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Phospholipid, Sphingolipid, and Fatty Acid Compositions of the Milk Fat Globule Membrane are Modified by Diet

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The phospholipid and sphingolipid composition of milk is of considerable interest regarding their nutritional and functional properties. The objective of this article was to determine the lipid composition of the milk fat globule membrane (MFGM) of milk from cows fed a diet rich in polyunsaturated fatty acids. The experiments were performed with 2 groups of 6 cows feeding on (i) maize silage ad libitum (+ grassland hay, mixture of cereals, soyabean meal) or (ii) the maize silage-based diet supplemented with extruded linseed (bringing a lipid proportion of 5% of dry matter). The phospholipid and sphingolipid composition of the MFGM was determined using HPLC/ELSD. The fatty acid (FA) composition of total lipids and phospholipids was determined using GC. As expected, the linseed-supplemented diet decreased the saturated FA and increased the unsaturated FA content in milk fat. MFGM in milk from cows fed the diet rich in polyunsaturated FA resulted in (i) a higher amount of phospholipids (+ 18%), which was related to a smaller size of milk fat globules (ii) an increase of 30% (w/w) of the concentration in sphingomyelin, (iii) a higher content in stearic acid (1.7-fold), unsaturated FA (1.36-fold), and C18:1 *trans* FA: 7.2 ± 0.5% (3.7-fold). The MFGM contained a higher concentration of unsaturated FA (C18:1, C18:2, and C18:3) and very long-chain FA (C22:0, C23:0, C24:0, EPA, DHA) compared with total lipids extracted from milk. The technological, sensorial, and nutritional consequences of these changes in the lipid composition of the MFGM induced by dietary manipulation remain to be elucidated.

KEYWORDS: Phospholipids; sphingomyelin; fatty acid composition; fat globule; cow diet

INTRODUCTION

Modification of the fatty acid (FA) profile of milk fat to yield greater (poly)unsaturated FA and lower saturated FA contents is a major research focus for the dairy industry. Moreover, the milk fat globule membrane (MFGM) has recently gained attention for the emulsifying and nutritional properties of its constituents, mainly the phospholipids.

Population studies have long established strong relationships between saturated FA in the diet and the incidence of coronary heart diseases (*1*), which is a leading cause of death in most industrialized countries. Whole milk and some dairy products have been listed as one of the risk factors in coronary heart disease due to their high content of saturated FA (*2*). Moreover, prospective studies have shown polyunsaturated FA to be negatively associated with coronary heart disease mortality (*3*). Particularly, polyunsaturated FA such as C18:2 (n-6) and C18:3 (n-3) cannot be formed de novo in humans and are essential for health. Then, they need to be ingested from the diet.

The main strategies available to increase the concentration of unsaturated FA in milk fat are (i) technology, mainly with dry fractionation of fat, (ii) chemical methods such as enzymatic interesterification, (iii) selection of cows (*4*), and (iv) dietary manipulation by means of feeding dairy animals (*5, 6*). Cows are fed diets rich in polyunsaturated lipids (e.g., rapeseed, linseed, etc.) to produce milk with a naturally more unsaturated FA profile and to manufacture innovative dairy products promoting human health. Most studies investigating the effect of cow diet on milk fat composition were carried out on its whole FA composition. Less attention has been given to the composition of the MFGM.

Fat is secreted in milk in the form of fat globules (mean diameter ~4 μm) mainly composed of triacylglycerols that account for ~98% of total lipids. Triacylglycerols are enveloped

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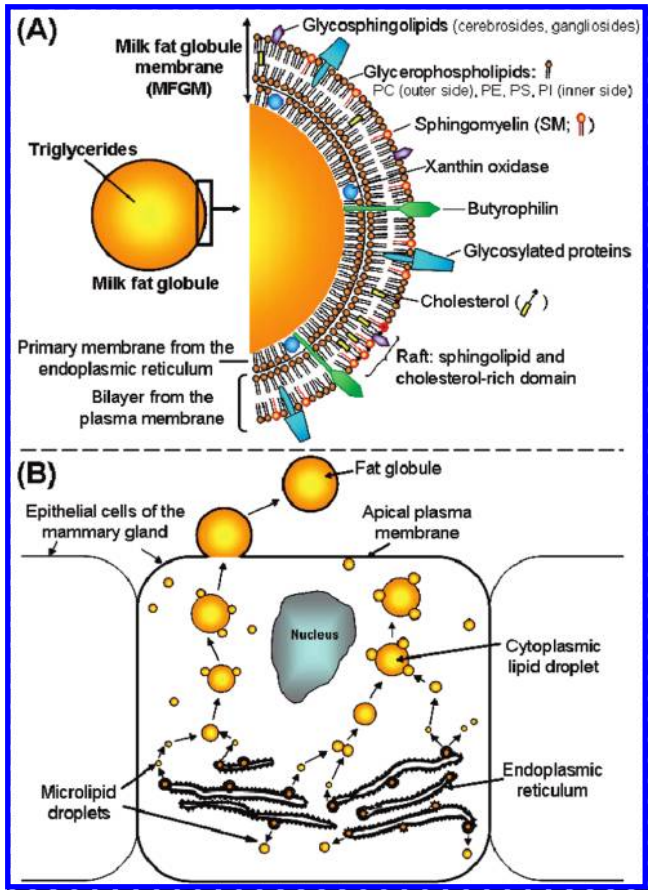

Figure 1. Schematic representation of (**A**) the structure of the milk fat globule membrane and (**B**) the pathways for the intracellular origin, growth, and secretion of milk fat globules.

by a biological membrane that results from the mechanisms of fat globule secretion. For further details, see the recent review of Heid and Keenan (*7*). From a structural point of view, the MFGM is organized as a trilayer (thickness = 10−50 nm), with an electron dense material on the inner membrane face composed of proteins and polar lipids from the endoplasmic reticulum, and a bilayer membrane derived from the apical plasma membrane of the mammary epithelial cells that surrounds fat globules when they are secreted. A schematic representation of the MFGM with the pathways for the origin, growth and secretion of fat globules is presented **Figure 1**. The MFGM consists of a complex mixture of (glyco)proteins (20 to 60%), triacylglycerols, glycerophospholipids (33% of the MFGM), sphingolipids (mainly sphingomyelin), glycolipids, cholesterol, enzymes, and other minor components (*8*). The five major classes of polar lipids in milk fat are the phosphatidylcholine (PC; 35%), the phosphatidylethanolamine (PE; 30%), the sphingomyelin (SM; 25%), the phosphatidylinositol (PI; 5%), and the phosphatidylserine (PS; 3%) (*9, 10*). The polar lipids are not organized homogeneously in the MFGM. Deeth (*10*) reported that PE, PS, and PI are mostly located on the inner surface of the MFGM, whereas PC, SM, and the glycolipids (i.e., cerebrosides and gangliosides) are mainly concentrated in the bilayer of the MFGM. SM is found primarily in the outer leaflet of the plasma membrane of most mammalian cells (*11*) (**Figure 1**).

The MFGM has interesting emulsifying properties that can be used in several applications (*12*). In addition to the use of the MFGM as a food ingredient for functional properties, it may also have an important role as an active ingredient in health-related diets (*13*). The amounts of phospholipids in milk and cream are small compared with neutral lipids, but they can be increased by the concentration of the MFGM fragments in buttermilk, e.g., the aqueous phase released during the churning of cream in butter manufacture, or in whey, e.g., the aqueous phase released during the manufacture of cheese. Buttermilk is considered as a low value byproduct of the butter industry and currently has a limited market. However, it has recently gained attention as a source of functional and nutritional ingredients because of its low cost and the presence of the MFGM.

Although there are no studies on the health implications of a diet rich in MFGM, several of its components have been related to health-enhancing functions (*14*). Milk fat, and particularly the MFGM, is a rich and convenient source of exogenous SM in the human diet (*14, 15*). The potential health benefits of bovine MFGM components as potential nutraceuticals have been recently reviewed by Spitsberg (*15*). Though there is no essential nutritional requirement for phospholipids and sphingolipids, research is revealing evidence suggesting a relationship between dietary consumption of these lipids and enhanced health (*15*).

The composition of the MFGM in milk can be altered by various factors, such as processing, environmental, and animal factors. Processing factors include cooling, drying, separation, agitation, heating, and homogenization. Among these factors, heat treatment causes denatured whey proteins to incorporate the MFGM, and homogenization causes the disruption of fat globules with an increase of the surface area leading to the adsorption of proteins, mainly caseins (*16*). Environmental factors include the presence of bacteria in milk from pre- or postpasteurization contamination or the presence of mastitis pathogens in mastitic milk (*17*). Animal factors include the changes in the chemical composition of the MFGM during lactation (*18*) and diet, season, and breed of cow (*19, 20*).

The functional and nutritional properties of the MFGM result from its chemical composition. Thus, the characterization of the lipid composition of the MFGM is important to better understand the properties of fat globules and to produce dairy products with improved nutritional properties. The objective of this study was to extend the current knowledge on the phospholipid, sphingolipid, and FA composition of the MFGM in milk from cows fed a diet rich in polyunsaturated FA.

MATERIALS AND METHODS

Animals and Diets. Twelve cows (6 Holstein and 6 Montbeliarde) calving between November 24, 2006 and January 7, 2007 were selected in the experimental herd of Marcenat (Cantal, France). During a 3-week pre-experimental period, each cow received daily a diet based on hay (6 kg) and maize silage ad libitum plus an individual concentrated mixture of cereals (70% barley and 30% maize; maximum 6 kg) and soybean meal, according to milk production. At the end of the pre-experimental period, the average live weight was 646 ± 54 kg, lactation stage was 62 ± 17 days, daily milk yield was 31.6 ± 5.9 kg, and milk fat and milk protein contents were 39.1 ± 2.9 and 31.3 ± 1.9 g/kg, respectively. Then, two groups of 6 cows (each of them with 3 Holstein + 3 Montbeliarde) with similar weight, lactation stage, milk production and composition were constituted. The animals were progressively adapted (3 days transition period) to the experimental diets that were offered during 5 weeks. The first group (noted maize silage) received maize silage ad libitum at 9:30 a.m. and grassland hay (1.7 kg DM/day) at 3:00 p.m., supplemented for each cow with an average of 3 kg DM/day of the cereal mixture used during the pre-experimental period and 2.7 kg DM/day of soybean meal. The second group (noted maize silage + linseed) received the same ration supplemented with an average of 1.9 kg DM/day of soybean meal and 3.4 kg extruded linseeds (INZO, Argentan, France), for each cow. Extruded linseed is rich in linolenic acid (n-3 polyunsaturated FA), which represents more than 50% of their FA content. The extruded linseed was prepared the week before

the beginning of the experiments to avoid problems with rancidity during storage. The quantities of extruded linseed were adjusted individually to bring a lipid proportion of 5% dry matter for each cow. Cows were housed in a tie-stall barn and were milked at 6:00 a.m. and 5:00 p.m. in a milking parlor. The milk from evening and morning milkings of each group of cows was mixed and refrigerated at 4 °C.

Cream of Concentrated Milk Fat Globules. Twenty liters of raw milk (evening + morning milkings) produced by the cows fed with the 2 diets were heated from 4 to 45 °C and skimmed by centrifugation with a plate-separator (Elecrem, Vanves, France) to concentrate milk fat globules. Three independent milk samples were skimmed during the 3 weeks of the experimental period (1 milk from cows fed each diet was skimmed per week). The samples of cream were stored at ambient temperature for particle size measurements and then stored at −20 °C until further analysis.

Fat Globule Size Measurements. The fat globule size distribution of the cream was measured by laser light scattering using a Mastersizer 2000 (Malvern, UK) with two laser sources. The refractive indexes used were 1.458 and 1.460 for milk fat at 633 and 466 nm, respectively, and 1.33 for water. The samples of cream were diluted in 100 mL of water directly in the measurement cell of the apparatus in order to reach 10% obscuration. Standard parameters were calculated by the software: the volume-weighted average diameter d_{43} defined as $\Sigma n_i d_i^4/\Sigma n_i d_i^3$ (where n_i is the number of fat globules of diameter d_i), the volume−surface average diameter d_{32} defined as $\Sigma n_i d_i^3/\Sigma n_i d_i^2$, the modal diameter that corresponds to the population of fat globules the most important in volume, the specific surface area $S = 6.\varphi/d_{32}$ (where φ is the volume fraction of milk fat), and the size distribution width, span $= (d_{v0.9} - d_{v0.1})/d_{v0.5}$ (where $d_{v0.9}$ is the diameter below which lies 90% of the globule volume, and 10% for $d_{v0.1}$ and 50% for $d_{v0.5}$).

Extraction and Analysis of the Lipids. *Chemicals and Reagents.* For HPLC analysis, chloroform stabilized with ethanol and methanol (both HPLC grade) were purchased from Carlo Erba Reagents (Val de Reuil, France). Triethylamine (purity >99%) and formic acid (purity >98%) were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). The phospholipid standards were supplied by Sigma Aldrich (Saint Quentin Fallavier, France): PE (L-α-phosphatidylethanolamine dipalmitoyl, *N*,*N*-dimethyl(C16:0); purity 99%), PI (L-α phosphatidylinositol ammonium salt, from soybean, purity 98%), PS (1,2-diacyl-*sn*-glycero-3 phosphoserin; chloroform/methanol 2:1 vol/vol solution, purity 98%), PC (1,2-dipalmitoyl-*sn*-glycero-3- phosphocholine; purity 99%), and sphingomyelin (SM from bovine brain; purity 99%). For GC analysis, methylene chloride and hexane were provided by Carlo Erba Reagents (Val De Reuil, France). Sodium methoxide 0.5 M and 10% BF_3-Methanol were provided by Sigma-Aldrich (St. Quentin Fallavier, France). Retention times were determined by injection of commercial mixes of FA methyl ester standards from C4:0 to C24:0 (FAME Mix C4−C24), from C14:0 to C22:0 (FAME Mix C14−C22), and from *trans* FA purchased from Sigma Aldrich (Saint Quentin Fallavier, France).

Extraction of Total Lipids. An adapted protocol of the cold extraction procedure developed by Folch et al. (*21*) was used for the extraction of total lipids from the cream. Two grams of cream was homogenized with chloroform/methanol (2:1, vol/vol) with a mixer (IKA, Fisher Bioblock). The extract was shaken and equilibrated with one-fourth its volume of a saline solution (NaCl 0.73%, w/w). The upper phase was washed three times with a mixture of 40 mL of chloroform/methanol (2:1, vol/vol) and 10 mL of NaCl 0.58% (w/w). The solvent phases containing total lipids were filtered (Whatman filter paper, 2.5 μm, Grosseron, France) through a pinch of anhydrous sodium sulfate and evaporated under vacuum. The extraction of total lipids was performed in duplicate or triplicate to obtain a coefficient of variation <5%. Total lipids extracted were stored at −20 °C until further GC and HPLC analysis.

Analyses of Total Fatty Acids. Methyl esters of FA were prepared from total lipids according to an adapted method (*22*). Briefly, in a screw-capped tube, 10 mg of milk fat was weighed; 200 μL of methylene chloride and 1 mL of 0.5 M sodium methoxide were added. After a flush of nitrogen, caps were closed, and tubes were placed in a water bath at 90 °C for 10 min. Tubes were cooled, and 1 mL of 10% BF_3-Methanol was added. After a flush of nitrogen, caps were closed, and tubes were placed again in a water bath at 90 °C for 10 min. Tubes were cooled before adding 1 mL of deionized water and 2 mL of hexane. After shaking, the upper layer was collected before injection to GC. FA methyl esters were measured on a Varian gas chromatograph (GC; model 3800, Varian, Walnut Greek, CA, USA) equipped with a flame ionization detector, a programmed temperature injector, and 2 capillary columns (50 m by 0.32 mm; film thickness 0.25 μm each one) coated with 70% Cyanopropyl polysilphenylene-siloxane (BPX-70, SGE, Ringwood, Vic., Australia) mounted in series. Experimental conditions were as follows: initial temperature of on-column injection (1 μL) was 40 °C for 0.2 min; the temperature injector was programmed to increase to 200 °C at a rate of 200 °C·min^{-1}, an isotherm at 200 °C for 6 min and a decrease in temperature to 40 °C at a rate of 200 °C·min^{-1}. Detector temperature was 250 °C; carrier gas was hydrogen at a pressure of 138 kPa. Oven temperature was programmed as follows: 50 °C for 10 min followed by an increase to 175 °C at a rate of 10 °C·min^{-1}; the oven was maintained at this temperature for 27 min. Then, temperature was increased to 215 °C at a rate of 4 °C/min and maintained during 60 min. Total analysis time was 75.50 min. The GC analysis was performed in triplicate for each sample.

Analysis of Polar Lipids: Glycerophospholipids and Sphingolipids. Phospholipids: Concentration and Individual Classes. The quantification of the phospholipids and sphingolipids and the determination of the polar lipid classes were performed using high-performance liquid chromatography (HPLC) combined with an evaporative light scattering detector (ELSD). The chromatographic method used for the separation of the polar lipids extracted from cream was adapted from the method of Rombaut et al. (*23*).

Phospholipids and sphingomyelin separations were performed on an Agilent HPLC chain (HP 1100, Agilent, Massy, France) with 4 solvent lines, a degasser, and a quaternary pump. The detector was Evaporative Light Scattering Detector (ELSD; PL-ELS1000, Polymer Laboratories, Marseille, France). A HPChem software (Agilent, Massy, France) permitted the acquisition of data from the ELSD detector. A silica column, 150 × 3 mm with 3 μm particle diameter (AIT, Houilles, France) and a precolumn in silica with the same packing and internal diameter were used. Dried and filtered compressed air was used as the nebulizing gas at a flow rate of 1.7 L/min and temperature of 50 °C. The evaporating temperature was 85 °C. The elution program was (i) isocratic conditions with chloroform/methanol/buffer (1 M formic acid, neutralized to pH 3 with triethylamine) 87.5:12:0.5 (vol/vol/vol) from $t = 0$ min to $t = 7$ min and then (ii) a linear gradient of chloroform/methanol/buffer 87.5:12:0.5 (vol/vol/vol) to 28:60:12 (vol/vol/vol) from $t = 7$ min to $t = 27$ min. The mobile phase was brought back to the initial conditions, i.e., 87.5:12:0.5 (vol/vol/vol), from $t = 27$ min to $t = 29$ min, and the column was allowed to equilibrate until the next injection at $t = 36$ min. The total chromatographic run time was 36 min per sample, which consisted of (i) the 7 min in isocratic conditions to elute nonpolar lipids, (ii) the linear gradient to elute the polar lipids, (iii) 2 min to restore the initial conditions, and (iv) the last 7 min to re-equilibrate the column. The flow rate was maintained at 0.5 mL/min. The column was maintained at 40 °C. The injection volume was 10 μL per sample. The lipids extracted were weighed (100 mg) and dissolved in 500 μL of chloroform/methanol (88:12, vol/vol) and transferred into capped test tubes for HPLC analysis. Each sample was injected three times.

HPLC/ELSD Calibration. The identification of phospholipids and sphingomyelin was carried out by comparison with the retention time of pure standards. To obtain a quantitative evaluation of the phospholipids and sphingomyelin, five calibration curves were determined from the area values obtained by injecting 10 μL of chloroform/methanol (88:12, vol/vol) serial diluted solutions of PE (0.1−1.5 μg), PC (0.5−2.5 μg), PS (0.1−1.75 μg), PI (0.1−1.75 μg), and SM (0.5−3 μg). Each solution was prepared and injected in triplicate. Calibration curves were calculated by applying the equations of the power model to the area and concentration values, PE: $y = 3420x^{1.836}$($r^2 = 0.996$); PI: $y = 1780x^{1.793}$($r^2 = 0.993$); PS: $y = 1152x^{1.597}$($r^2 = 0.991$); PC: $y = 2270x^{1.819}$($r^2 = 0.989$); SM: $y = 996x^{1.535}$($r^2 = 0.990$). The sum of glycerophospholipids (PE, PI, PS, and PC) and sphingomyelin concentration was regarded as total phospholipid concentration.

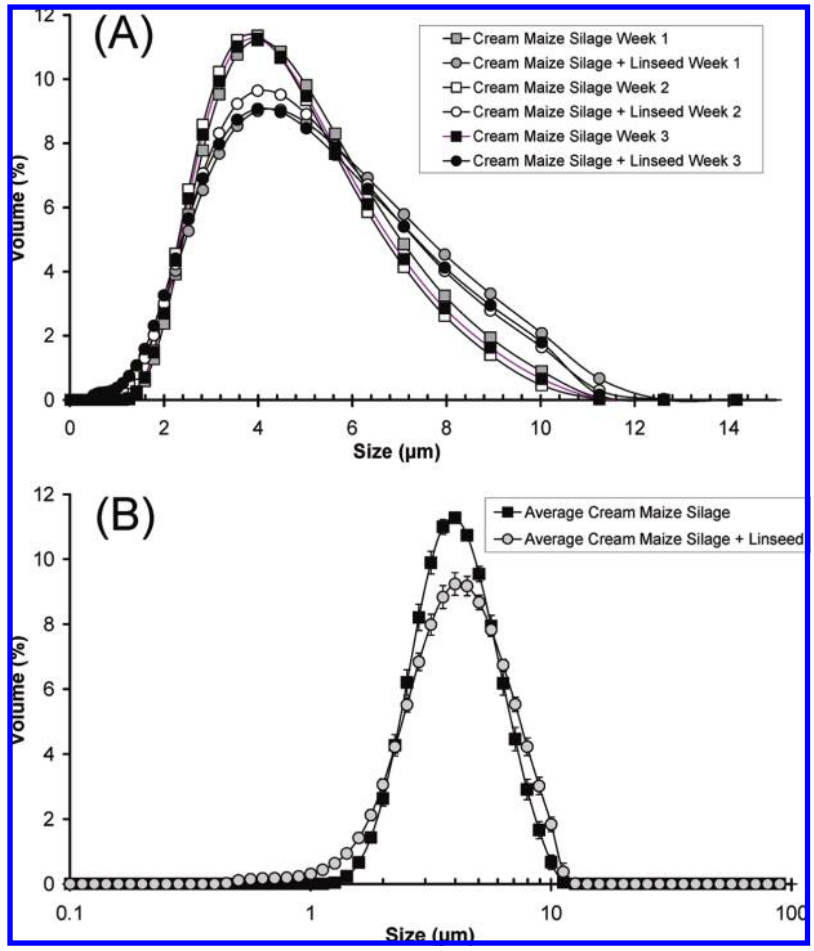

Figure 2. Fat globule size distribution in the cream obtained from the milk of cows fed the two different diets (**A**) during the 3 weeks of experiments ($n = 3$ measurements for each type of milk) and (**B**) mean values calculated for the 3 weeks of experiments ($n = 9$; 3 independent milk samples from cows fed with each diet and 3 measurements for each type of milk).

Fatty Acid Composition of Glycerophospholipids and Sphingomyelin. Prior to the analysis of the FA composition of phospholipids, the separation of nonpolar to polar lipids was performed using small solid-phase extraction (SPE) cartridges according to an adapted method (*24*). Total lipid sample (100 mg) was dissolved in 500 μL of chloroform/methanol (2:1, v/v). Silica gel bonded columns (Supelco LC-Si 0.5 g, Supelco Bellefonte, USA) were used for SPE. The nonpolar lipids were eluted with 3 mL of hexane/diethyl ether (8:2, v/v) and then 3 mL of hexane/diethyl ether (1:1, v/v). The recovery of the phospholipids and the sphingomyelin was performed with the addition of 2 mL of methanol and then 2 mL of chloroform/methanol/water (3:5:2, v/v/v). The recovered fraction containing the glycerophospholipids and sphingolipids in chloroform was stored at 4 °C until further analysis. The SPE experiments were performed in duplicate for each sample.

FA methyl esters of phospholipids were prepared with a method adapted from ref *25*. Glycerophospholipids and sphingolipids in chloroform were transferred into a screw capped-tube, and the solvent was evaporated under a flow of nitrogen to determine the exact weight of the polar lipids (from 5 to 100 μg). Three hundred microliters of 10% BF_3-methanol was added and once closed, the tube was heated at 95 °C overnight (because of amide bonds of sphingomyelin). Six hundred microliters of filtered hexane and 1.5 mL of deionized water were added. The content of the tube was mixed and centrifuged at 1300*g* for 5 min at 20 °C (Heraeus Cryofuge M7000, Osterode, Germany). After removing the lower phase, 1.5 mL of deionized water was added, and the tube was mixed and centrifuged again in the same conditions. This step of washing/centrifugation was repeated a third time. A pinch of anhydrous sodium sulfate was added, and the tube was stored at 4 °C for 30 min. After a new centrifugation at 1300*g* for 20 min at 20 °C, the upper layer was collected and injected into the GC. FA methyl esters of phospholipids were analyzed for total FA.

Statistical Analysis. Analyses of variance (ANOVA) was performed using the General Linear Model procedure of Statgraphics Plus version 5 (Statistical Graphics Corp., Englewood Cliffs, NJ). Differences between the treatment means were compared at the 5% level of significance using Fisher's least significance difference (LSD) test.

RESULTS AND DISCUSSION

Size Distribution of Fat Globules. Figure 2 shows the size distributions of the milk fat globules dispersed in the cream obtained after skimming of the milk from cows fed the two diets. **Figure 2A** shows the good reproducibility of the size distributions of fat globules in the 3 samples of cream obtained from cows fed each diet, during the 3 weeks of the experiments. **Figure 2B** shows the average of the fat globule size distribution in the cream from cows fed the 2 diets. The size distributions were monomodal with fat globules ranging from about 1.2 to 11 μm and from 0.4 to 12 μm for the cream from cows fed the maize silage diet and the linseed supplemented diet, respectively. The parameters extracted from the particle size distributions are presented in **Table 1**. The span of the size distribution was significantly ($P < 0.0001$) higher for cream from cows fed the diet supplemented with linseed, with the presence of smaller and larger fat globules compared with the cream from cows fed the maize silage diet. As a result, d_{32}, which is sensitive to the presence of smaller particles, was significantly ($P < 0001$) lower for the cream from cows fed the diet supplemented with linseed, whereas the d_{43}, which is sensitive to the largest particles, was significantly ($P < 0.001$) higher. The specific

Table 1. Parameters of the Size Distribution of Fat Globules in Cream (Mean ± Standard Deviation)

Size distribution parameters[a]	cow diet: maize silage + linseed	cow diet: maize silage	statistics[b]
mode (μm)	4.15 ± 0.08	4.41 ± 0.08	***
d_{32} (μm)	3.93 ± 0.07	3.66 ± 0.10	***
d_{43} (μm)	4.56 ± 0.09	4.73 ± 0.08	**
span	1.07 ± 0.01	1.29 ± 0.05	***
specific surface area (m^2 per g of fat)	1.66 ± 0.03	1.78 ± 0.05	***

[a] Diameters $d_{43} = \Sigma n_i d_i^4/\Sigma n_i d_i^3$, $d_{32} = \Sigma n_i d_i^3/\Sigma n_i d_i^2$, where n_i is the number of fat globules of diameter d_i. Specific surface area $S = 6.\varphi/d_{32}$ (where φ is the volume fraction of milk fat). Size distribution width: span = $(d_{v0.9} - d_{v0.1})/d_{v0.5}$ (where $d_{v0.9}$ is the diameter below which lies 90% of the globule volume and 10% for $d_{v0.1}$ and 50% for $d_{v0.5}$). [b] Results of the analysis of variance. Propability of *F*-test: *** $P < 0.001$; ** $P < 0.001$.

surface area was significantly ($P < 0.0001$) higher for the cream from cows fed the diet supplemented with linseed (**Table 1**). The centrifugation of milk performed to concentrate fat globules in the cream lead to the removal of the smallest fat globules ($d < 1$ μm) in skimmed milk. We measured the residual fat in skimmed milk, which was 2.63 ± 0.05 g/kg for milk from cows fed the maize silage diet and 2.85 ± 0.08 g/kg for milk from cows fed the linseed supplemented diet, which corresponded to 6 − 7.5% of total fat in milk.

Glycerophospholipid and Sphingolipid Content. The chromatograms presented **Figure 3** show that the neutral lipids (mainly triacylglycerols) were first eluted and did not interfere with the separation of the glycerophospholipids, i.e., PE, PI, PS, PC, and sphingomyelin (SM). PC eluted as 4 peaks (**Figure 3**, **insert**), and SM eluted as 3 peaks (**Figure 3**) because of the partial separation of molecular species. From the analysis of all of the chromatograms recorded (e.g., 18 for each milk), we observed that the relative intensities of the 4 peaks recorded for PC were different in the milk from cows fed the maize silage diet compared with the linseed-supplemented diet. These different relative intensities mean that PC species in milk were affected by cow diet (**Figure 3**, **insert**). Since these different PC species have not yet been reported in the literature, further experiments are required to elucidate their composition. Considering SM, peak 1 was observed at much lower intensities than were peaks 2 and 3 (**Figure 3**, **insert**). Christie et al. (*26*) and Rombaut et al. (*27*) reported 2 peaks for SM that were interpreted as the absence or the presence of an extra hydroxyl group, whereas Fong et al. (*28*) reported 3 peaks corresponding to various sphingoloid bases and FA.

The sum of glycerophospholipids (PE, PI, PS, and PC) and SM concentration was regarded as total phospholipid concentration. The amount of polar lipids (glycerophospholipids + SM) that we determined in this study, i.e., 0.25−0.3% (w/w) (**Table 2**), is a little bit lower than the results found in the literature. Fat-rich products such as cream, obtained by concentration of the fat globules from milk, have a polar lipid content of less than 1% (w/w) of total lipids. More precisely, authors found in cream 0.35 to 0.53 g of polar lipids per 100 g of total lipids (*23, 24, 27*). Previous authors reported that the amount of polar lipids in raw milk is far from constant, ranging from 9.4 to 35.5 mg per 100 g of product (*18, 24, 27*). Variations in the polar lipid content of raw milk can be ascribed to differences in the methods of preparation and analysis of the samples (*27*). Moreover, environmental factors such as the stage of lactation, feeding of the cow, and milk processing may also explain the differences found in the literature. The concentration of phospholipids was significantly ($P < 0.0001$) higher in the milk from cows fed the linseed-supplemented diet, with an increase of 18% (**Table 2**, **Figure 4**).The significantly greater surface area (+7.2%; $P < 0.0001$), which was related to the smaller milk fat globule size (mainly the d_{32}) measured for milk from cows fed the linseed-supplemented diet (**Table 1**), may be responsible for the greater PL-containing membrane per unit of milk fat. The concentration of all of the individual phospholipids significantly increased in the milk from cows fed the linseed-supplemented diet, with the following percentage: PE + 19.8% ($P < 0.0001$), PI +10.8% ($P < 0.05$), PS +18.6% ($P < 0.001$), and PC + 11.7% ($P < 0.0001$) (**Table 2**). The content of SM was significantly ($P < 0.0001$) higher for milk from cows fed the diet supplemented with linseed, with an increase of 30% (**Table 2**). Thus, the amount of the choline-containing phospholipids (sum of SM and PC) increased in the milk produced by cows fed the linseed-supplemented diet. During the secretion of milk fat globules from mammary cells, the droplets of triacylglycerols were enveloped by the plasma membrane, thereby incorporating SM, which is primarily found in the outer leaflet of the bilayer, in the MFGM (*11*) (**Figure 1**). The content of the individual phospholipids did not increase in the same proportions, which may indicate that cow diet affected the composition of phospholipids in the MFGM. This increase in the concentration of SM in milk may be of benefit healthwise

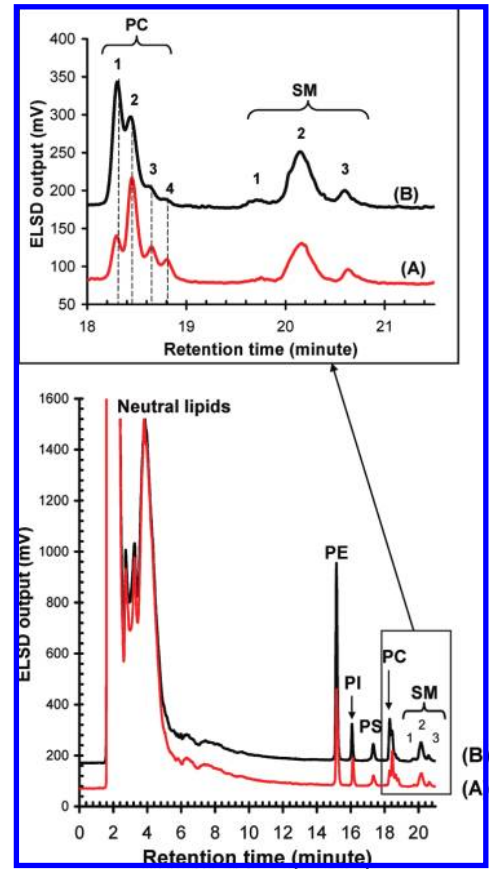

Figure 3. Normal-phase liquid chromatography (LC) evaporative light-scattering detector (ELSD) chromatogram of the total lipid fraction of the cream obtained from cows fed the two different diets: (**A**) maize silage and (**B**) maize silage supplemented with extruded linseed. These 2 chromatograms are representative of the chromatograms obtained for all of the milk ($n = 18$ for each diet; 3 independent milk samples, 2 extractions of fat, and 3 injections). The polar lipids from the milk fat globule membrane are phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), and sphingomyelin (SM).

Table 2. Concentration of Polar Lipids (Glycerophospholipids and Sphingolipids) in Cream Obtained from Two Different Diets and the Relative Proportion of Each Class of Phospholipids

	concentration in phospholipids (μg polar lipids per g total lipids)			relative proportion of phospholipids (% of polar lipids)	
	Cow diet			Cow diet	
phospholipids[a]	maize silage	maize silage + linseed	stat[b]	maize silage	maize silage + linseed
PE	658 ± 71	788 ± 53	***	26.8 ± 1.6	26.8 ± 0.9
PI	343 ± 45	380 ± 22	*	13.6 ± 1.4	12.7 ± 1.0
PS	409 ± 53	485 ± 37	**	16.1 ± 1.7	16.1 ± 1.1
PC	556 ± 32	621 ± 22	***	22.0 ± 0.9	21.0 ± 0.5
SM	548 ± 69	713 ± 63	***	21.6 ± 2.6	23.4 ± 0.8
polar lipids/total lipids	2531 ± 139	2986 ± 86	***		

[a] PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin. [b] Results of the analysis of variance. Probability of F-test: *** $P < 0.001$; ** $0.001 < P$; * $P < 0.05$.

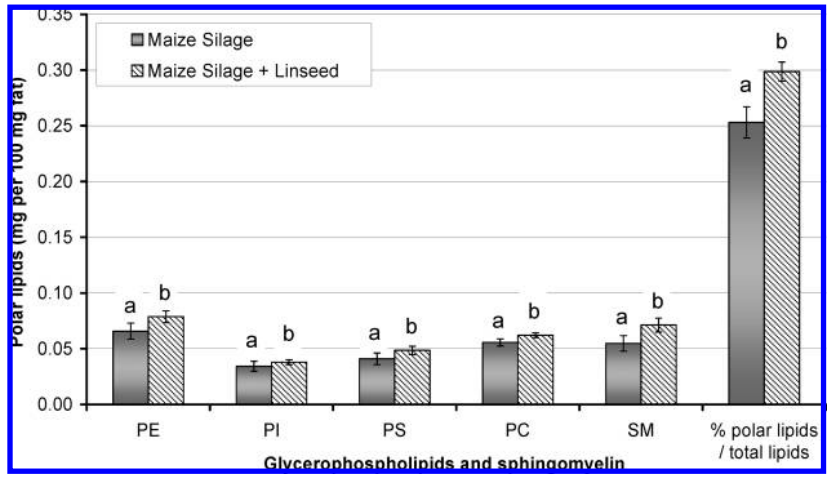

Figure 4. Comparison of the polar lipid composition of the milk from the two diets: maize silage and maize silage supplemented with extruded linseed ($n = 18$ for each diet; 3 independent milk samples, 2 extractions of fat, and 3 injections). For each class of glycerophospholipid and sphingomyelin, the different letters show that the concentrations were significantly different according to the LSD test ($\alpha < 0.05$).

to consumers (*13*). The factors that may affect the concentration of SM in bovine milk, e.g., breed, physiological, dietary, and environmental factors, have been recently studied (*20*). The authors reported that the variations in the SM content were mainly related to the milk fat globule size and then to the amount of the MFGM.

The relative proportion of each class of phospholipid is presented **Table 2**. The most abundant polar lipids were the PE, the PC and the SM with more than 20% (w/w). According to the data reported in the literature, the major milk phospholipids are PE (19.8−42.0%, w/w), PC (19.2−37.3%, w/w), PS (1.9−10.5%, w/w), and PI (0.6−11.8%, w/w), and the major sphingolipid is SM (18.0−34.1%, w/w) (*18, 23, 24, 26, 27*). Authors reported that SM contributes approximately one-quarter to one-third of the phospholipid portion (*14, 18*), although more recent reports have indicated that SM represents only 18 to 20% of the total phospholipids in milk (*23, 24*). Regarding PS and PI, our results are higher than those reported in the literature. This may be due to the resolution of the LC peaks and the signal/noise ratio that affect the quantification of these minor phospholipids (*24, 28*). Glycolipids such as glucosyl-ceramide and the lactosyl-ceramide were not detected in the cream (**Figure 3**). Other authors determined that they correspond to about 0.3−5.0% (w/w) and 2.8−6.7% (w/w) of the polar lipids, respectively (*23, 26–28*).

Glycerophospholipids and Sphingomyelin Fatty Acid Composition. The FA composition of the phospholipids in the milk from cows fed the two diets were determined and compared. **Table 3** shows the individual FA composition of the polar lipids, both glycerophospholipids and sphingolipids, which were located in the MFGM. The long-chain FA, i.e., from C16:0 to C18:3 (n-3), represented the main FA of the MFGM with amounts >74%. The supplementation of the maize silage-based diet with extruded linseed resulted in a significant ($P < 0.0001$) decrease in the saturated FA in the MFGM (−11.8% of total FA) and a significant ($P < 0.0001$) increase in the unsaturated FA (1.34-fold; **Table 3**). We found that the main FA of the MFGM was C16:0 in the milk from cows fed the maize silage diet, whereas it was C18:1 (n-9) for the milk from cows fed the linseed-supplemented diet in which C16:0 and C18:0 were present in quite similar proportions (17−19%) (**Table 3**).

In the phospholipids of milk from cows fed the diet rich in polyunsaturated FA, (i) the monounsaturated FA significantly increased (1.43-fold; $P < 0.0001$), the C18:1 c9 increased (1.32-fold), and the C18:1 *trans* FA were significantly higher (3.73-fold; $P < 0.0001$), mainly, vaccenic acid, i.e., C18:1 t11 FA (6-fold); (ii) the polyunsaturated FA slightly but significantly increased (1.1-fold; $P < 0.001$) with variations between the n-6 and n-3 FA; the C18:2 (n-6) significantly ($P < 0.0001$) decreased, whereas the C18:3 (n-3) significantly increased (3.8-fold; $P < 0.0001$); (iii) the most important very long chain polyunsaturated FA, e.g., C20:5 n-3 (EPA), significantly increased (1.92-fold; $P < 0.0001$) (**Table 3**).

Regarding the composition in saturated FA of the phospholipids contained in milk from cows fed the diet rich in polyunsaturated FA, (i) the long-chain saturated FA were significantly higher ($P < 0.0001$); the C16:0 decreased of about 45.4%, whereas the C18:0 increased (1.7-fold) (**Table 3**), (ii) the very long-chain FA, i.e., from C20 to C24, were significantly

Table 3. Fatty Acid Composition of the Glycerophospholipids and Sphingolipids from the Milk Fat Globule Membrane of Cream Corresponding to the Maize Silage Diet and the Maize Silage Diet Supplemented with Linseed (% Area of Total Methyl Esters; Mean ± Standard Deviation)

	cow diet		
fatty acids	maize silage	maize silage + linseed	stats[a]
C4:0	0.67 ± 0.13	0.73 ± 0.16	NS[c]
C6:0	0.85 ± 0.16	0.76 ± 0.16	*
C8:0	0.77 ± 0.12	0.65 ± 0.12	**
C10:0	2.27 ± 0.29	1.58 ± 0.27	***
C11:0	0.05 ± 0.01	0.00 ± 0.01	***
C12:0	3.09 ± 0.29	1.95 ± 0.27	***
C13:0	0.10 ± 0.04	0.04 ± 0.02	***
C14:0	9.99 ± 0.70	6.93 ± 0.64	***
C14:1 c9	0.64 ± 0.07	0.32 ± 0.05	***
C15:0	0.91 ± 0.07	0.57 ± 0.04	***
C15:1 c10	0.24 ± 0.01	0.23 ± 0.02	NS
C16:0	33.19 ± 1.45	18.50 ± 0.92	***
C16:1 c9	1.45 ± 0.32	0.64 ± 0.04	***
C17:0	0.43 ± 0.02	0.38 ± 0.02	***
C17:1 c10	0.18 ± 0.01	0.13 ± 0.01	***
C18:0	10.11 ± 0.42	17.29 ± 0.23	***
C18:1 t6+t7+t8+t9	0.57 ± 0.04	1.20 ± 0.07	***
C18:1 t10	0.36 ± 0.02	0.86 ± 0.21	***
C18:1 t11	0.75 ± 0.09	4.46 ± 0.38	***
C18:1 t12	0.26 ± 0.01	0.72 ± 0.45	**
C18:1 c9 (n-9)	21.20 ± 1.18	27.98 ± 0.81	***
C18:2 t9,t12	0.22 ± 0.02	0.88 ± 0.07	***
C18:2 c9,c12 (n-6)	5.11 ± 0.57	4.13 ± 0.52	***
C18:3 c9,c12,c15 (n-3)	0.43 ± 0.06	1.65 ± 0.11	***
C20:0	0.26 ± 0.04	0.28 ± 0.02	NS
C20:1 c11	0.01 ± 0.01	0.14 ± 0.00	***
C21:0 + C20:2 c11,c14	0.14 ± 0.02	0.15 ± 0.03	NS
C20:3 c8,c11,c14 (n-6)	0.57 ± 0.08	0.38 ± 0.07	***
C20:3 c11,c14,c17	0.56 ± 0.08	0.47 ± 0.08	***
C20:4 c5,c8,c11c14 (n-6)	traces[b]	traces	
C22:0	1.59 ± 0.26	2.21 ± 0.45	***
C22:1 c13	0.00 ± 0.01	0.01 ± 0.01	**
C20:5 c5,c8,c11,c14,c17 (n-3; EPA)	0.13 ± 0.02	0.25 ± 0.05	***
C23:0	1.48 ± 0.24	1.78 ± 0.37	**
C22:2 c6,c13	0.00 ± 0.00	0.00 ± 0.00	NS
C24:0	1.22 ± 0.18	1.60 ± 0.34	***
C24:1 c15	0.20 ± 0.03	0.16 ± 0.05	**
C22:6 c4,c7,c10,c13,c16,c19 (n-3; DHA)	0.04 ± 0.03	0.01 ± 0.01	**
Σ(C4−C8)	2.3 ± 0.4	2.1 ± 0.4	NS
Σ(C10−C15)	17.3 ± 1.3	11.6 ± 1.3	***
Σ(C16−C18)	74.5 ± 0.9	79.1 ± 0.4	***
Σ(C20−C24)	5.9 ± 0.9	7.2 ± 1.4	**
saturated FA	67.0 ± 2.1	55.2 ± 1.4	***
unsaturated FA	33.0 ± 2.1	44.8 ± 1.4	***
-monounsaturated FA	25.8 ± 1.4	36.9 ± 0.7	***
-polyunsaturated FA	7.2 ± 0.7	7.9 ± 0.8	**
-C18:1*trans*	1.94 ± 0.17	7.24 ± 0.51	***

[a] Results of the analysis of variance. Probability of F-test: *** $P < 0.0001$; ** $0.0001 \leq P < 0.01$; * $P < 0.05$. [b] Traces <0.04%. [c] NS: nonsignificant difference.

higher ($P \leq 0.0001$) with an increase of C22:0, C23:0, and C24:0 (1.2- to 1.4-fold) (**Table 3**). Considering the MFGM composition, authors agree with the presence of long and very long saturated FA chains, which were attributed to the SM. Graves et al. (*20*) reported that the major FA of the SM are the C16:0, C18:0, C18:1 (n-9), C22:0, C23:0, and C24:0. Bitman and Wood (*18*) found that approximately 90% of the SM FA were long-chain saturated FA, with the following five FA: C16:0, C18:0, C22:0, C23:0, and C24:0. Fong et al. (*28*) found significantly more long-chain FA with carbon chain lengths greater than 20, particularly C22:0, C23:0, and C24:0 in the FA composition of SM. The latter authors reported that this FA composition appeared to be unique for SM because these very long-chain FA (C > 20) were present only in small to trace levels in the glycerophospholipids (PE, PI, PS, and PC). We showed that the linseed-supplemented diet increased the amount of SM in the fat globules until 713 ± 63 μg per g of total lipids (+ 30% compared with the maize silage-based diet), which corresponded to 23.4 ± 0.8% of the polar lipids of the MFGM (**Table 2**). This increase in the SM concentration may be related to the larger amount of very long-chain saturated FA in the MFGM (**Table 3**).

Large differences were observed in the FA compositions of the phospholipids in the milk from cows fed the two diets, showing that the diet rich in polyunsaturated FA affected the FA composition of the MFGM. Bitman and Wood (*18*) reported changes in the FA composition of the individual phospholipid classes during lactation. Palmquist and Schanbacher (*19*) reported changes in the FA composition of the MFGM due to the dietary FA composition, but did not characterize milk from cows fed the linseed-supplemented diet. Moreover, the latter authors analyzed the FA composition after isolation of the MFGM and did not analyze the FA composition of total polar lipids of fat globules that may lead to artifacts due to the

Table 4. Fatty Acid Composition of Creams Corresponding to the Maize Silage Diet and the Maize Silage Diet Supplemented with Linseed (% Area of Total Methyl Esters; Mean ± Standard Deviation)

	cow diet		
fatty acids (FA)	maize silage	maize silage + linseed	stats[a]
C4:0	1.46 ± 0.27	1.50 ± 0.20	NS[b]
C6:0	1.54 ± 0.28	1.33 ± 0.19	*
C8:0	1.31 ± 0.21	1.05 ± 0.14	**
C10:0	3.55 ± 0.29	2.50 ± 0.22	***
C11:0	0.06 ± 0.03	0.02 ± 0.02	***
C12:0	4.49 ± 0.22	2.88 ± 0.19	***
C13:0	0.12 ± 0.01	0.07 ± 0.02	***
C14:0	13.86 ± 0.49	9.54 ± 0.25	***
C14:1 c9	1.01 ± 0.05	0.52 ± 0.03	***
C15:0	1.06 ± 0.08	0.65 ± 0.03	***
C15:1 c10	0.26 ± 0.01	0.25 ± 0.03	NS
C16:0	41.85 ± 1.00	22.05 ± 0.44	***
C16:1 c9	1.40 ± 0.37	0.74 ± 0.04	***
C17:0	0.43 ± 0.02	0.38 ± 0.02	***
C17:1 c10	0.15 ± 0.01	0.13 ± 0.01	**
C18:0	8.01 ± 0.40	16.41 ± 0.56	***
C18:1 t6 + t7 + t8 + t9	0.49 ± 0.03	1.28 ± 0.06	***
C18:1 t10	0.34 ± 0.05	0.86 ± 0.33	***
C18:1 t11	0.79 ± 0.14	5.63 ± 0.24	***
C18:1 t12	0.49 ± 0.42	0.91 ± 0.26	**
C18:1 c9	14.57 ± 0.71	25.21 ± 0.71	***
C18:2 t9,t12	0.25 ± 0.02	1.10 ± 0.06	***
C18:2 c9,c12 (n-6)	1.70 ± 0.02	1.59 ± 0.05	***
C18:3 c9,c12,c15 (n-3)	0.28 ± 0.03	1.25 ± 0.06	***
C20:0	0.25 ± 0.18	1.86 ± 0.06	***
C20:1 c11	0.00 ± 0.00	0.09 ± 0.03	***
C21:0 + C20:2 c11,c14	0.01 ± 0.01	0.00 ± 0.01	NS
C20:3 c8,c11,c14 (n-6)	0.09 ± 0.02	0.03 ± 0.03	***
C20:3 c11,c14,c17	0.12 ± 0.03	0.06 ± 0.03	***
C20:4 c5,c8,c11,c14 (n-6)	0.00 ± 0.00	0.00 ± 0.00	NS
C22:0	0.01 ± 0.02	0.02 ± 0.03	NS
C22:1 c13	0.00 ± 0.00	0.00 ± 0.00	NS
C20:5 c5,c8,c11,c14,c17 (n-3; EPA)	0.03 ± 0.02	0.07 ± 0.02	***
C23:0	0.01 ± 0.01	0.00 ± 0.01	NS
C22:2 c6,c13	0.00 ± 0.00	0.00 ± 0.00	NS
C24:0	0.01 ± 0.01	0.00 ± 0.01	NS
C24:1 c15	0.00 ± 0.00	0.00 ± 0.00	NS
C22:6 c4,c7,c10,c13,c16,c19 (n-3; DHA)	0.00 ± 0.00	0.00 ± 00	NS
Σ(C4−C8)	4.3 ± 0.7	3.9 ± 0.5	*
Σ(C10−C15)	24.4 ± 0.6	16.4 ± 0.7	***
Σ(C16−C18)	70.8 ± 0.7	77.5 ± 1.0	***
Σ(C20−C24)	0.5 ± 0.3	2.1 ± 0.1	***
saturated FA	78.0 ± 0.6	60.3 ± 0.7	***
unsaturated FA	22.0 ± 0.6	39.7 ± 0.7	***
-monounsaturated FA	19.5 ± 0.6	35.6 ± 0.7	***
-polyunsaturated FA	2.5 ± 0.1	4.1 ± 0.2	***
-C18:1 *trans*	2.11 ± 0.37	8.69 ± 0.42	***

[a] Results of the analysis of variance. Probability of F-test: *** $P < 0.0001$; ** $0.0001 \leq P < 0.01$; * $P < 0.05$. [b] NS: non significant difference.

presence of residual triacylglycerols. The different protocols used by the authors to analyze the MFGM composition does not facilitate the comparison of the data.

Fatty Acid Composition of Cream. The diet rich in polyunsaturated FA significantly ($P < 0.0001$) decreased the saturated FA content in milk from 78.0 ± 0.6% to 60.3 ± 0.7% and significantly ($P < 0.0001$) increased the unsaturated FA (1.8-fold) (**Table 4**). Also, this diet led to a highly significant ($P < 0.0001$) decrease of the palmitic, myristic, and lauric acids and an increase of the FA ≥ 18 atoms of carbon, mainly stearic acid (2.05-fold; $P < 0.0001$) and the C20:0 (7.4-fold; $P < 0.0001$). Moreover, in the milk from cows fed the diet rich in polyunsaturated FA, (i) the monounsaturated FA were significantly higher (1.83-fold; $P < 0.0001$); oleic acid was significantly higher (C18:1 c9; 1.73-fold; $P < 0.0001$); the concentration in C18:1 *trans* FA significantly increased (4.12-fold; $P < 0.0001$), mainly *trans*-vaccenic acid (C18:1 t11; 7.13-fold); (ii) the polyunsaturated FA content significantly increased (1.64-fold; $P < 0.0001$); the C18:3 (n-3) significantly increased (3.6-fold; $P < 0.0001$), whereas the C18:2 (n-6) significantly decreased ($P < 0.0001$); the rumenic acid, which is the major conjugated linoleic acid found in milk, significantly ($P < 0.0001$) increased from 0.39 ± 0.02% to 1.96 ± 0.11% (w/w) with the linseed-supplemented diet (CLA: C18:2 c9 t11; 5.03-fold).

Milk fat composition was greatly affected by the supplementation of linseed to the maize silage-based diet, mainly with the increase in mono- and polyunsaturated FA. The FA composition of milk fat results from the composition of the diet, mainly the lipid composition of the supplementation, the metabolism of lipids in the rumen, which is due to microorganisms, and the desaturation of FA in the mammary gland. According to the literature, the increase in FA ≥ 18 atoms of carbon in milk fat, mainly stearic acid and *trans* C18:1 FA, result from isomerization and biohydrogenation of the unsaturated FA performed by ruminal bacteria (*29*).

The increase in the oleic acid content mainly results from desaturation of the stearic acid in the mammary gland and the increase in the rumenic acid results from the desaturation of the *trans*-vaccenic acid performed by the delta 9 desaturase (*30*).

Comparison of Fatty Acid Composition: Whole Fat Globules versus MFGM. Differences in FA compositions were characterized between the total fat of cream (**Table 4**), mainly composed by triacylglycerols, and the biological membrane of the milk fat globules in which the phospholipids correspond to about 0.25−0.3% of total fat (**Tables 2** and **3**). **Figure 5** exhibits the relative differences between the FA compositions of the polar lipids and the whole FA composition of cream. Our results are in agreement with previous studies that reported that the content of unsaturated FA is significantly higher in the MFGM, while the content of saturated FA is lower (*31*). Short-chain (C4:0 − C8:0) FA were detected in the polar lipid fraction with significantly ($P < 0.0001$) lower content than in total lipids (**Figure 5**). Jensen and Nielsen (*31*) also detected short-chain (C4:0 − C8:0) FA in the MFGM, in significantly ($P < 0.05$) lower amounts than in the total FA of cream and reported that they were present in the MFGM with a concentration of 51 to 90 g/kg of total FA. Most of the authors who analyzed the FA composition of the MFGM started the GC experiments with the detection of C10:0 (*28, 32*). Whatever the diet of the cows, the medium-chain saturated FA (C10:0, C12:0, C14:0) in the milk were detected in significantly ($P < 0.0001$) lower content in the polar lipids compared with total lipids (**Tables 3** and **4**). The concentration of C16:0 was significantly ($P < 0.0001$) lower in the polar lipids. The content in C18:0 was 1.05−1.26-fold more important in the MFGM ($P < 0.0001$) and depended on the diet of the cows that produced the milk. Previous research reported that C18:0 is in equal proportions in cream and in the MFGM (*31*). Others authors found more C18:0 in the MFGM (*32*).

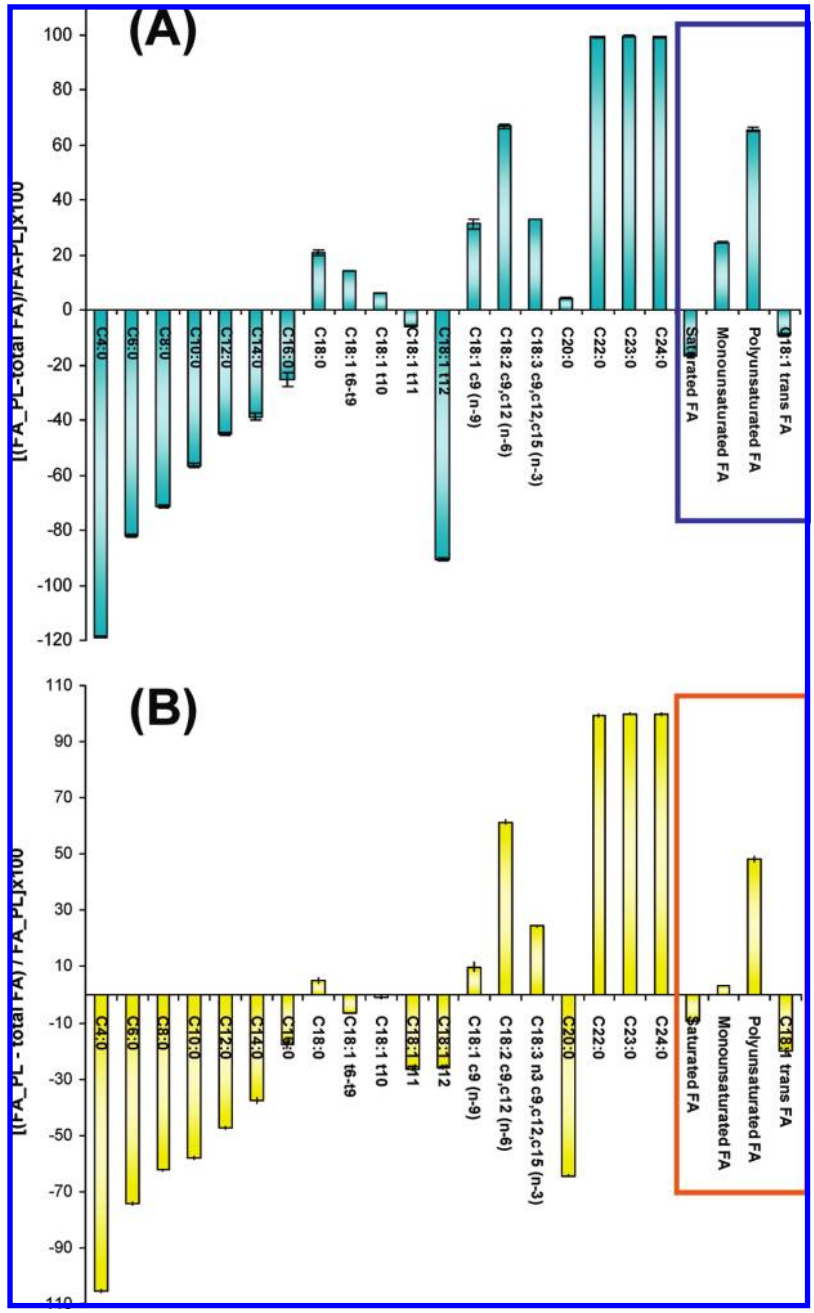

Figure 5. Relative compositional differences in fatty acids between the polar lipids located in the milk fat globule membrane (FA_PL) and the total fatty acids (total FA) of milk from cows fed with (**A**) maize silage-based diet and (**B**) maize silage diet supplemented with extruded linseed.

Figure 5 shows that the FA composition of the MFGM is significantly ($P > 0.0001$) richer in mono- and polyunsaturated FA than total lipids. Whatever the diet, we found that the C18:2 (n-6) was 2.6 to 3-fold more important in the MFGM and that the C18:3 (n-3) was 1.3 to 1.5-fold more concentrated in the MFGM. These results are consistent with previous studies that reported that the principal precursors of the n-6 FA and the n-3 FA, i.e., C18:2 (n-6) and C18:3 (n-3), were 2.3−2.7-fold and 1.2−1.7-fold higher in the MFGM than in the corresponding cream (*31*). The total C18:1 *trans* FA were significantly higher in the total lipids compared with the MFGM (**Tables 3** and **4**).

The very long-chain saturated FA (C22:0, C23:0, and C24:0) and polyunsaturated FA (mainly EPA and DHA) were significantly ($P < 0.0001$) higher in the MFGM compared with total lipids (**Figure 5**). Jensen and Nielsen (*31*) reported these very long-chain polyunsaturated FA were 3 to 10-fold more concentrated in the MFGM than in cream ($P < 0.001$).

In conclusion, using a combination of techniques such as HPLC/ELSD, SPE and GC, we have been able to provide evidence that a diet rich in polyunsaturated FA affects the lipid composition of the MFGM. These changes in FA composition of the phospholipids and triacylglycerols of milk fat as a function of cow diet reflect changes in FA synthesis in the mammary gland. Moreover, because the MFGM is derived from the plasma membrane of the lactating mammary cells, it is likely that changes in the FA of the MFGM correspond to similar changes in intracellular membrane composition. These changes in the composition of the MFGM are consistent with the high turnover of cell membranes associated with milk fat globule secretion. The effect of cow diet on the protein composition of the MFGM remains to be elucidated using proteomic methodologies. The complexity of the MFGM suggests that a number of minor components (proteins, lipids, vitamins, antioxydants, enzymes, etc.) are yet to be identified, particularly as a function of cow diet and genetic characteristics. A better understanding of these components of the MFGM will enable us to take advantage of their properties, from a technological, sensory, and nutritional point of view.

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LITERATURE CITED

(1) Keys, A. Coronary heart disease in seven countries. *Circulation* **1970**, *41*, 1162–1175.
(2) Noakes, M.; Nestel, P. J.; Clifton, P. M. Modifying the fatty acid profile of dairy products through feedlot technology lowers plasma cholesterol of humans consuming the products. *Am. J. Clin. Nutr.* **1996**, *63*, 42–46.
(3) Ascherio, A.; Rimm, E. B.; Giovannucci, E. L.; Spiegelman, D.; Stampfer, M. J.; Willett, W. C. Dietary fat and risk of coronary heart disease in men: cohort follow-up study in the United States. *Brit. Med. J.* **1996**, *313*, 84–90.
(4) Chen, S.; Bobe, G.; Zimmerman, S.; Hammond, E. G.; Luhman, C. M.; Boylston, T. D.; Freeman, A. E.; Beitz, D. C. Physical and sensory properties of dairy products from cows with various milk fatty acid compositions. *J. Agric. Food Chem.* **2004**, *52*, 3422–3428.
(5) Palmquist, D. L.; Beaulieu, A. D.; Barbano, D. M. Feed and animal factors influencing milk fat composition. *J. Dairy Sci.* **1993**, *76*, 1753–1771.
(6) Chilliard, Y.; Ferlay, A.; Doreau, M. Effect of different types of forages, animal fat or marine oil in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid and polyunsaturated fatty acids. *Livestock Prod. Sci.* **2001**, *70*, 31–48.
(7) Heid, H. W.; Keenan, T. W. Intracellular origin and secretion of milk fat globules. *Eur. J. Cell. Biol.* **2005**, *84*, 245–258.
(8) Keenan, T. W.; Patton, S. The Milk Lipid Globule Membrane. In *Handbook of Milk Composition*; Jensen, R. G., Ed.; Academic Press: San Diego, CA, 1995; pp 5−50.
(9) Christie,W. W. Composition and Structure of Milk Lipids. In *Advanced Dairy Chemistry, Vol. 2, Lipids*; Fox, P. F., Ed.; Chapman & Hall, New York, 1995; pp 1−37.
(10) Deeth, H. C. The role of phospholipids in the stability of milk fat globules. *Aust. J. Dairy Technol.* **1997**, *52*, 44–46.
(11) Timmen, H.; Patton, S. Milk fat globules: fatty acid composition, size and in vivo regulation of fat liquidity. *Lipids* **1988**, *23*, 685–689.
(12) Corredig, M.; Roesch, R. R.; Dalgleish, D. G. Production of a novel ingredient from buttermilk. *J. Dairy Sci.* **2003**, *86*, 2744–2750.
(13) Vesper, H.; Schmelz, E. M.; Nikolova-Karakashian, M. N.; Dillehay, D. L.; Lynch, D. V.; Merrill, A. H. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.* **1999**, *129*, 1239–1250.
(14) Parodi, P. W. Cow's milk fat components as potential anticarcinogenic agents. *J. Nutr.* **1997**, *127*, 1055–1060.
(15) Spitsberg, V. L. Bovine milk fat globule membrane as a potential nutraceutical. *J. Dairy Sci.* **2005**, *88*, 2289–2294.
(16) Lopez, C. Focus on the supramolecular structure of milk fat in dairy products. *Reprod., Nutr. Dev.* **2005**, *45*, 497–511.
(17) Erwin, R. E.; Randolph, H. E. Influence of mastitis on properties of milk. XI. Fat globule membrane. *J. Dairy Sci.* **1975**, *58*, 9–12.
(18) Bitman, J.; Wood, D. L. Changes in milk fat phospholipids during lactation. *J. Dairy Sci.* **1990**, *73*, 1208–1216.
(19) Palmquist, D. L.; Schanbacher, F. L. Dietary fat composition influences fatty acid composition of milk fat globule membrane in lactating cows. *Lipids* **1991**, *26*, 718–722.
(20) Graves, E. L. F.; Beaulieu, A. D.; Drackley, J. K. Factors affecting the concentration of sphingomyelin in bovine milk. *J. Dairy Sci.* **2007**, *90*, 706–715.
(21) Folch, J.; Lees, M.; Stanley, H. S. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, 497–509.
(22) Park, P. W.; Goins, R. E. In situ preparation of fatty acid methyl esters for analysis of fatty acid composition in foods. *J. Food Sci.* **1994**, *59*, 1262–1266.
(23) Rombaut, R.; Camp, J. V.; Dewettinck, K. Analysis of phospho- and sphingolipids in dairy products by a new HPLC method. *J. Dairy Sci.* **2005**, *88*, 482–488.
(24) Avalli, A.; Contarini, G. Determination of phospholipids in dairy products by SPE/HPLC/ELSD. *J. Chromatogr., A* **2005**, *1071*, 185–190.
(25) Morrison, W. R.; Smith, L. M. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride methanol. *J. Lipid Res.* **1964**, *5*, 600–608.
(26) Christie, W. W.; Noble, R. C.; Davies, G. Phospholipids in milk and dairy products. *J. Soc. Dairy Technol.* **1987**, *40*, 10–12.
(27) Rombaut, R.; Dewettinck, K. Properties, analysis and purification of milk polar lipids. *Int. Dairy J.* **2006**, *16*, 1362–1373.
(28) Fong, B. Y.; Norris, C. S.; MacGibbon, A. K. H. Protein and lipid composition of bovine milk-fat-globule membrane. *Int. Dairy J.* **2007**, *17*, 275–288.
(29) Chilliard, Y.; Frelay, A.; Mansbridge, R. M.; Doreau, M. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated,

trans and conjugated fatty acids. *Ann. Zootech.* **2000**, *49*, 181–205.

(30) Corl, B. A.; Baumgard, L. H.; Dwyer, D. A.; Griinari, J. M.; Phillips, B. S.; Bauman, D. E. The role of delta 9 desaturase in the production of cis-9, trans-11 CLA. *J. Nutr. Biochem.* **2001**, *12*, 622–630.

(31) Jensen, S. K.; Nielsen, K. N. Tocopherols, retinol, beta-carotene and fatty acids in fat globule membrane and fat globule core in cow's milk. *J. Dairy Res.* **1996**, *63*, 565–574.

(32) Fauquant, C.; Briard, V.; Leconte, N.; Michalski, M.-C. Differently sized native milk fat globules separated by microfiltration: fatty acid composition of the milk fat globule membrane and triglyceride core. *Eur. J. Lipid Sci. Technol.* **2005**, *107*, 80–86.

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