same material. An empty, hermetically sealed pan was used as a reference. The thermograms were analyzed using Pyris data Analysis (Perkin Elmer).

Two different crystallization conditions were used to study the effect of crystallization history on MF melting behaviour, namely isothermal and non-isothermal crystallization (Figure 1). The isothermal crystallization conditions were as follow: MF was melted to 70°C for 10 min to remove all crystals in MF. Then, MF was rapidly cooled (20°C/min) to either 20, 15 or 10°C, and that temperature was kept for 120 min. Finally, the melting behavior was determined by heating at 5°C/min from the crystallization

temperature to 70°C. The non-isothermal crystallization conditions were as follows: MF was melted to 70°C for 10 min to remove all crystals. Then, MF was cooled at 5°C/min from 70°C to -60°C and then, the fat was stored at that temperature for 10 min. Next, the melting behavior was determined by heating at 5°C/min from the -60 to 70°C. The maximum point of a curve was considered as the melting point of a fraction.

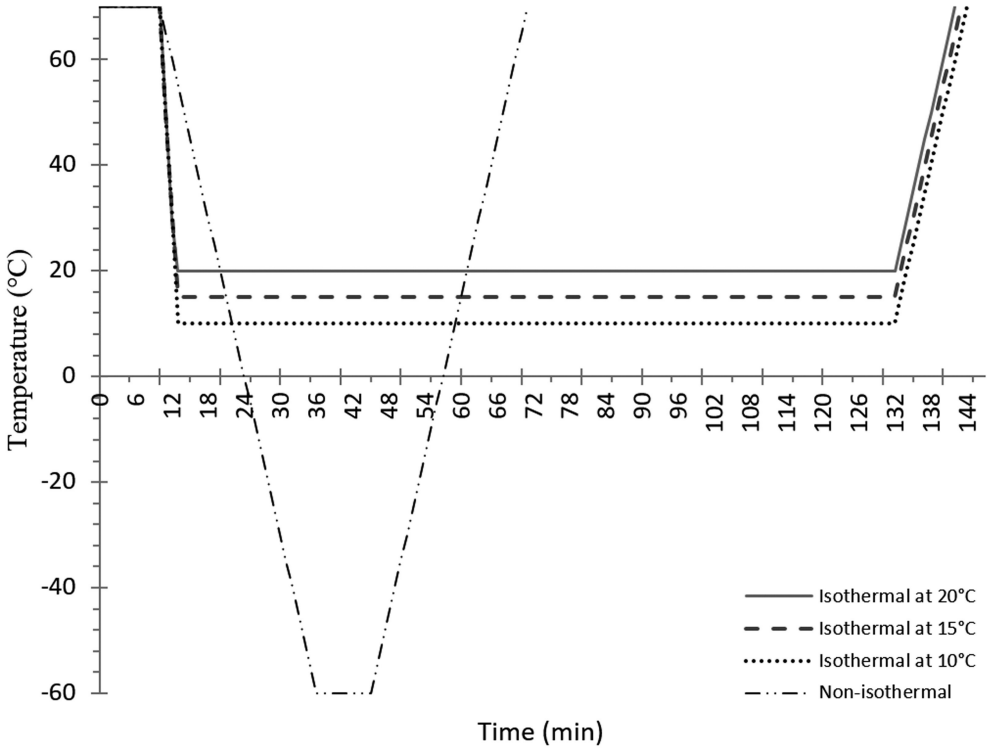

Figure 1. Time-temperature program of crystallization and melting treatments.

Statistical analysis

The effect of fat content, saturation index and the ratio C16:0/C18:1cis-9 on TAGs was analysed using the model

$$y_{ij} = \mu + \beta\, x_{ij} + e_{ij} \quad (1)$$

where y_{ij} are observations for individual TAG species; μ is the overall mean, β is the regression coefficient; x_{ij} is the covariable describing the effect of fat content, saturation index or the ratio C16:0/C18:1cis-9 and e_{ij} is the residual. Data analysis were performed using IBM SPSS statistics software (Version 21; Armonk, NY).

Results and Discussion

FA and TAG profile were used as parameters to characterize MF composition. The MF used in this study had a wide range of FA and TAG profiles (Table 1 and 2). The biggest difference among samples was found in the most abundant FAs C16:0 and C18:1cis-9. The difference for C16:0 and C18:1cis-9 were 9.7 and8.4 mol%, respectively. These two FA are negatively correlated (Schennink *et al.*, 2007) and have an opposite effect on physical properties of MF because C16:0 has a melting point of 62.9°C whereas C18:1cis-9 has a melting point of 13°C. We previously used the ratio C16:0/C18:1cis-9 to describe the changes on TAG profile in MF (Tzompa-Sosa *et al.*, 2016). For these reason, we calculated the ratio C16:0/C18:1cis-9 in our samples. This ratio summarizes the relation between the most abundant saturated FA and the most abundant unsaturated FA in MF. Furthermore, saturation index was calculated. This index is used in industry for estimation of the physical properties of the fats. It index describes the degree of saturation in fats. It is a ratio between the sum of all saturated FA and the sum of all unsaturated FA.

The MF samples used in this study show a wide range of different TAG. Identification of TAG in MF was done by MALDI-TOF. Using this approach, we identified 69 different TAG ranging from 28 to 55 carbons. Twenty nine of the TAGs were saturated and 40 were unsaturated (Table 2). We also identified TAGs with up to four double bonds. The most abundant TAG species with an average abundance higher than 3% were CN34:0, 35:0, 36:0, 37:0, 38:0, 39:0, 41:0, and 38:1. The relative abundances of all the TAG species were used to test the effect of fat content and FA composition, as described by the ratio C16:0/C18:1cis-9 and saturation index, on TAG profile (Table 3).

Our findings showed that the ratio C16:0/C18:1cis-9 and saturation index have a significant positive effect ($P \leq 0.05$) on the relative abundance of saturated TAG CN31:0, 34:0, 36:0 and a significant negative effect on unsaturated TAG CN38:1, 40:2, 41:1, 47:1, 51:1, 52:1, 53:1, 54:2 and54:3 (Table 3, Figure 2). Saturation index and the ratio C16:0/C18:1cis-9 showed similar effects, however the effect of the ratio C16:0/C18:1cis-9 on TAG species was greater than the one showed by saturation index. This indicates that the concentration of C16:0 and C18:1cis-9 is more important to explain the changes in relative abundance of TAG species. Because saturation index and the ratio C16:0/C18:1cis-9 are so closely related, we will further discuss our results only in terms of the ratio C16:0/C18:1cis-9.

Table 1. Fatty acids (mol%), saturation index and ratio C16:0/C18:1cis-9 of milk fat samples used in this study.

	LS1	LS2	IS1	IS2	HS1	HS2
Parameters						
Fat content	4.19	3.43	3.75	5.09	4.21	5.68
Saturation Index[1]	2.52	2.73	2.99	3.13	4.32	4.90
ratio C16:0/C18:1cis-9	1.35	1.47	1.62	1.80	2.74	3.27
Fatty acids						
C4:0	10.31	9.44	7.65	8.43	9.21	10.17
C5:0	0.06		0.06	0.05	0.09	
C6:0	3.99	4.02	3.88	3.95	4.57	4.73
C7:0	0.03		0.06	0.04	0.09	
C8:0	1.56	1.87	2.17	1.90	2.36	2.27
C9:0	0.03		0.07	0.04	0.10	
C10:0	2.33	3.30	4.72	3.34	4.77	4.34
C10:1	0.29	0.44	0.46	0.51	0.68	0.34
C11:0	0.03	0.05	0.12	0.05	0.15	0.04
C12:0	2.32	3.85	5.03	3.90	5.14	4.60
C12:1	0.05	0.12	0.14	0.14	0.04	0.08
C13:0	0.09	0.08	0.16	0.08	0.16	0.06
C14:0iso	0.16	0.09	0.11	0.09	0.09	0.06
C14:0	8.97	11.29	12.85	10.99	12.55	11.46
C15:0iso					0.21	
C14:1cis-9	1.12	1.26	1.48	1.61	1.09	0.82
C15:0anteiso	0.58	0.48	0.47	0.49	0.55	0.31
C15:0	1.40	1.06	1.31	1.05	1.29	0.66
C16:0iso	0.26	0.18	0.22	0.19	0.23	0.14
C16:0	26.25	26.99	26.01	30.11	30.72	35.93
C16:1trans9	0.10	0.05	0.05	0.05	0.04	0.04
C16:1cis-9	1.56	1.68	1.30	1.34	1.24	1.16
C17:0iso	0.35	0.27	0.27	0.28	0.27	0.22
C17:0anteiso	0.48	0.47	0.34	0.52	0.06	0.37
C17:0	0.57	0.34	0.43	0.40	0.40	0.36
C17:1cis-9	0.31	0.17	0.17	0.19	0.14	0.14
C18:0	9.12	6.85	6.10	7.51	5.05	5.84
C18:1cis-9	19.39	18.36	16.06	16.69	11.20	10.98
C18:1cis-11	0.48	0.38	0.34	0.25	0.30	0.32
C18:1cis-12	0.10	0.13	0.25	0.11	0.13	0.12
C18:1cis-13	0.09	0.07	0.08	0.07	0.07	0.06
C18:2cis-9.12	0.72	0.85	1.09	0.70	0.94	1.02
C18:2cis-9.trans11	0.56	0.44	0.42	0.32	0.31	0.21
C18:3cis-9.12.15	0.44	0.42	0.48	0.43	0.39	0.18
C18:1trans (total)	2.05	1.38	1.66	1.06	1.34	1.15
C19:0	0.09	0.06		0.05	0.05	0.05
C20:0	0.13	0.08	0.08	0.10	0.07	0.07
C20:3cis-8.11.14	0.03	0.03	0.05	0.04	0.05	0.05
C20:1cis-11	0.03	0.03	0.03	0.03	0.04	0.03
C20:5cis-5.8.11.14.17	0.05	0.03	0.04		0.04	
C21:0	0.03		0.02	0.01		0.03
C22:0	0.11	0.09	0.09	0.09	0.08	0.08
C22:1cis-13					0.03	
C22:5cis-7.10.13.16.19	0.06	0.08	0.06		0.05	
C22:6cis-4.7.10.13.16.19						
C24:0	0.02	0.03	0.02			

[1] Saturation Index = total saturated / total unsaturated fatty acids

Table 2. Relative abundance of saturated, monounsaturated and polyunsaturated triacylglycerol species in milk fat with different fatty acid profiles.

Saturated triacylglycerols						
TAG	LS1	LS2	IS1	IS2	HS1	HS2
CN36:0	7.11	5.72	6.14	8.35	9.54	11.40
CN37:0	7.03	8.11	4.53	7.05	7.20	8.35
CN39:0	7.04	7.71	4.18	5.65	5.23	5.25
CN38:0	4.40	3.40	3.64	5.17	5.10	5.13
CN35:0	3.49	4.53	3.23	4.32	4.35	4.26
CN34:0	3.01	2.90	3.83	3.96	4.55	4.70
CN41:0	3.53	4.14	2.72	3.09	2.78	2.74
CN51:0	2.58	2.51	1.71	2.25	2.06	2.00
CN40:0	1.76	1.87	2.70	2.37	2.66	2.44
CN43:0	1.79	2.63	2.12	2.35	2.37	2.02
CN33:0	1.85	2.44	1.86	1.91	2.15	2.78
CN49:0	1.70	2.54	2.11	2.06	2.22	2.06
CN45:0	1.95	2.39	1.98	1.96	2.05	1.92
CN31:0	1.69	1.61	2.21	1.82	2.04	2.81
CN47:0	1.55	2.53	1.78	1.70	2.30	1.87
CN42:0	0.97	1.24	2.18	1.76	2.61	2.05
CN32:0	1.02	1.36	2.05	1.34	1.72	1.92
CN46:0	0.97	1.13	1.67	1.62	2.47	1.26

Monounsaturated triacylglycerols						
TAG	LS1	LS2	IS1	IS2	HS1	HS2
CN38:1	3.49	2.90	3.13	3.33	2.29	2.07
CN50:1	2.16	1.54	2.31	2.55	2.01	1.69
CN40:1	2.06	1.56	2.10	1.88	1.61	1.42
CN48:1	1.39	1.51	2.37	1.89	1.51	1.33
CN36:1	1.52	1.45	1.82	1.77	1.27	1.14
CN46:1	1.06	0.98	1.90	1.38	1.31	1.05
CN44:1	0.82	1.05	1.74	1.38	1.17	0.89
CN42:1	0.93	1.02	1.59	1.32	1.14	0.96
CN52:1	1.50	1.05	0.93	1.20	0.79	0.70
CN53:1	1.25	1.13	0.72	0.58	0.58	0.33
CN51:1	1.07	0.91	0.81	0.60	0.54	0.53
CN34:1	0.69	0.79	0.84	0.76	0.59	0.78
CN39:1	0.85	0.89	0.63	0.54	0.64	0.71
CN41:1	0.96	0.97	0.64	0.56	0.46	0.46
CN49:1	0.74	0.61	0.76	0.63	0.61	0.58
CN37:1	0.69	0.75	0.63	0.44	0.48	0.55
CN32:1	0.60	0.50	0.63	0.41	0.55	0.61
CN43:1	0.61	0.74	0.55	0.44	0.46	0.40

Polyunsaturated triacylglycerols						
TAG	LS1	LS2	IS1	IS2	HS1	HS2
CN52:2	1.24	1.03	1.42	1.43	0.71	0.70
CN50:2	0.99	0.96	1.22	1.00	0.72	0.77
CN40:2	0.91	0.95	0.76	0.62	0.54	0.50
CN48:2	0.69	0.62	0.85	0.62	0.58	0.47
CN38:2	0.85	0.64	0.59	0.54	0.53	0.62
CN42:2	0.64	0.44	0.62	0.49	0.38	0.58
CN46:2	0.54	0.35	0.79	0.44	0.48	0.39
CN52:3	0.66	0.52	0.61	0.40	0.29	0.39
CN44:2	0.40	0.39	0.67	0.48	0.42	0.39
CN54:2	0.64	0.50	0.49	0.34	0.28	0.20
CN51:2	0.48	0.45	0.51	0.32	0.28	0.39
CN53:2	0.59	0.54	0.42	0.28	0.25	0.31
CN50:3	0.30	0.39	0.48	0.26	0.45	0.34
CN54:3	0.52	0.43	0.51	0.28	0.19	0.20
CN55:2	0.47	0.43	0.30	0.18	0.13	0.22
CN54:4	0.28	0.27	0.32	0.21	0.15	0.28
CN52:4	0.19	0.21	0.35	0.14	0.23	0.20

Saturated triacylglycerols							Monounsaturated triacylglycerols							Polyunsaturated triacylglycerols						
TAG	LS1	LS2	IS1	IS2	HS1	HS2	TAG	LS1	LS2	IS1	IS2	HS1	HS2	TAG	LS1	LS2	IS1	IS2	HS1	HS2
CN44:0	0.95	0.96	1.94	1.46	2.25	1.29	CN47:1	0.62	0.65	0.70	0.43	0.38	0.28							
CN48:0	0.86	1.14	1.43	1.61	2.08	1.60	CN45:1	0.52	0.65	0.48	0.42	0.38	0.44							
CN50:0	1.47	0.99	1.29	1.74	1.78	1.46	CN33:1	0.46	0.43	0.54	0.39	0.34	0.36							
CN30:0	0.79	0.84	1.55	0.86	0.90	1.10	CN54:1	0.60	0.43	0.38	0.37	0.21	0.32							
CN36:0F	0.74	1.10	0.79	0.86	0.84	1.29	CN55:1	0.61	0.47	0.26	0.26	0.21	0.33							
CN28:0	0.57	0.65	1.02	0.60	0.66	0.85														
CN29:0	0.50	0.82	0.66	0.54	0.54	1.02														
CN52:0	0.87	0.62	0.55	0.72	0.53	0.76														
CN34:0F	0.36	0.47	0.63	0.43	0.43	1.12														
CN53:0	0.60	0.82	0.61	0.64	0.28	0.49														
CN55:0	0.41	0.30	0.19	0.30	0.20	0.21														
HMW[2]	10.03	14.96	13.32	14.59	15.98	13.63	HMW	8.54	6.78	6.85	6.62	5.33	4.77	HMW	7.05	6.34	7.48	5.46	4.25	4.47
MMW[3]	11.91	14.35	15.31	14.60	17.19	13.71	MMW	6.96	6.96	8.99	7.38	6.52	5.62	MMW	2.50	2.13	2.83	2.03	1.82	1.86
LMW[4]	39.60	41.65	36.33	42.84	45.23	51.98	LMW	8.30	7.72	8.23	7.63	6.16	6.23	LMW	0.85	0.64	0.59	0.54	0.53	0.62

[1]Relative abundance set to the sum of all triacylglycerols

[2]HMW- Triacylglycerols with 47 to 54 carbons;
[3]MMW- Triacylglycerols with 40 to 46 carbon;
[4]LMW- triacylglycerols with 28 to 39 carbons

The relation between TAG profile and FA composition found in our study is in accordance with a previous reported study (Tzompa-Sosa *et al.*, 2016). They showed that an increased in C16:0/C18:1cis-9 ratio in MF increased the proportion of TAG CN34 and 36 and decreased the proportion of TAG CN52 and 54. In this previous study MF TAG profile was described with 16 different TAG, whereas in the present study we distinguished 69 TAG species with up to four unsaturation levels.

The effect of fat content on TAG species was tested because it is an important MF production trait. The fat content in milk had a significant positive effect on TAG CN36:0 and C38:0, whereas it had a significant negative effect on TAG CN43:1 and C47:1. TAG CN38:0 and CN36:0 are two of the most abundant TAG (Table 3, Figure 2). Therefore, breeding strategies aiming to increase fat content will also impact TAG composition, which can further influence the physical properties of MF.

Table 3. Effect of fat content, C16:0/C18:1cis-9 ratio and saturation index on triacylglycerol species and on groups of triacylglycerol. Only TAG species with a significant effect ($p \leq 0.05$) are shown.

TAG	Fat content	Saturation index	C16:0/C18:1cis9 ratio
CN31:0		0.38*	0.51*
CN34:0		0.72*	0.90*
CN36:0	2.22*	2.08**	2.74**
CN38:1		-0.55**	-0.69*
CN38:0	0.79 *		
CN40:2		-0.18*	-0.22*
CN41:1		-0.20*	
CN43:1	-0.12*		
CN47:1	-0.17*	-0.15*	-0.20*
CN51:1		-0.20*	
CN52:1		-0.25*	
CN53:1		-0.31*	-0.40*
CN54:2		-0.16*	-0.20*
CN54:3		-0.14*	-0.17*
Grouped TAG[1,2]			
S HMW			
S MMW			
S LMW		4.88*	6.56*
M HMW		-1.28**	-1.61*
M MMW			
M LMW		-0.95**	-1.17*
P HMW		-1.20*	-1.50*
P MMW			
P LMW			

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

[1]S- saturated; M- monounsaturated; P- polyunsaturated

[2]HMW- Triacylglycerols with 47 to 54 carbons; MMW- Triacylglycerols with 40 to 46 carbon; LMW- triacylglycerols with 28 to 39 carbons.

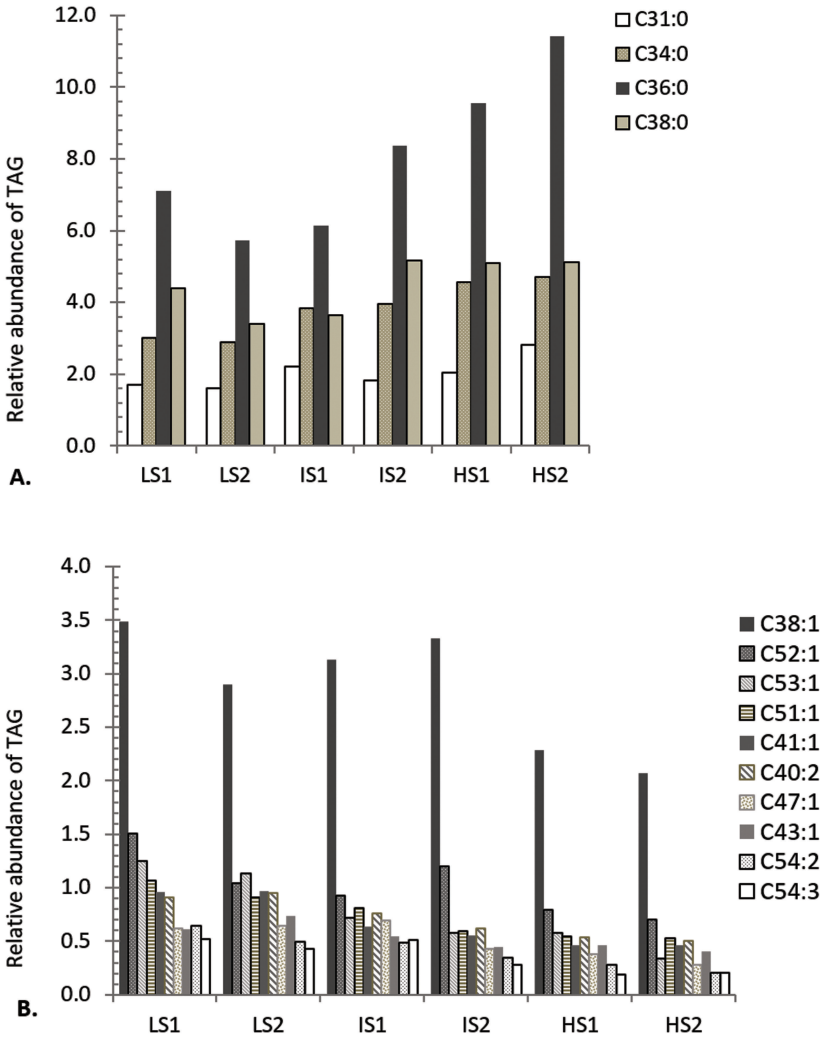

Figure 2. Relative abundance of TAG species which are significant for fat content, C16:0/C18:1cis-9 ratio and saturation index. A. Saturated TAG species. B. Unsaturated TAG species.

Melting behavior of milk fat after isothermal solidification at 10, 15 and 20°C for 120 min

Milk fat with variate FA and TAG profile was isothermally crystallized at 10, 15 and 20°C for 120 min. After isothermal crystallization, MF was melted at 5°C/min. We selected 120 min of storage time to allow the growth and transformation of the crystals. We expect that a mix of α, β' and β polymorphs will be present. This is expected because α polymorphs are formed at the beginning of crystallization. Part of the α crystals transform to β' after 35 min of isothermal crystallization if the

crystallization temperature range from 10°C to 20°C (ten Grotenhuis *et al.*, 1999). Also β crystals are expected in some samples because in a recent study it was shown that β crystals occur after isothermal crystallization at 20°C in MF with an unsaturated FA profile (Tzompa-Sosa *et al.*, submitted).

The endothermic effects, among fats were recorded during heating (Figure 3). The temperature at the maximum point of each endothermic peak was used to describe the melting process. Normalized curves for heat flow were used to compare the intensities of each endothermic effect. During melting four endothermic effects were recorded with an average melting point of 19.5, 26.4, 32.7 and 37.7°C (Table 4). The number of endothermic effects, the melting temperatures and the intensity of these effects varied with MF composition. Table 4 describes the endothermic effects in order of appearance and melting point.

Table 4. Melting point of the endotherms recorded during melting process (5°C/min) after isothermal crystallization at 10, 15 and 20°C for 120 min.

Sample	Isothermal temperature	I	II	III	IV
LS1	10°C	17.7	25.0	33.0	-
LS2		17.6	30.0	32.5	-
IS1		19.8	26.2	-	36.3
IS2		-	23.6	-	37.9
HS1		-	24.4	-	37.7
HS2		-	26.7	-	38.3
	average	18.4	26.0	32.7	37.6
LS1	15°C	19.3	27.9	33.2	-
LS2		19.3	27.6	32.9	-
IS1		20.6	28.9	-	36.5
IS2		-	30.0	-	38.0
HS1		-	25.1	-	37.7
HS2		-	27.0	-	38.3
	average	19.8	27.8	33.1	37.6
LS1	20°C	20.8	24.7	32.5	-
LS2		20.7	24.7	31.7	-
IS1		-	24.5	32.1	36.9
IS2		-	25.0	32.8	38.3
HS1		-	26.6	33.0	38.2
HS2		-	27.8	33.0	38.6
	average	20.7	25.6	32.5	38.0
	Total average	19.5	26.4	32.7	37.7

The first endothermic effect during isothermal crystallization at 10, 15 and 20°C had an average melting temperature of 19.5°C (Table 4). This effect was evident mainly in MF with low C16:0/C18:1cis-9 ratio. These MF samples are decreased in

saturated TAG (CN31:0, 34:0 and 36:0) and increased in unsaturated TAG (CN38:1, 40:2, 47:1, 52:1, 53:1, 54:2, 51:1 and 54:3). The TAG with melting points close to this temperature are the unsaturated high molecular weight TAG (mean Mw = 809 g/mol) (Taylor *et al.*, 1978). Therefore, we suggest that most of the TAGs melting in this first endotherm are CN52:1, 53:1, 54:2, 51:1 and 54:3. Furthermore, the melting temperature of this first effect decreased with a decrease in crystallization temperature. The intensity of the effect, also called heat of fusion, increased as the temperature of isothermal crystallization decreased. Both observations are the effect of the increased inclusion of unsaturated high molecular weight TAG into the crystals as the temperature of crystallization is decreased.

The second endothermic effect seen after isothermal crystallization at 10, 15 and 20°C had an average melting temperature of 26.4°C. This peak was present in all samples, its intensity increased with an increase in the C16:0/C18:1cis-9 ratio. In MF with high C16:0/C18:1cis-9 ratio this was the most intense endothermic effect. MF samples with this high ratio are increased in saturated TAG (CN31:0, 34:0 and 36:0) and are decreased in unsaturated TAG (CN38:1, 40:2, 47:1, 52:1, 53:1, 54:2, 51:1 and 54:3). The melting point of this endotherm was positively correlated to TAG CN34:0 and 36:0 when MF was isothermally crystallized at 20°C (Supplementary information). Moreover, Taylor *et al.* (1978) showed that the melting point of medium molecular weight TAG (mean Mw = 710 g/mol) was between -18 and 23°C and the melting point of saturated low molecular weight TAG (mean Mw = 644 g/mol) was between 8 and 30°C. Both types of TAG had a similar endothermic shape as the one seen in our study for the second endotherm. Therefore, we suggest that the main TAG melting in this endotherm are CN40:2, 47:1 and 31:0, 34:0, 36:0.

The third endothermic effect had an average melting temperature of 32.7°C. In MF with low C16:0/C18:1cis-9 ratio this was the last effect recorded. Thus, it was the final melting point for this type of MF. In MF with intermediate and high ratio C16:0/C18:1cis-9 this effect was recorded only when MF was isothermally crystallized at 20°C. Moreover, when this occurred, the melting point of the second endotherm was affected. This indicates that segregation of TAG with a melting point of about 32°C occurred during isothermal crystallization at 20°C. The melting point of this endotherm was positively correlated to TAG CN36:0 and 38:0 when MF was isothermally crystallized at 20°C (Supplementary information). It is possible that when MF is isothermally crystallized at 20°C, TAG CN40:2, 47:1 segregate from CN31:0, 34:0, 36:0. Therefore, after isothermal crystallization at 20°C, TAG CN40:2, 47:1 would melt in the second endotherm and CN31:0, 34:0, 36:0 would melt in the third endotherm.

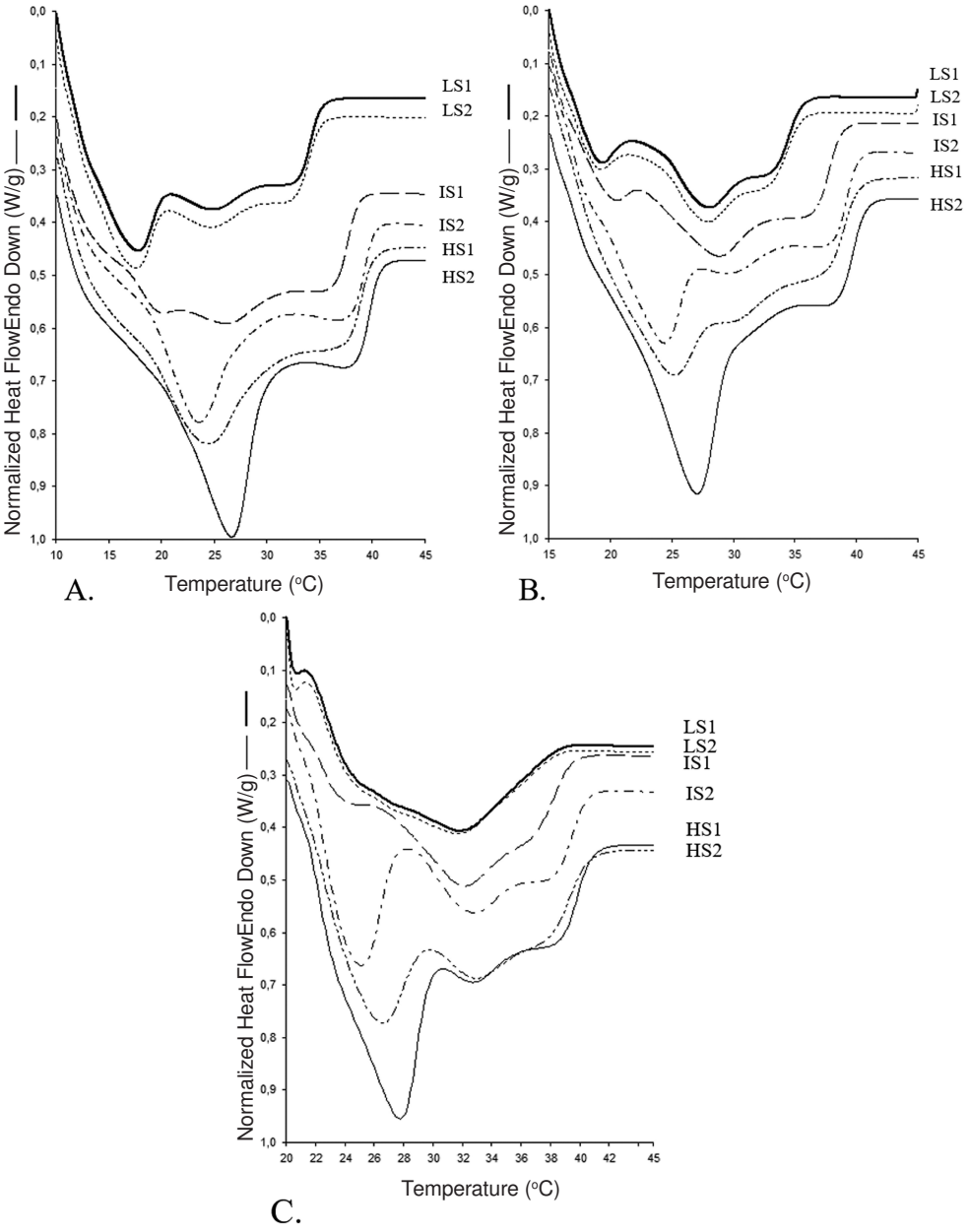

Figure 3. DSC curves of milk fat with variate composition heated at 5°C/min after isothermal crystallization at 10°C (A), 15°C (B) and 20°C (C) for 120 min.

The fourth (last) endothermic effect had an average melting temperature of 37.7°C and was recorded as a broad shoulder at the end of the heating curves. The melting temperature of this effect slightly decreased as the crystallization temperature

decreased. This fourth endothermic effect was present in MF with intermediate and high C16:0/C18:1cis-9 ratio and was absent in MF with low C16:0/C18:1cis-9 ratio. According to Taylor, *et al* (1978) the TAG with the highest melting points are saturated high molecular weight TAG having a melting point between 14 and 45°C. In our samples, these saturated high molecular weight TAG were not correlated to the melting point of the fourth endotherm but TAG CN37:0 and 38:0 were. TAG CN37:0 and 38:0 are two of the most abundant TAG. The correlation between the melting point of this last endotherm and TAG CN37:0 and 38:0 indicates that these TAGs species are the main species melting in this endotherm and are responsible for the increase in the melting point. But this does not exclude the possibility that also saturated high molecular weight TAG are melting in this endotherm.

The four endothermic peaks recorded after isothermal crystallization corresponded to the melting of crystals thermostable at 10, 15 or 20°C. After isothermal crystallization at 20°C, we expect that most of these crystals are in β' and in β form. This is expected because β' polymorphs are formed after 20-35 min of isothermal crystallization at temperatures as low as 10°C (ten Grotenhuis *et al*., 1999) Moreover, in our previous study (Tzompa-Sosa, *et al*., submitted) we showed that after 120 min of isothermal crystallization at 20°C, all crystals were in the β' or β form. However, after isothermal crystallization at 10 and 15°C also α crystals are expected to be present. The first endotherm recorded in our study (melting point = 19.5) can be related to the melting of α crystals, since α crystals have a clear point around 20°C (ten Grotenhuis *et al*., 1999). This first endotherm was present only in MF with low and intermediate C16:0/C18:1cis-9 ratio. The second, third and fourth endotherm recorded in our samples are likely to correspond to the melting of β' and β crystals.

TAG segregated during crystallization at 20°C in MF with intermediate and high ratio C16:0/C18:1cis-9. This was shown by the appearance of an endotherm between 26.4 and 37.7°C when MF was isothermally crystallized at 20°C. This endotherm was absent in these MF after isothermal crystallization at 10 and 15°C. Isothermal crystallization at 10 and 15°C causes strong supersaturation of high melting TAG. Strong supersaturation causes co-crystallization of TAGs with similar chain length (Mortensen, 1982). During isothermal crystallization at 20°C, supersaturation of high melting TAG was lower than that at 10 or 15°C. The crystallization kinetics proceed at slower rates during isothermal crystallization at 20°C allowing high melting TAG to crystallize as separate crystals (Breitschuh and Windhab, 1996). The high melting TAG segregating at isothermal crystallization at 20°C could have co-crystallized with other lower melting TAG and formed the endotherm melting at 26.4°C when MF was isothermally crystallized at 10 and 15°C.

The temperature of isothermal crystallization affected the melting temperature of the endotherms in different ways. The melting point of the first endotherm decreased as the temperature of isothermal decreased, whereas the melting point of the second and third endotherm did not show a pattern. The melting points of these endotherms were lower after isothermal crystallization at 10°C and 20°C as compared with isothermal crystallization at 15°C. The unexpected decrease in melting point after isothermal crystallization at 20°C can be related to segregation of TAG, as previously discussed. In contrast with the first, second and third endotherm, the melting point of the last endothermic effect was consistent within a sample (Figure 4). With these observations we conclude that the final melting point is not affected by the final temperature of isothermal crystallization.

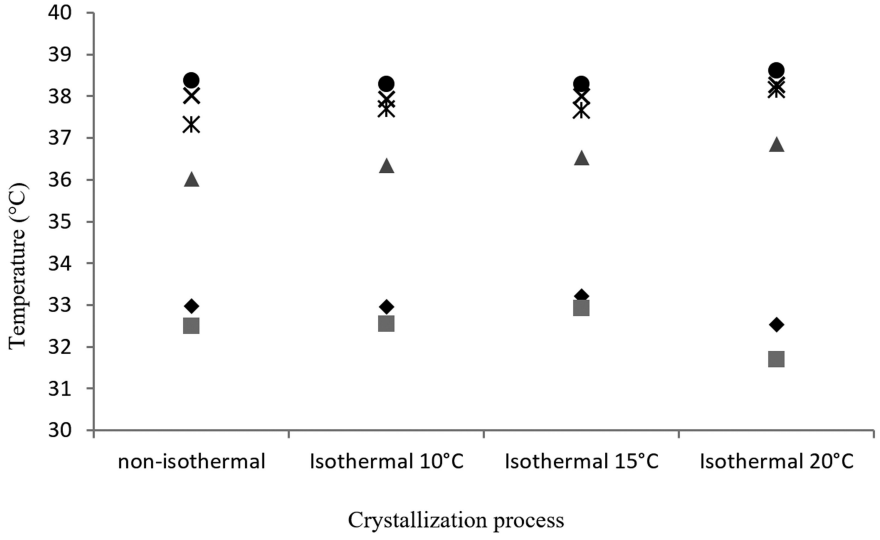

Figure 4. Final melting temperature of milk fat either non-isothermally crystallized or isothermally crystallized at 10, 15 or 20C for 120 min.

Melting behavior of milk fat after non-isothermal solidification from 70 to -60C.

Milk fat with variate FA and TAG profile was non-isothermally crystallized from 70 to -60°C at 5°C/min. Then, MF was melted at the same rate from -60 to 70°C. As previously reported, when MF is crystallized rapidly (> 1°C/min) all crystals are in α form. Therefore, we expect mainly α crystals in our crystallized fat. During the melting process, we recorded three endothermic peaks (Figure 6), which corresponds to what has been depicted as low melting fraction (LMF; average melting point = 8.9°C), middle melting fraction (MMF; average melting point = 17.6°C), and high melting fraction (HMF; average melting point = 35.9°C). The melting points of MF fractions seen in our study were within the previously reported temperatures (Deffense, 1993) but we found large variability among samples.

The first endothermic effect recorded after non-isothermal crystallization showed a clear point between 3.9 and 14.2°C. This effect has been depicted as low melting fraction and corresponds to the melting of low and middle melting TAG in α form (ten Grotenhuis *et al.*, 1999). After this first endothermic effect, we observed a recrystallization process and a second endothermic effect appeared afterwards, showing a melting point between 13.8 and 21.4°C. This endothermic effect was the most intense in all MF samples (Figure 6). Ten Grotenhuis *et al.* (1999) showed that this second endothermic effect corresponds to the melting of middle melting TAG in β' form. The last endothermic effect observed in our study showed a clear point between 33.0 and 38.4 °C. This third and last endothermic effect has been related to the melting of high melting TAG in the β' form, which were formed after re-crystallization during the melting process (ten Grotenhuis *et al.*, 1999).

Milk fat composition affects the melting point of all endotherms seen when MF was non-isothermally crystallized. An increase in the ratio 16:0/18:1cis-9 increased the melting point of the three endothermic effects (Figure 5). Previously Buldo, P. *et al.,* (2013) showed that the melting point of the medium melting fraction was positively correlated with C16:0 and negatively correlated to C18:1cis-9 and other unsaturated C18 FA.

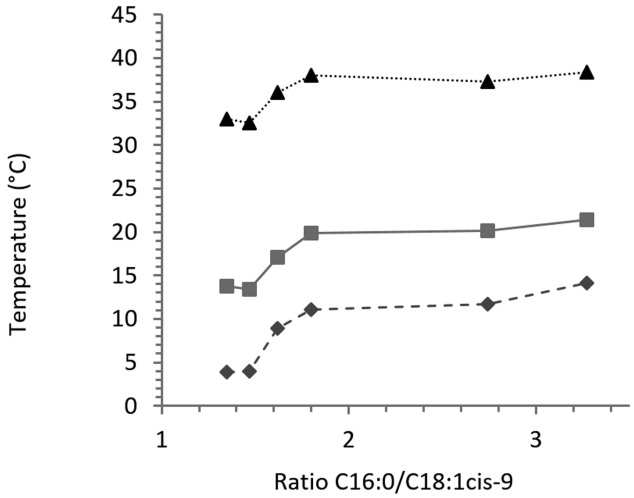

Figure 5. Relation between C16:0/C18:1cis9 and melting temperature of low, middle and high melting fractions (heating rate 5°C/min) determined by DSC after non-isothermal crystallization (cooling from 70 to -60°C at 5C/min).

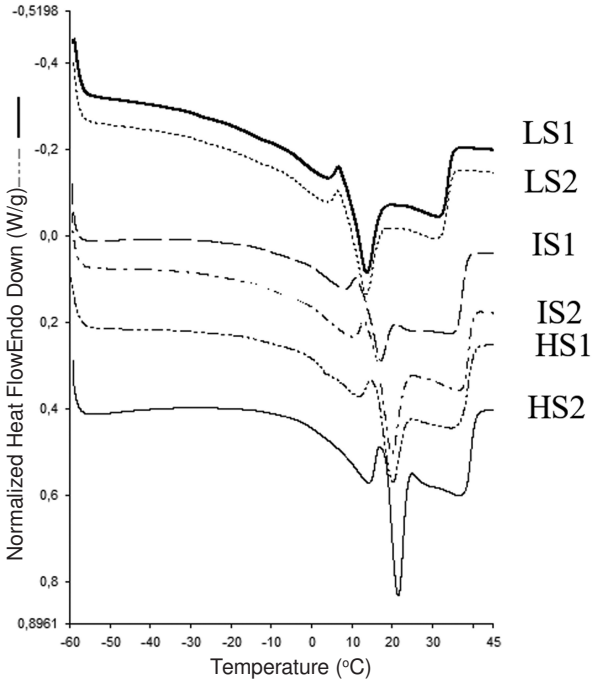

Figure 6. DSC curves of milk fat with variate composition heated at 5°C/min after cooling from 70 to -60°C at 5°C/min.

The kinetics of crystallization during isothermal and non-isothermal crystallization are different. Therefore, the endothermic effects seen after both crystallization processes represent the melting of different types of crystals with different TAG composition. Non-isothermal crystallization produces large supersaturation of TAG, resulting in co-crystallization of TAG. Moreover, the timespan of crystallization is short, thus hindering the formation of metastable and stable crystals, because crystallization of the more stable crystals take a longer time to form. In contrast, during isothermal crystallization the timespan is longer, which promotes the transformation of unstable crystals to metastable and stable ones. After isothermal crystallization at 20°C we saw an endothermic peak which was absent the other isothermal crystallization temperatures. We suggest that at 20°C low supersaturation of the highest melting TAG occurred, thus allowing segregation of the high melting TAGs. A last observation regarding the last endothermic effect is that similar melting points were observed, regardless of the crystallization history. This indicates that these crystals are less likely to be affected by the crystallization process.

The importance of the TAG composition is evident in the samples LS1 and LS2. LS1 has a lower C16:0/C18:1cis-9 ratio (1.35) than LS2 (2.47). Therefore, we expected

lower melting points for LS1 as compared with LS2. But as seen for most melting points, LS1 has a higher melting point than LS2. This difference can be explained by the fact that LS1 is more abundant in saturated TAG (CN31:0, 34:0, 36:0, 38:0, 50:0, 51:0, 52:0 and 55:0) as compared with LS2.

We showed that the shape of the melting curve depends on the crystallization history, on the composition of MF and on the final temperature of crystallization. The three main endothermic effects after non-isothermal crystallization are only observed when the DSC curve is recorded in a specific way (ten Grotenhuis *et al*., 1999) but if MF is isothermally crystallized, then the endothermic effects will differ. The composition of the fat is relevant because it determines the melting temperature and height of the endotherm. And finally, the final melting temperature influences supersaturation of TAGs that further influence if the TAGs co-crystallize or segregate.

Conclusions

Milk fat composition varies within samples and is characterized by its FA and TAG composition. MF with low C16:0/C18:1cis-9 ratio and low saturation index are decreased in saturated low molecular weight TAG (CN31:0, 34:0 and 36:0) and are increased in unsaturated high molecular weight TAG (CN38:1, 40:2, 47:1, 51:1, 52:1, 53:1, 54:2 and 54:3) as compared with MF with an high C16:0/C18:1cis-9 ratio and high saturation index.

Milk fat composition affected the melting point and the number of endotherms observed after isothermal crystallization whereas MF composition affected the melting point of the endotherms but not the number of endotherms observed after non-isothermal crystallization. Segregation of high melting TAGs occurred when MFs with intermediate and high C16:0/C18:1cis-9 ratio were isothermally crystallized at 20°C. This was the result of low supersaturation of high melting TAG and slow crystallization kinetics occurring at 20°C. Finally, the final melting point was influenced by MF composition but not by crystallization history.

Aknowledgements

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Supplementary information

Table S1. Correlation between the clear points of each fraction and the triacylglycerol species in milk fat[1]. Milk fat was isothermally crystallized at 20, 15 or 10C for 120 min and then it was melted at 5°C/min.

	Peak II				Peak III				Peak IV			
TAG	Isothermal at 20°C (N = 6)	Isothermal at 15°C (N = 6)	Isothermal at 10°C (N = 6)		TAG	Isothermal at 20°C (N = 6)	Isothermal at 15°C (N = 2)	Isothermal at 10°C (N = 2)	TAG	Isothermal at 20°C (N = 4)	Isothermal at 15°C (N = 4)	Isothermal at 10°C (N = 4)
C34:0	0.82*				C36:0	0.88*	N.C.	N.C.	C37:0	0.99*	0.97*	0.98*
C36:1	-0.84*	0.82*			C37:1	-0.81*	N.C.	N.C.	C38:0	0.97*		0.96*
C36:0	0.94**				C38:0	0.97**	N.C.	N.C.	C43:1	-0.99*	-0.99**	-0.99**
C38:1	-0.91*				C40:2	-0.84*	N.C.	N.C.	C44:2	-0.97*		-0.96*
C45:1			0.85*		C43:1	-0.89*	N.C.	N.C.	C46:2	-0.99**	-0.99*	-0.99**
C47:1	-0.90*				C45:1	-0.90*	N.C.	N.C.	C46:1	-0.98*	-0.96*	-0.97*
C48:2	-0.81*				C47:1	-0.85*	N.C.	N.C.	C47:1	-0.98*	-0.96*	-0.97*
C50:2	-0.82*				C50:0	0.89*	N.C.	N.C.	C48:2	-0.97*	-0.95*	-0.97*
C50:0			-0.93**		C53:0	-0.82*	N.C.	N.C.	C49:1	-0.99*	-0.96*	-0.98*
C52:2	-0.85*	0.89*							C51:1	-0.97*		-0.95*
C54:3	-0.83											
C54:2	-.84*											

*p < 0.05; **p < 0.01

N.C. – not calculated.

[1] Peak I was not calculated because N = 2

Chapter 6

General discussion

Two research questions are the foundation of the work described in this thesis. The first research question was: “what is the normal variation of TAG in milk fat (MF) and how *DGAT1* K232A polymorphism affects the variability and structure of these TAG”. The second research question was: “how does the variability of TAG composition affect the thermal behavior of MF”. The results described in **chapter 2** showed that TAG profile greatly varies within a dairy cattle population. The variation in TAG profile was explained by fatty acid (FA) concentrations and *DGAT1* K232A polymorphism. Part of the variation caused by FA was explained by the ratio C16:0/C18:1cis-9. In contrast, days in milk and MF productive traits (fat content and morning milk yield) did not influence TAG profile. Further, *DGAT1* K323A K allele was associated with an increase in TAG CN38. In **chapter 3** we described the regiospecific structure of MF TAG. We showed that the variation in TAG structure was explained again by the concentration of FA and by *DGAT1* K232A polymorphism. Part of the variation of TAG structure was explained by the concentration of C16:0 in the TAG. An increase in C16:0 in the TAG increased the proportion of C18:1cis-9 at sn-2 while decreasing the proportion of C14:0, C16:0 and other long chain saturated FA positioned at sn-2. We showed that *DGAT1* K232A polymorphism affects TAG structure. *DGAT1* KK genotype was associated with an increase in the rate at which C16:0 is esterified at sn-2. In **chapter 4** we explored the relation between MF composition (FA and TAG) and the polymorphisms that are thermodynamically stable at 20°C. We showed that the concentration of TAG is relevant for the formation of TAG polymorphs. MF with high concentrations of TAGCN52-54 and low concentrations of TAG CN34-38 showed β polymorphs when isothermally crystallized at 20°C. This TAG profile corresponds to MF with an unsaturated FA profile. Finally, in **chapter 5** we explored the effect of MF composition and crystallization history on melting behavior. We showed that MF composition (FA and TAG profile) is relevant to explain the final melting point of MF and its melting fractions.

In this last chapter we discuss the considerations taken for the experimental design and the importance of using individual samples of MF. Moreover, we describe the relevance of studying MF TAG and further discuss the results of the entire thesis.

Considerations for this study

Three considerations were taken into account for the experimental design, sample selection and analysis of the results of the second and third chapter of this thesis. The first consideration was the relation between FA composition and fat content. The second consideration was the units of measurement used to describe the concentration of FA. And the third consideration was the way of presenting data of FA composition in the sn-2 position of TAG.

The first consideration in this work was the relation between FAs and fat content and between *DGAT1* K232A polymorphism and fat content. When exploring the effects of MF production traits on FA concentration, we found that 20 FA were significantly affected by fat content (Table 1). Moreover, it is known that *DGAT1* K232A polymorphism has a mayor effect on fat content and FA profile. Therefore, a random selection from the population of cows with *DGAT1* AA and KK genotype would have resulted in two different populations: one with high fat content and an increased amount of saturated FAs (KK genotype) and another with low fat content and lower amount of saturated FAs (AA genotype). Consequently, these two populations would have shown different TAG profiles not only due to the *DGAT1* genotype as such but also due to the difference in the FA profile. In Figure 1 we show the relation between fat content and FA profile, as shown by saturation index. As seen in Figure 1, the population of cows with *DGAT1* KK genotype has an increased fat content and saturation index, whereas the milk of the population of cows with *DGAT1* AA genotype shows a lower average fat content and higher variation than the milk of cows with KK or AK genotype. We approach this variation in fat content and FA composition by grouping the cows according to fat content (from 3 to 3.9, from 4 to 4.9 and from 5 to 6%) and selecting cows with *DGAT1* genotype AA and KK within each fat group (Chapter 3 and 4). In this way we could determine if the differences in TAG profile and structure were related to FA composition or to *DGAT1* genotype.

The second consideration taken into account in this work was the units expressing the concentration of FA in MF. MF FA profile is typically expressed on weight basis, however, in this thesis FA composition is expressed in mole basis (mol%). When studying MF synthesis and the effects of *DGAT1* K232A polymorphism, the FA composition in mol basis should be used because it gives information about the number of molecules (FA) produced in the mammary gland and not about the size of these molecules. The information of FA composition in mol basis can be later linked to the mechanisms of lipid synthesis. Thus, in reaction kinetics studies, FA composition should be shown in mole basis.

Table 1. Significant effect of fat content on fatty acids, saturated fatty acids, unsaturated fatty acids, saturation index and C16:0/C18:1cis9 ratio (P < 0.05). Milk fat from Dutch-Holstein Friesian cows under a winter diet (N = 1760).

Saturated fatty acids	Effect size	p	Unsaturated fatty acids	Effect size	P	Grouped fatty acids	Effect size	p
C4:0			C10:1	0.01	0.041	SFA	1.20	< 0.001
C5:0			C12:1			UFA	-1.08	< 0.001
C6:0	0.03	0.001	C14:1cis-9			Saturation Index	0.22	< 0.001
C7:0			C16:1cis-9	0.09	< 0.001	ratio16:0/18:1cis-9	0.20	< 0.001
C8:0	0.02	0.006	C17:1cis-9					
C9:0	0.00	0.035	C18:1trans6	-0.02	< 0.001			
C10:0			C18:1trans9	-0.01	< 0.001			
C11:0			C18:1trans10	-0.06	< 0.001			
C12:0			C18:1trans11	-0.03	< 0.001			
C13:0			C18:1cis-9	-0.78	< 0.001			
C14:0	-0.34	< 0.001	C18:1trans15 (likely)	-0.01	< 0.001			
C15:0			C18:1cis-11	-0.03	< 0.001			
C16:0	1.17	< 0.001	C18:1cis-12	-0.02	< 0.001			
C17:0			C18:1cis-13	-0.01	< 0.001			
C18:0			C18:2cis-9,12	-0.12	< 0.001			
C20:0			C18:3cis-9,12,15	-0.03	< 0.001			
C21:0			C18:2cis-9,trans11	-0.04	< 0.001			
C22:0			C20:3cis-8,11,14	0.00	< 0.001			

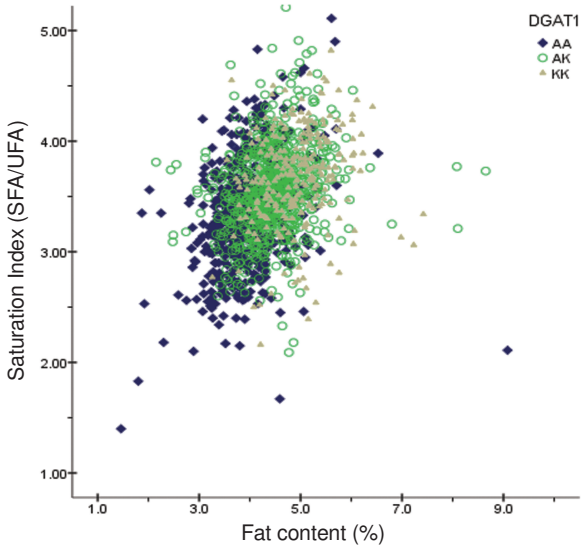

Figure 1. Relation between fat content (%) and saturation index (SFA/UFA) (N = 1760; $p < 0.001$) on milk fat from Dutch Holstein-Friesian cows under winter diet. Cows are depicted with different genotypes of *DGAT1*.

The third consideration taken into account in this work was the way of presenting the FA composition at sn-2 position. The results for the FA composition at sn-2 were shown with an intra- and an interpositional approach. An intrapositional approach shows the proportion of FAs at sn-2 without taking into account the total percentage of the FAs in the original sample, which in our study varied among individuals. An interpositional approach shows the proportion of FA at sn-2 position taking into account the total percentage of each FA. For comparison, our results in chapter 3 were also shown with an intrapositional approach because most studies only show their results with this approach. However, by this approach concentrations of FAs at sn-2 can only be compared when samples have equal FA composition. Due to variation in FA composition of the samples we studied FA composition at sn-2 position with an interpositional approach . This approach helped us to understand the dynamics of the positioning of FA within the TAG.

Relevance of the study of milk fat composition on individual cows

In this work the variability of MF TAG was determined in MF from individual cows. The advantage of using MF from individual cows over bulk milk is that information on FA composition, M F productive traits and the genetic background of each individual cow is available and can be related to changes in TAG profile and structure. When studying thermal behaviour of MF, the use of MF from individual cows allows the

selection of samples with differences in MF composition. Most of the studies in the past used MF from bulk milk. Pooling milk from different cows creates a blind spot to the natural variation in MF TAG composition. Using MF from individual cows, we showed for the first time, that TAG profile and TAG structure varies within a population of cows. We further explained the variability of MF using the concentration of FAs, productive traits and *DGAT1* K232A polymorphism. Moreover, we showed that MF from individual cows is useful to study the kinetics of MF crystallization and melting because it makes possible to study MF with a broad range of FA and TAG profiles.

Importance of the study on triacylglycerols

Characterization of MF is typically based on FA composition, which determine TAG structure. MF FA have been thoroughly characterized for cow breeds, feeding regimes, lactation stages, regions, etc. (DePeters *et al.*, 2001, Jensen, 2002, Heck *et al.*, 2012). However, this information is not enough to characterize a fat because one FA blend can yield different TAG profiles. In this study we investigated MF composition in terms of FA and TAG composition. We showed how FA and *DGAT1* affect the TAG profile and structure. We also investigated the importance of TAG profile on the crystallization and melting behavior of MF.

Studying MF TAG becomes of relevance for the study of *DGAT1* K232A polymorphism because this enzyme catalyzes the last step of TAG synthesis. Therefore, we hypothesized that this polymorphism would influence either the concentration of TAG species synthesized or the structure of TAG. In chapter 2 we described the distribution of MF TAG in a cow population under a winter diet and showed that *DGAT1* K232A polymorphism affected the concentration of TAG profile. In chapter 3 we further showed the influence of *DGAT1* K232A polymorphism on TAG structure. Cows with *DGAT1* KK genotype appear to place C16:0 at sn-2 at higher rates than cows with AA genotype.

Triacylglycerol composition is also relevant for the thermal behavior of MF because TAG constitute the fundamental unit of crystals and changes in TAG composition modify the nanostructure of TAG crystal networks (Acevedo and Marangoni, 2010). We hypothesized that the abundance of each TAG and its FA profile were influencing the crystallization kinetics of MF and, consequently, the melting behavior. In chapter 4, we showed that TAG and FA profile determine the type of polymorphs thermally stable at 20°C. Moreover, we showed that the presence of β polymorphs in MF is indeed related to TAG and FA profile, as it was hypothesized in previous studies. TAGs can also help to understand some characteristics of melting behavior such as melting point and number of melting fractions (Chapter 5). Due to the relation between MF composition and thermal behavior, the studies of crystallization and melting kinetics of MF should include profiling of FAs and TAGs.

Relation between FA and TAG in milk fat

The distribution of FA within MF TAG is not random as demonstrated in figure 2 where the random and the observed TAG profile are plotted. The differences seen between the observed and the random TAG profile are largely determined by the FA specificity of acyltransferases in the mammary gland, such as *DGAT1* (Gresti *et al.*, 1993). TAG synthesis is regulated by the enzymes involved in lipid synthesis, which respond to the availability of FA present in the system and probably this preference changes with genetic factors (Bionaz and Loor, 2008, Smiddy *et al.*, 2012). It has been shown that GPAT, which is the first enzyme involved in TAG synthesis, has preference for SFA at the expense of UFA. GPAT shows a higher activity in the presence of C16:0 as compared with other FA (Kinsella, 1976). The FA preference of other enzymes related to TAG synthesis is still unknown. However the FA specificity of the enzymes involved in TAG synthesis explains that the TAG profile observed differs from a profile based on random distribution.

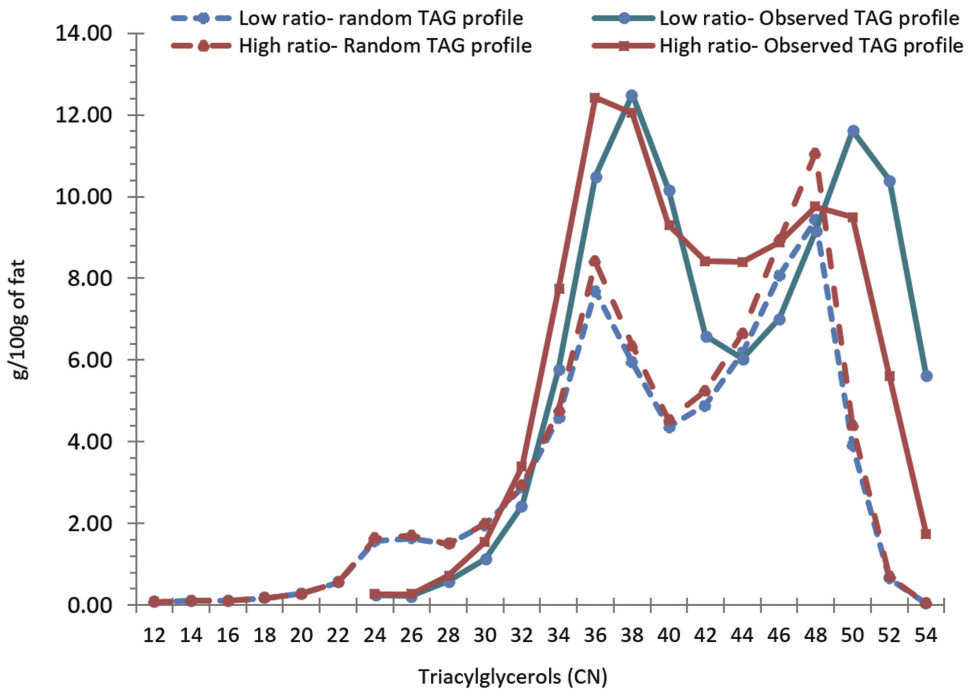

Figure 2. Random and observed distribution of milk fat triacylglycerols. Low ratio corresponds to milk fat with ratio C16:0/C18:1cis-9 = 1.2; high ratio corresponds to milk fat with ratio C16:0/C18:1cis-9 = 2.7.

The most abundant FA C16:0 and C18:1cis-9, are negatively correlated and have a major impact on the physical properties of MF. In chapter 2 and 3 we showed that these two FA are related to the concentration and to the structure of TAGs.

In the study described in chapter 2 the ratio C16:0/C18:1cis-9 was used to show the variation in concentration of TAG within our cow population, and in the study described in chapter 3 the concentration of C16:0 was used to show the variation in TAG structure. When studying the effect of FA concentration on TAG profile, we found that MF with high ratio C16:0/C18:1cis-9 showed an increased concentration of TAG CN34 and 36 and a decreased concentration of TAG CN 52 and 54, as compared with MF with low ratio C16:0/C18:1cis-9. We hypothesized that the increase in TAG CN 34 and 36 was a response of the mammary gland to the overall increase in C16:0. These TAG are composed of one small chain FA (C4:0 or C6:0), and medium and long chain FAs. Esterifying C16:0 together with other short chain FA, such as C40:0 or C6:0, prevents MF from having a melting point above the body temperature of the cow (36.5°C). We suggest that regulation of MF TAG synthesis in the mammary gland occurs either by a change in the activity of enzymes related to TAG synthesis or by a change in FA preference of the enzymes or its polymorphs.

Effect of *DGATI* K232A polymorphism on TAG variability and structure

Diacylglycerol acyltransferase is the enzyme that catalyzes the positioning of a FA at sn-3. The *DGAT1* K232A polymorphism was previously shown to have a significant influence on bovine milk characteristics, namely milk yield, protein content, fat content, FA composition, milk proteome and metabolome (Schennink *et al.*, 2007, Duchemin *et al.*, 2013, Lu *et al.*, 2015). In this study we investigated the TAG profile and structure of MF samples from cows with the *DGAT1* KK and AA genotypes. Cows with *DGAT1* KK genotype were increased in TAG CN38 and showed less variability in the concentration of TAG. This increase was not influenced by fat content neither by the concentration of FA C16:0 which suggests that the increase in TAG with CN 38 is mainly due to *DGAT1* K232A polymorphism. Regarding TAG structure, cows with *DGAT1* KK genotype showed an increased proportion of C16:0 esterified at sn-2.

Regulation of the positioning of FA within the TAG could be occurring via glycerol-3-phosphate acyltransferase (GPAT), which regulates the first step in triacylglycerol synthesis in the glycerol-3-phosphate pathway. Mitochondrial GPAT has preference for C16:0 FA and it is 8 to 10 times more active in the presence of C16:0 than in the presence of other FAs. This may explain our observations on the increased proportion of C16:0 esterified at sn-2 in cows with *DGAT1* KK genotype. A genetic predisposition in cows with *DGAT1* KK genotypes may increase the concentration of C16:0 in the pool of FA available for TAG synthesis. This increase in C16:0 can further trigger a rise in the activity of GPAT causing a higher esterification of C16:0 at sn-1 with the consequent decrease of C16:0 at sn-2, as shown in chapter 3. The preference of GPAT for C16:0 and its increase activity in the presence of C16:0,

can further regulate the positioning of other FAs in the TAG. Additional support for the increase in the activity of GPAT can be obtained by studying lipids with a similar synthesis pathway, such as phospholipids. Argov-Argaman *et al.* (2013) found that an increase in sphingomyelin content was affected by fat content and *DGAT1*. This phospholipid was increased in cows with *DGAT1* KK genotypes as compared with cows with *DGAT1* AA genotype. This observation supports our hypothesis that GPAT activity is being increased in cows with *DGAT1* KK genotype.

Several changes in MF composition have been associated to *DGAT1* K232A polymorphism. However, the mechanism behind this effect has not been elucidated. One common characteristic among studies is that less variability in milk components (FA, TAG, proteins, etc.) occurs in cows with the *DGAT1* K allele. Suggesting that this allele is responsible for an increase in the overall regulation of milk synthesis.

The key to unravel the specific effect of *DGAT1* K232A polymorphism on TAG synthesis is studying TAG individually. Because, as shown in chapter 3, *DGAT1* K232A polymorphism affects only a few TAGs. The current FA and TAG structure analysis performed to investigate *DGAT1* K232A polymorphism involves the deacylation of FA in all the MF TAG, thereby losing the information of the original TAG species. We recommend that further studies aiming to unravel the mechanism behind *DGAT1* K232A polymorphism should include information of FA composition, concentration and structure of individual TAG species.

Implication of natural variation in Triacylglycerol profile on thermal behavior of milk fat

Triacylglycerols constitute the fundamental unit of crystals. In MF three types of crystals have been described, α, β'and β. The formation of these crystals is affected by factors such as cooling rate, crystallization temperature and amount of liquid fat (Lopez *et al.*, 2001, Lopez *et al.*, 2005, Cisneros *et al.*, 2006). Most studies have described the presence of α and β' in MF, however β crystals have not always been detected. It has been suggested that fat composition is responsible for the differences in polymorphs seen among studies, however the relation between MF composition and polymorphs in MF has not been understood (ten Grotenhuis *et al.*, 1999) . We hypothesized that the concentration of individual TAG species were responsible for the types of crystals formed in MF. To test this hypothesis we selected MF with variable TAG profiles and analyzed the types of crystals formed when MF was crystallized at 20°C (Chapter 4). Variation of MF TAG profiles was described as the ratio TAG CN 34-38/52-54. These TAGs were selected because in Chapter 2 we showed that these TAG were describing the variability of MF TAGs. In chapter 4 we further described the effect of the variation of MF TAG profile in the melting behavior after it was crystallized at 10, 15 and 20°C.

In this study we showed that TAG profile affects induction time, intensity and type of crystals formed in MF. These differences further affect the melting behavior of MF by changing the quantity of endothermic peaks and the final melting temperature. We also observed that the concentration of TAG is responsible for segregation and co-crystallization of TAG during crystallization and this further affects the melting behavior.

FA and TAG composition are closely related, as shown by the effect of saturation index and the ratio C16:0/C18:1cis-9 on TAG profile (Chapter 2). One FA blend may result in different TAG profiles, as is shown in chapter 4. Samples with a similar saturation index (saturation index = 2.1) showed differences in the TAG ratio 34-38/52-54. These differences in TAG composition further affected the types of crystals formed. MF with a low TAG ratio 34-38/52-54 (ratio = 1.6) showed clear peaks of β crystals and MF with a high TAG ratio 34-38/52-54 (ratio = 2.2) showed only traces of this type of crystal. This is one example of the importance of TAG for the study of crystallization behavior of MF.

Implications for technological properties and breeding strategies

Understanding the variability of TAGs and the role of FA composition, *DGAT1* K232A polymorphism and milk productive traits on the variation of MF TAGs helps to find opportunities to modify physical properties of MF. For instance, MF with low saturation index and low ratio 34-38/52-54 TAG, crystallize in β forms. Whereas MF with an opposite MF profile form exclusively β' polymorphs. The latter type of crystals is desirable in food industry for its plasticity, β crystals have less desirable physical properties. However, they can be relevant in fractionation technologies.

Nowadays, feeding strategies are used to modify FA composition, and hence TAG composition. But as shown in this research, *DGAT1* could also be used to further modify TAG composition, which influences the crystallization and melting behavior of MF. In chapter 2 we showed that MF with similar FA content but different *DGAT1* genotypes (AA and KK) had small difference in the amount of TAG CN38. In chapter 2 we used MF from cows with different *DGAT1* genotypes but similar fat content to determine the effect of *DGAT1* K232A polymorphism alone. However, the average cow population with *DGAT1* KK genotype has a more saturated profile and is increased in fat content. Therefore, the differences on TAG profile and on the physical properties of MF from cows with *DGAT1* KK and AA genotype are much larger. This variation can be used in breeding strategies to further modify MF TAG profile.

Conclusions

- The study of MF TAGs is necessary to understand the effect of *DGAT1* K232A polymorphism on MF synthesis. Mainly because this enzyme catalyzes the last step of TAG synthesis in the mammary gland. Moreover, the study of TAGs help to understand the mechanism of crystal formation in MF and to explain its melting behavior.
- Individual samples of MF prove to be useful to understand the effects of *DGAT1*, FA composition, and productive traits on the variability of TAGs.
- MF TAG profile is related to the FA composition and *DGAT1* K232A polymorphism but not to days in milk and milk production traits (fat content and morning milk yield).
- Cows with *DGAT1* KK genotype had an increased concentration of TAG with CN38.
- MF with low saturation index and low concentration of FA C16:0 will be decreased in TAG CN34 and 36 and will be increased in TAG CN52 and 54 as compared with MF with high saturation index and high concentration of FA C16:0. Moreover, MF with low saturation index and low concentration of FA C16:0 will tend to have an increased proportion of FA C16:0 at sn-2. Under crystallization, this type of fat will tend to form β crystals and will show a melting point at about 32°C.
- The concentration of TAG is relevant to form fat polymorphs because for a crystallization to proceed at a reasonable rate at a given temperature, an appreciable number of TAGs supersaturated should be present to form a nuclei that can further grow.
- Prediction of MF physical and functional properties requires understanding of the variability of MF triacylglycerols. This knowledge may help to control functional and compositional differences between MFs.

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Summary

Milk fat (MF) is one of the major components of milk. Milk contains 3.0 to 6.0% of fat, but typically is in the range 3.5 to 4.7%. Due to its unique taste and nutritional value, MF is used as ingredient in many food products. More than 400 FA have been identified in MF resulting in a wide range of TAG. The concentration of these FAs can be modified by nutrition and management, by genetics or by industrial processes. MF FA composition has been traditionally characterized based on FA profile. However, knowing the FA profile of a fat is not enough for its characterization because one FA blend can produce different TAG profiles.

Understanding the variability of MF TAG and the role of FA composition, milk productive traits and enzymes related to TAG synthesis helps to find opportunities to modify physical properties of MF. The study of TAG is of relevance for thermal behavior of MF because TAGs constitute the fundamental unit of crystals, which define the functionality of MF in the products. We hypothesized that the abundance of TAG species, its FA profile and the configuration of TAG influence the crystallization kinetics of MF, which further influence its melting behavior.

The effect of dietary FA on MF TAG composition has been previously reported, but the effect of genetic means to modify TAG composition remains unexplored. Therefore, in this thesis we studied the effect of a mutation of diacylglycerol acyltransferase (DGAT) on MF TAG. DGAT catalyses the last step of TAG synthesis in the mammary gland. A polymorphism (mutation) of *DGAT1*, namely K232A polymorphism, was previously shown to have a significant influence on bovine milk characteristics, namely milk yield, protein content, fat content, FA composition, milk proteome and metabolome. Since *DGAT1* is involved in TAG synthesis, we hypothesized that this polymorphism would have an effect on TAG synthesis and not only on FA composition.

Aim and outline of this thesis

The objectives of the present thesis were to describe the variability and structure of MF TAGs within the Dutch Holstein-Friesian cow population; to study the effect of variability of FA, milk productive traits, days in milk and *DGAT1* K232A polymorphism on MF TAGs. And to understand the effect of MF TAG and FA profile on the thermal behavior of MF. In **Chapter 1** the general composition of MF is described and relevant information related to MF TAGs and *DGAT1* K232A polymorphism is presented. Moreover, a brief introduction to thermal behavior of MF is given.

Chapter 2 "Milk fat triacylglycerols and their relations with milk fat acid composition, *DGAT1* K232A polymorphism and milk production traits",

describes the variability of TAG on MF from individual cows. In this chapter we investigated the effect of FA composition, *DGAT1* K232A polymorphism and milk production traits (fat content, morning milk yield) and DIM on MF TAG profile in the Dutch Holstein-Friesian dairy cattle population. Large differences in MF TAG profile were seen among cows that imply differences in MF physical and functional properties. Part of the variation caused by FAs was described using the ratio C16:0/C18:1cis-9. In contrast, MF productive traits did not influence TAG profile. Further, *DGAT1* KK genotype was associated to an increased in TAG CN38. The implications of these differences in fat composition were studied in Chapter 4 and 5.

Chapter 3 "Influence of C16:0 and long-chain FA on normal variation of bovine MF TAG structure" reports the TAG structure of individual cows with variate FA profiles. We investigated the relation of MF FA profile and *DGAT1* K232A polymorphism with TAG structure. Once again we showed that the variation in TAG structure was explained by the concentration of FAs and by *DGAT1* K232A polymorphism. Part of the variation of TAG structure was described by the concentration of C16:0 in the TAG. An increased in C16:0 in the TAG (mol %) increased the proportion of C18:1cis-9 at sn-2 while decreasing the proportion of C14:0, C16:0 and other long chain saturated FA positioned at sn-2. Moreover, *DGAT1* KK genotype was associated to an increase in the proportion of C16:0 esterified at sn-2 (in cows with low concentration of C16:0 in the TAG).

In the first part of this thesis (Chapter 2 and 3) the effect of *DGAT1* K232A genotype on TAG variability and structure is shown. These observations could provide direction for further understanding the mechanisms by which the *DGAT1* K232A polymorphism influences MF synthesis.

In **Chapter 4 "Formation of β polymorphs in milk fats with large differences in triacylglycerol profiles"** we studied the effect of MF TAG on the formation of polymorphs thermodinamically stable at 20°C. We aimed to better understand the relation between MF TAG profile and crystals thermodinamically stable at 20°C, such as β' and β. We showed that the concentration of TAG is relevant for the formation of TAG polymorphs. MF with high concentration of TAG CN52-54 and low concentrations of TAG CN34-38 showed β polymorphs when isothermally cooled at 20°C. In this way we explain the presence of β polymorph in MF and solve the controversy surrounding this polymorph.

In **Chapter 5 "Melting point of milk fat fractions: the importance of triacylglycerol profile"** we studied the relation between MF composition and melting fractions. We showed that four melting fractions are recorded when heating MF after being isothermally crystallized at 10, 15 or 20°C for 120 min. Not all four melting fractions are present in the fats. The presence and melting point of these fractions was related

to the FA and TAG profile of MF. For instance, MF increase in unsaturated TAG CN51-54 and decreased in saturated TAG CN31, 34 and 36 showed a final melting point of 32.6°C and lack a melting peak corresponding to TAG with the highest melting point. Whereas MF with an opposite TAG profile showed a final melting point above 38°C and lack a melting peak corresponding to TAG with the lowest melting point.

In this thesis we showed that understanding the variability of MF TAG and the role of FA composition, milk productive traits and enzymes related to TAG synthesis helps to understand the thermal behavior of MF and to find opportunities to modify physical properties of MF.

Acknowledgements

This book represents the end of a journey. A journey full of joy, hard work and (some) tears. It is also the probe to me that I could re-invent myself and become a researcher. Something that seemed impossible just 7 years ago when my husband and I moved to the Netherlands. Because, how could I ever succeed in science in a country with 19 Nobel prices??!!

This journey could not have been possible without Hein van Valenberg. He took the risk of taking me as PhD student almost without knowing my capabilities. Hein guided me, supported me, was next to me in each step of this journey and showed me the way thought science in his very particular way. Hein, I am grateful to you for all what you have done for me during these years. Also very important is George van Aken. George shared his immense knowledge in lipids with me unreservedly. He guided me thought the hardest times of my PhD studies. I will never forget one time when I was completely lost in my research. On that day I felt like if I was in a “dark hole” without possibilities of coming out. We had then one meeting and in a couple of hours he guided me and pulled me out of “my hole”. And how could I forget Henk Bovenhuis? I am thankful to him for his great help in statistics and genetics. He was always there to help me and give me his kind advice and always with a smile on his face.

I was in this journey with many other PhD student at FQD (and before PDQ, remember?), which whom I share office, coffee breaks, lunch breaks, lab work, lab trips, PhD trips, congresses, courses, WE Days, meetings, etc. They make this experience incredible. We shared frustrations, joy, but also recipes and everyday life. With them I was able to learn a bit of many cultures in this world. Since most of my PhD student colleagues were women, I was able to know a bit of the thoughts and feelings of them. There is where I realize that the essence of women around the world is the same. And this helped me to write a preposition “Women around the world are similar. We all have similar dreams, similar worries and similar complaints”. Sadly this preposition was not accepted.

In this journey, Victor, my husband, was always next to me. He encouraged me to pursuit this PhD degree, and was always my support. He was happy whenever a paper was accepted, and was there to hug me whenever I had a bad day. Victor was always my link to “the real world”, especially during the last six months before finishing this manuscript, when I became a hermit in order to finish writing. I feel that a part of this degree also belongs to him. Also next to me has always been my family, Mom, Dad, Mundo, Aly, Zitely and Aunt Amelia. They had follow this journey via WhatsApp of by phone. Always interested of what was happening and always supporting me from the distance.

I am grateful to the Mexican government and to CONACYT for the financial support given to my PhD studies. I hope that I can pay back their support by educating others. Because I believe that education is the only way to overcome most of the problems that my country faces, such as poverty, corruption, and drug trafficking. Mexico is a country full of opportunities, but most people do not have the capabilities to grab them simply because they do not know how to do it.

Every journey has a start and an end point. And I am now about to get to my final destination together with my paranimphs, Ruben and Elsa. Guys, thank you both for accepting being my paranimphs. I appreciate the time spent to share our thoughts, problems, gossips, discussions, dinners, etc. Together with Teresa we made a nice office environment, het was altijd gezellig in ons kantoor. We have shared many good and bad moments during these years and I feel that we became a bit more than colleagues.

I am finally at the end of this journey, or as I always said “I arrived to the end of the tunnel”.

Curriculum Vitae

Daylan Amelia Tzompa Sosa was born on July 25, 1980 in the sunny Mexico City in Mexico. She is Animal Science Engineer and has a Master degree in Dairy Science and Technology, which she studied in her home country. At the beginning of her career she worked in the primary production sector and in agricultural business in companies located in Mexico and in the U.S.A. When she moved to the Netherlands she worked in the food industry and later she started her PhD studies at Wageningen University. During her time as researcher in Wageningen University, she has been involved in projects on lipidomics, nutrigenomics, physical properties of fats and on extraction and characterization of insect lipids. Daylan is currently post-doc researcher at the Dairy Science Group in Wageningen University in the Netherlands.

Specialties- Lipid analysis (lipid extraction; separation and characterization of lipids with MALDI-TOF, HPLC, GC, TLC); triglyceride characterization and structure; thermal characterization of oils and fats with DSC, pNMR, XRD; dairy science; animal breeding; agricultural business.

PhD student Training and Education

Programme of education

a. Discipline specific activities (courses, workshops, symposia, summer schools etc.)

Name of the course	Graduate school/institute	Year	Credits
Advanced Food Analysis	VLAG, NL	2010	1.4
Plant and dairy fat functionality in foods	NIZO Foods, NL	2011	0.6
Fatty Acids and lipids-Chemistry and Analysis	Mylnefield Lab, UK	2012	0.9
Food and biorefinery Enzymology (FBE)	VLAG, NL	2011	1.2
Fatty acids in dairy cattle in relation to product quality and health	WIAS- Ghent University, Be	2012	1.4
Milk Genomics Symposium	WIAS, NL	2012	0.9
Compositional Analysis of Lipids	Ghent University, Be	2013	0.6
Short training in Japan	Hokkaido University, Jp	2013	3.0
Milk Genomics Symposium + poster	UC Davis, USA	2013	1.9
Lipid Analysis course	Instituto de la grasa, Sp	2014	1.4
Insect to feed the World + oral presentation	WUR/FAO, NL	2014	1.9
			Total ECTS 15.2

b. General courses (e.g. PhD week, language courses, presentation courses, statistics, etc.) >6

Name of the course	Graduate school/institute	Year	Credits
VLAG PhD week	VLAG, NL	2010	1.5
Techniques for Writing and Presenting Scientific Papers	WGS, NL	2011	1.2
Multivariate Analysis	PE&RC, NL	2011	1.2
Applied Statistics	VLAG, NL	2012	0.6
Scientific Writing	Language Center WUR, NL		1.8
II Symposium CONACYT students in Europe + oral presentation	CONACYT, Fr	2013	0.6
Voice matters	WGS, NL	2014	0.6
			Total ECTS 7.5

Optional (participation in discussion groups, PhD excursions, MSc courses, etc.) >8

Name of the course	Graduate school/institute	Year	Credits
Preparation of Research proposal	VLAG, NL	2010	4.0
PhD trip UK	PDQ, UK	2012	2.5
PhD trip organization	PDQ	2012	1.0
Milk fat ring	WUR- PDQ, Animal Science, NL	2010	1.0
PhD Trip Thailand and Singapore	FQD	2014	2.8
VLAG PhD Council	VLAG, NL	2012-2014	1.0
			Total ECTS 12.3

Supervision of master students

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  Thesis topic: Influence of the Regiospecific structure of the triacylglycerols on the crystallization and melting behaviour of milk fat
- Megala Palanisamy
  Thesis topic: Influence of Free fatty acids and their interaction with calcium on product Inhibition of raw milk Lipolysis
- Roel van der Vaart
  Thesis topic: Milk fat triacylglycerol structure and its effect on crystallization and melting behaviour
- Alexia Tilotti
  Thesis topic: Physical and chemical characteristics of insect fat.
- Thinh Nguyen
  Thesis topic: Modelling of crystallization and melting behaviour of milk fat.
- Hanneke de Rond
  Thesis topic: Physical characteristics and Regiospecific analysis of insect fat.
- Serafiani Gabriela Nuryani
  Thesis topic: Thermal behaviour and structure analysis of triacylglycerols in milk fat fractions
- Pere Randy Ramel.
  Thesis Topic: Isothermal Crystallization Kinetics of Milk Fat as Affected by its Triacylglycerol Composition
- Simone Knoef
  Thesis Topic: Separation of molecular species of milk fat triacylglycerols by molecular weight and mass-to-charge ratio.

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