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Multiscale Characterization of the Organization of Triglycerides and Phospholipids in Emmental Cheese: From the Microscopic to the Molecular Level

CHRISTELLE LOPEZ,*,† VALERIE BRIARD-BION,† ERIC BEAUCHER,† AND MICHEL OLLIVON‡

UMR Science et Technologie du Lait et de l'Oeuf, INRA-Agrocampus, 65 rue de Saint-Brieuc, 35042 Rennes Cedex, France, and UMR 8612 CNRS, Equipe physico chimie des systèmes polyphasés, 5 rue J.-B. Clément, 92296 Châtenay-Malabry, France

The chemical composition and properties of lipids, both triglycerides and phospholipids, play a major role in the functional and nutritional properties of food products. In this study, the suprastructure of fat, solid fat content, and crystallographic properties of triglycerides were investigated in hard-type cheeses from the microscopic scale to the molecular level using the combination of relevant techniques. Two industrial cheeses with different oiling off properties were compared with experimental cheeses manufactured in the laboratory. Microstructural analysis performed using confocal laser scanning microscopy showed that milk processing led to the disruption of fat globules with the formation of nonglobular fat. For a similar fatty acid composition, oiling off was mainly related to the fat in dry matter content and to the suprastructure of fat in cheese. An exogenous fluorescent phospholipid permitted the localization of milk phospholipids in the cheese matrix, which mainly remain around fat inclusions after disruption of the milk fat globule membrane, and to show heterogeneities. We also showed using differential scanning calorimetry that the suprastructure of fat did not affect the solid fat content in cheese at 4 °C: 71.6 ± 4.9%. The organization of triglyceride molecules in fat crystals, elucidated at a molecular level using X-ray diffraction, corresponded to the coexistence of 2 lamellar structures (2L 40.5 Å and 3L 54.6 Å) with four polymorphic forms: α, two β' and β. A schematic representation of the multiscale organization of triglycerides and phospholipids in cheese is proposed.

KEYWORDS: Milk fat globule membrane; solid fat content; fat crystal; X-ray diffraction; fluorescence microscopy

INTRODUCTION

The different levels of the organization of fat in food products, i.e., from the microscopic (suprastructure of fat, localization of phospholipids) to the molecular (organization of triglycerides in fat crystals) scale, are highly complex to characterize. However, increasing the knowledge in the suprastructure of fat and both its physical and thermal properties (i.e., solid fat content and type of crystals as a function of temperature) in situ in food products is of tremendous importance with respect to technological, functional, sensorial, and nutritional properties (*1*).

Fat is dispersed in milk in the form of lipid droplets, called the milk fat globules, which have a size distribution ranging from about 0.02 to 10 μm with a mean diameter of 4 μm. Milk fat globules are enveloped by a biological membrane, the milk fat globule membrane (MFGM), which is rich in phospholipids (PL ~ 0.2 to 1% of total lipids), (glyco)proteins, enzymes, and cholesterol (*2, 3*). The MFGM is organized as a trilayer (monolayer of PL for the inner MFGM + bilayer of PL for the outer MFGM), which results from the mechanism of secretion of fat globules (*3*). The interior of fat globules is constituted by triglycerides (TG; esters of fatty acids and glycerol), which represent 98% of total milk lipids.

Information on the suprastructure of fat in food products is scarce because of the difficulty of the investigations in optically dense and complex products. The use of confocal laser scanning microscopy (CLSM) provides important potentialities to characterize food products, without disturbing the internal structure and to determine the spatial distribution of different chemical components, e.g., fat, proteins, and bacteria (*4–6*). Recently, the organization of fat has been investigated in dairy products and revealed various suprastructures using CLSM (*1, 4*). The effects of various treatments on the microstructure of cheeses, mainly mozzarella and cheddar, have been investigated using

* Author to whom correspondence should be addressed. E-mail: Christelle.Lopez@rennes.inra.fr. Phone: +33 2 23 48 56 17. Fax: +33 2 23 48 53 50.

† UMR Science et Technologie du Lait et de l'Oeuf.

‡ UMR 8612 CNRS, Equipe physico chimie des systèmes polyphasés.

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CLSM with a view to characterizing the organization of fat in the casein matrix (*7–9*). Lopez et al. (*10*) focused on the changes in the suprastructure of fat globules during the manufacture and ripening of Emmental cheese and showed that pressing of curd grains leads to the disruption of fat globules with the formation of free fat. The mechanical and thermal treatments applied during the processing of milk change the suprastructure of fat globules and may alter the composition and the organization of their interface after disruption of the MFGM (*4*). However, the localization of milk phospholipids in dairy products has not yet been investigated.

Milk fat has the particularity to be partially crystallized (e.g., a mixture of crystals and oil) over a wide range of temperatures, including the temperature of storage (4–7 °C) and consumption of dairy products. The amount, size, and type of crystals are of primary importance for the functional and sensory characteristics of food products. This thermal behavior of milk fat, characterized by a melting range which spans from −40 to +40 °C, results from its TG and fatty acid composition (*11, 12*). Moreover, the fat-composition-related complexity of milk fat thermal properties is dramatically enhanced by the existence of a polymorphism of monotropic type for each TG (*13–15*).

The techniques most frequently used for the study of the thermal and crystallographic properties of TG are differential scanning calorimetry (DSC) and X-ray diffraction (XRD), respectively. DSC studies have given an insight into the thermodynamics of fat phase transition in bulk (*16–18*), in emulsions (*19*), and in a complex food product such as cheese (*8, 20–22*). Authors showed that the suprastructure of fat may affect the thermal and crystallographic properties of TG. Studies monitored on milk fat globules showed that the temperature of the beginning of crystallization is delayed as a function of the decrease of their size (*19, 23*). Lopez et al. (*22*) showed that the formation of nonglobular fat during the manufacture of Emmental cheese affects the liquid to solid phase transition recorded by DSC on cooling. Moreover, using DSC, Lopez et al. (*22*) developed a protocol to determine the solid fat content in cheese at 4 °C and the evolution of the ratio of solid to liquid fat as a function of temperature. The identification of the crystalline structures formed at low temperature requires the use of XRD. XRD is an essential tool for elucidating the molecular packing of triglycerides in the solid state and their polymorphism. Recently, the use of DSC coupled to synchrotron radiation XRD allowed identification of the crystalline structures formed by TG molecules as a function of temperature and time in anhydrous milk fat and fractions (*17, 24, 25*), and in milk fat globules (*19, 23, 26, 27*). Lopez et al. (*19, 26*) showed that the dispersion state of milk fat, for example in bulk as anhydrous milk fat or dispersed in fat globules, alters both its thermal and structural properties. However, to date, the effect of the organization of fat (fat globule size; presence of free fat) on its crystallographic and thermal properties in complex food products such as cheeses has not yet been elucidated.

The objective of this work was to perform a deep and multiscale characterization of the organization of fat, both triglycerides and phospholipids, in industrial Emmental cheeses with different oiling off characteristics in comparison with experimental cheeses. The hypothesis of this study was the following: the suprastructure of fat governs the oiling off properties of cheeses as well as the solid fat content and the type of crystals formed by triglycerides at a molecular level. The combination of techniques used to investigate the properties of fat from the microscopic (several μm; 10^{-6} m) to the molecular (several Å; 10^{-9} m) scale, mainly CLSM, DSC, and XRD, increased the knowledge on the suprastructure of fat, the solid fat content, and the molecular packing of TG in fat crystals at 4 °C.

MATERIALS AND METHODS

Industrial and Experimental Emmental Cheeses. The organization and thermal properties of fat were compared in two different industrial cheeses and experimental cheeses manufactured in the laboratory.

Industrial hard-type cheeses, i.e., Emmental cheeses, manufactured by various French dairy companies were previously analyzed in terms of the quantity of free oil exuded at high temperature (oiling off properties) in order to determine the two extremes. The Emmental cheeses chosen for this study were noted high free oil (HFO) cheeses and low free oil (LFO) cheeses. They corresponded to industrial cheeses that were manufactured in winter by two different French companies. The cheeses were packaged with a rectangular format of 250 g (28% fat in cheese; 45% fat in dry matter). Three distinct manufactures of each were bought in the local supermarket (Géant, Rennes, France), about 1 month before the date for limit optimal consumption indicated on the packaging.

The experimental cheeses were manufactured using a specialized pilot plant based in the research center of INRA, Rennes (Chalon Megard, La Cluse, France). Two separate trials of Emmental cheese (80 kg each) were manufactured in January, from separate milk batches, as follows. Day P−1: Raw whole milk, purchased from a local dairy plant (CLE, Montauban, France), was collected the day before Emmental production (P−1). The milk was adjusted to a fat content/total nitrogen ratio (fat/TN) of 0.86 using skimmed milk, then stored overnight at 4 °C. Day P: Standardized milk was pasteurized, prepared, and inoculated on the day of manufacture of the cheese (P), as previously detailed in Lopez et al. (*6*). Day P+1: The curd was demolded, weighed, and placed in a cold brine bath for 48 h (saturated NaCl solution 350 g L^{-1}; 12 °C; pH 5.2). P+1 < Day ≤ P+70: Cheese was ripened for 21 d at 12 °C, 85% relative humidity, then 28 d at 21 °C, 80% relative humidity, and finally for 21 d at 4 °C, for a total ripening time of 70 days. Two Emmental cheeses of about 80 kg each were manufactured in total.

Biochemical and Physicochemical Analysis. About 200 g of three industrial HFO cheeses and LFO cheeses bought in the local supermarket and originating from independent manufactures were analyzed. Three samples of 20 × 6 × 4 cm (∼500 g) of experimental cheeses were cut at half-radius and half-height and analyzed after 70 days' ripening for compositional analysis. Dry matter (DM) was measured by drying 2 g of cheese mixed with sand at 102 °C (±2 °C) for 7 h (*28*). Fat content was determined using the Van Gulik butyrometric method, specially adapted for cheese (*29*). Total nitrogen (TN) in cheeses was obtained from nitrogen analysis using the Kjeldahl method (*30*). The protein breakdown was measured by the evolution of soluble nitrogen at pH 4.6 (NCN, noncasein nitrogen), and 12% TCA-soluble nitrogen (NPN, non-protein nitrogen), according to the method described by (*31*). To quantify the evolution of proteolysis, the following ratios were calculated: NPN/TN, NCN/TN, and (NCN − NPN)/TN, expressed as percentage of the cheese TN content. Fat in dry matter (FDM, %) was calculated as follows: FDM = fat/DM × 100. Moisture in nonfat substance (MNFS, %) was calculated as follows: MNFS = (1000 − DM)/(1000 − fat content) × 100. All analyses were performed in triplicate.

Oiling Off. The oiling off was characterized by a specialized institute (Institut Technique Français des Fromages, Rennes, France) as described in ref (*32*). Briefly, to quantify the oiling off, shredded cheese was heated to 65 °C in a butyrometer. After water addition and centrifugation, free oil was quantified at 65 °C.

Fatty Acid Composition. Samples of cheeses were stored at −80 °C until required for analysis and then stored at 4 °C during 24 h before analysis. The protocol used for the extraction of fat has been previously described in Lopez et al. (*6*). Methyl esters of fatty acids of milk fat were prepared according to a method adapted from ref (*33*). Fatty acid methyl esters were measured on a Varian gas chromatograph (Model

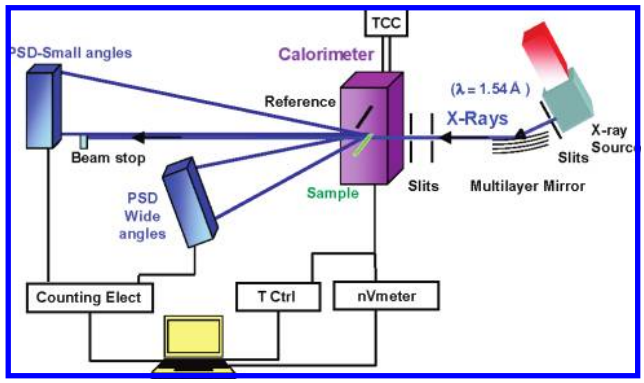

Figure 1. Experimental setup of the calorimeter cell, called Microcalix, in the X-ray diffraction environment. The X-ray source is a Diffractis 586 generator equipped with Cu sealed tube, which operated at 10 mA and 40 kV. A multilayer mirror (OSMIC-Rigaku, Troy, MI) is placed between a series of slits in order to get the X-ray focused approximately at the position-sensitive small-angle detector (PSD) (HECUS-Braun, Graz, Austria) after passing through calorimeter head (Calorimeter). A second PSD is located on the opposite side of the X-ray beam in order to collect wide angle diffraction data. X-ray data collection at both wide and small angles is obtained thanks to ASA24 software (HECUS-Braun, Graz, Austria). The temperature-controlled cryostat (TCC) was kept at constant temperature (e.g., 4 °C). Counting electronic (Counting Elect), nanovoltmeter (nVmeter), and temperature controller (T Ctrl) are monitored by the computer.

3800, Varian, Walnut Greek, CA) equipped with a flame ionization detector and a programmed temperature injector, using the experimental conditions detailed in Lopez et al. (*22*).

Microstructural Analysis. Emmental cheese microstructure was examined using confocal laser scanning microscopy (CLSM). Thin slices of cheese, measuring approximately 5 mm × 5 mm × 3 mm thick, were prepared from the freshly cut samples, using a scalpel.

The protein network was stained with the acridine orange fluorescent dye (3,6-acridinediamine, *N*,*N*,*N*′,*N*′-tetramethyl-, monohydrochloride; Aldrich Chemical Co., Inc., Milwaukee, WI), prepared in ethanol with a concentration of 100 μg/mL. Neutral lipids were stained with a lipid-soluble Nile red fluorescent dye (5*H*-benzo-α-phenoxazine-5-one, 9-diethylamino-; Sigma-Aldrich, St Louis, MO), prepared in acetone with a concentration of 42 μg/mL. Phospholipids were labeled with *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (Rh-PE; Avanti Polar Lipids Inc., Birmingham, England), dispersed in chloroform.

The samples were prepared as follows. About 300 μL of the staining solution of acridine orange was put in a coverslip and the ethanol was evaporated. To stain both the protein network and the neutral lipids, about 300 μL of the staining solution of Nile red was added in the coverslip and mixed with acridine orange until total evaporation of the acetone. To stain both the protein network and the phospholipids, about 200 μL of the staining solution of Rh-PE was added in the coverslip and mixed with acridine orange until total evaporation of the chloroform. Then, the respective coverslip was put on the slice of cheese to permit the diffusion of the stains, acridine orange + Nile red or acridine orange + Rh-PE, for 30 min in the dark at 4 °C.

Microstructural analysis was made using a confocal Leica TCS NT microscope (Leica Microsystems, Heidelberg, Germany), which employed an argon/krypton laser in dual-beam fluorescent mode, with excitation wavelengths of 568 nm for fat and phospholipids and 488 nm for proteins. Micrographs were taken at different magnifications: ×40, ×80 (×40, zoom 2) and ×200 (×100, zoom 2). The two-dimensional images had a resolution of 1024 × 1024 pixels, and the pixel scale values were converted into micrometers using a scaling factor. In the double-stained samples, the fat phase or phospholipids were coded in red and the protein phase was coded in green.

X-Ray Diffraction Experiments. XRD experiments were performed with the new setup which is illustrated **Figure 1**. The calorimeter, called Microcalix (*34*), was inserted in the laboratory X-ray bench (Châtenay-Malabry, France) to identify the crystalline structures formed at 4 °C by the triglycerides molecules dispersed in fat inclusions of the cheeses. Briefly, the XRD patterns were recorded by transmission through Lindeman glass capillaries (GLAS, Muller, Berlin, Germany), with 0.01 mm of wall thickness and diameter $\Phi = 1.40 \pm 0.10$ mm. The samples of cheese stored at 4 °C were cut using a home-developed device allowing sampling at controlled temperature precooled to 4 °C. Then, the piece of cheese of about 10−15 mg was pushed into preweighted capillaries and introduced in the calorimeter precooled to 4 °C. To identify the crystalline structures formed at 4 °C by triglycerides in the different cheeses, XRD patterns were recorded at small and wide angles for a total acquisition time of 3600 s. The channels of the small- and wide-angle detectors were calibrated as previously detailed in (*24*) to express the XRD data in scattering vector **q**.

Solid Fat Content in Emmental Cheeses. The solid fat content was determined by differential scanning calorimetry (DSC) using a TA Q-1000 calorimeter (TA Instruments, New Castle, DE). Calibration was made with indium standard (melting point = 156.66 °C; ΔH melting = 28.41 J/g). The enthalpy of melting of cheese fat ($\Delta H_{partial}$ cheese fat) and fat extracted from the cheeses (ΔH_{total}) were determined as previously detailed in Lopez et al. (*22*). The enthalpy of melting (ΔH expressed in $J \cdot g^{-1}$ sample), were calculated by the software (Advantage Software version 2, TA Instruments, Saint-Quentin-en-Yvelines, France). As the enthalpy of melting of triglycerides is proportional to the amount of fat in the samples of cheese, the results were divided by the concentration of fat determined for each sample.

Statistical Analysis. Analyses of variance (ANOVA) were 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

Compositional Analysis of Emmental Cheeses. The composition of ripened Emmental cheeses is shown in **Table 1**. For industrial cheeses and the experimental cheeses at the end of ripening (P+70), the compositions of the cheeses were within specified limits (*35*) for Emmental and were consistent with the literature (*36*). However, differences were observed between the cheeses considered. DM and TN were significantly lower ($P < 0.001$) for HFO cheeses (**Table 1**). FDM was significantly larger ($P < 0.05$) for experimental cheeses compared to industrial cheeses (**Table 1**) and to the literature (*36*). The MNFS were not significantly ($P > 0.05$) different. The soluble protein content ([NCN-NPN]/TN) was significantly larger ($P < 0.001$) for experimental cheeses, which indicated a greater hydrolysis of caseins (primary proteolysis). Peptide and amino acid content (NPN/TN), related to the degree of secondary proteolysis, was significantly lower ($0.001 < P < 0.01$) for LFO cheese. Since the TN/DM were similar between LFO and HFO cheese, the fact that the ratio NCN/TN was significantly lower ($P < 0.01$) for LFO cheese may indicate a larger proportion of intact caseins, i.e., pH 4.6 insoluble nitrogen and a lower proportion of soluble proteins in this cheese. As the experimental cheeses were ripened for 70 days, the lower proteolysis observed for the LFO cheeses may result from different conditions for ripening, such as a shorter period and/or different temperature.

The fatty acid composition of the Emmental cheeses was determined (results not shown). No significant differences ($P > 0.05$) were observed for the global composition in saturated (about 72.3%) and unsaturated (about 27.7% with 25.4% of monounsaturated) fatty acids, even if some significant differences were observed for some individual fatty acids (e.g., lauric acid, myristic acid, stearic acid). Since the experimental cheeses were manufactured in January, the similar fatty acid composition characterized for the industrial cheeses confirm that they were

Table 1. Composition of Ripened Emmental Cheeses: Low-Free Oil (LFO) Cheeses, High-Free Oil (HFO) Cheeses, and Experimental Cheeses (Mean Values ± Standard Deviation)[a]

components[c]	LFO cheese[b]	HFO cheese[b]	exptl cheese[b]	stat[c]
DM ($g \cdot kg^{-1}$)	618.80 a ± 0.27	606.92 b ± 3.42	620.65 a ± 6.28	***
fat ($g \cdot kg^{-1}$)	287.50 a ± 2.74	281.67 b ± 2.58	310.00 c ± 5.77	***
FDM (% w/w)	46.46 a ± 0.42	46.41 a ± 0.44	49.96 b ± 1.76	*
MNFS (% w/w)	53.50 ± 0.22	54.72 ± 0.48	54.99 ± 1.68	NS
TN ($g \cdot kg^{-1}$)	275.86 a ± 4.12	269.25 b ± 2.55	279.57 a ± 1.53	***
TN/DM (% w/w)	44.58 ± 0.53	44.36 ± 0.42	45.05 ± 0.45	NS
NCN ($g \cdot kg^{-1}$)	34.69 a ± 1.91	51.91 b ± 2.36	57.08 b ± 5.90	**
NPN ($g \cdot kg^{-1}$)	26.52 a ± 1.92	43.35 b ± 1.82	40.71 b ± 4.54	**
NPN/TN (% w/w)	9.61 a ± 0.66	16.11 b ± 0.84	14.56 b ± 1.59	**
NCN/TN (% w/w)	12.57 a ± 0.66	19.29 b ± 1.07	20.41 b ± 2.06	**
(NCN-NPN)/TN (% w/w)	2.96 a ± 0.05	3.18 a ± 0.23	5.85 b ± 0.47	***
pH	5.56 ± 0.08	5.62 ± 0.03	5.58 ± 0.02	NS
oiling off				
g per 100 g of cheese	8.8 a ± 0.8	12.8 b ± 0.6	13.4 b ± 1.0	***
g per 100 g of fat in cheese	30.7 a ± 3.0	45.3 b ± 2.1	43.2 b ± 4.3	**

[a] Abbreviations: DM = dry matter; FDM = fat in dry matter; MNFS = moisture in non-fat substance; TN = total nitrogen; NCN = non-casein nitrogen; NPN = non-protein nitrogen. [b] Mean ± standard deviation, calculated with $n = 3$ for each industrial cheese, $n = 6$ for the experimental cheeses (2 manufactures of cheeses × 3 samples characterized for each cheese). Values in the same row with the same letter were not significantly different according to the LSD test ($\alpha < 0.05$). [c] Results of the analysis of variance. Probability of F-test: ***, $P < 0.001$; **, $0.001 < P < 0.01$; *, $P < 0.05$; NS: nonsignificant difference.

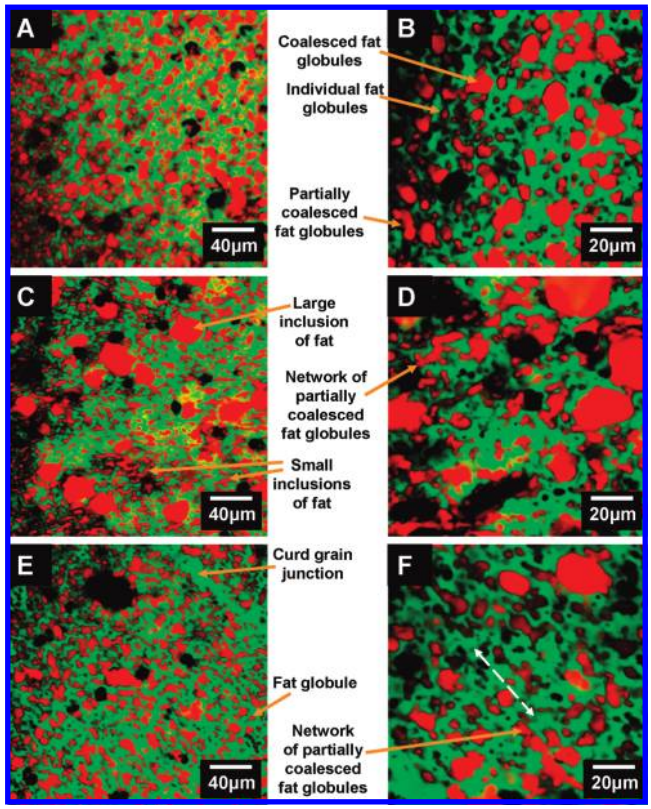

Figure 2. Confocal laser scanning micrographs of Emmental cheeses taken at the magnifications of ×40 (**A**, **C**, **E**) and ×80 (**B**, **D**, **F**). (**A**, **B**) Low free oil cheese, (**C**, **D**) high free oil cheese, and (**E**, **F**) experimental cheese. Fat is coded in red and the protein network is coded in green. The black areas correspond to pockets of gas produced during propionic acid fermentation, which are the precursor to the typical eyes formed in the Emmental cheese body. The arrow in picture **F** indicates the direction of the preferential organization of fat in the cheese matrix.

manufactured during the same season, i.e., in winter. Indeed, it is well-known that seasonal variations and cow feeding affect milk fat composition.

Suprastructure of Fat. Figure 2 shows the microstructure of the ripened Emmental cheeses, characterized at two different magnifications. Whatever the Emmental cheese considered (industrial or experimental), CLSM showed that fat particles of various shapes and sizes were entrapped in a compact casein network. However, different suprastructures of fat were observed in the different Emmental cheeses characterized (**Figure 2**). In LFO cheese (**Figure 2**, **A** and **B**), fat was dispersed as (i) fat globules with a spherical globular shape and a diameter of about 2 to 4 μm and (ii) inclusions of fat with various shapes and sizes (~10–25 μm) resulting from the aggregation and/or coalescence of fat globules after disruption of the MFGM. Similar suprastructure of fat were observed in experimental cheeses (**Figure 2**, **E** and **F**). Moreover, CLSM images showed aggregates of fat globules with a long shape and a preferential orientation in the protein network, which may be the opposite direction of the force exerted during pressing of curd grains (**Figure 2F**). In HFO cheese (**Figure 2**, **C** and **D**), two main organizations of fat were characterized: (i) large inclusions of fat, with a spherical shape and a diameter in the range of 15−45 μm which may result from the fusion of fat globules by coalescence and (ii) thin and long areas of fat (about 3 μm thick and until 40 μm long) that were preferentially oriented in the cheese matrix (horizontally in **Figure 2C**). These long-shaped inclusions of fat showed connectivity between fat globules which may correspond to their aggregation and/or partial coalescence.

Fluorescence confocal microscopy showed that processing of milk performed to manufacture Emmental cheeses affected the integrity of native fat globules (~4 μm in cheese milk; Lopez et al. (*10*)) and that different suprastructures of fat delimited by casein strands coexist in the cheese matrix. Similar observations were previously reported by Lopez et al. (*10*). Moreover, the size of the inclusions of fat was larger for HFO cheeses compared to LFO cheeses and experimental cheeses which may be a consequence of the technological parameters applied by dairy industrials during processing of milk. Like all the emulsions, cheese milk is a nonequilibrium system subjected to physical instabilities, leading to aggregation and coalescence of fat globules, and at the extreme to the formation of nonglobular fat, also called free fat. Moreover, changes in the organization of fat may be favored by mechanical (e.g., pumping, pressing, stirring, shearing of the MFGM) and/or thermal treatments, or by physicochemical parameters applied during milk processing. Aggregation and coalescence of fat globules were also evidenced in cheddar cheese (*7*) and mozzarella cheese (*40*). On the contrary, aggregates of fat particles with a diameter lower than 4 μm also observed for

HFO cheese may be natural small fat globules or may result from homogenization of a fraction of cheese milk or high shear stress able to decrease the size of fat globules. The dynamic changes in the organization of fat during the main steps involved in the manufacture and ripening of Emmental cheese were previously investigated and the physicochemical and technological (mainly temperature and pressure) parameters implicated in the disruption of fat globules have been already discussed (*10*).

Oiling Off as Explained by the Amount and the Suprastructure of Fat. The experimental determination of the oiling off confirmed the characteristics of the industrial cheeses in terms of free oil exuded (**Table 1**). As expected, the quantity of oiling off measured for LFO cheese was significantly ($P < 0.001$) lower than the oiling off determined for HFO cheese (**Table 1**). Regarding the experimental cheeses, the oiling off was significantly larger ($P < 0.001$) compared to LFO cheeses, but not significantly different to HFO cheeses (**Table 1**). The oiling off determined for HFO cheeses and experimental cheeses, expressed in g per 100 g of cheese, was consistent with the literature (*36*), whereas the oiling off determined for LFO cheeses was lower.

The comparison performed between LFO and HFO cheeses showed that the amount of FDM, which was ~46.4 ± 0.4% (w/w), was not significantly different (**Table 1**) whereas the oiling off was significantly ($P < 0.01$) different. The larger oiling off measured for HFO cheeses may be explained by (i) the larger fat inclusions observed in the cheese matrix (**Figure 2**) and (ii) the lesser content of intact para-caseins due to a larger proteolysis (**Table 1**). LFO cheeses and experimental cheeses had similar suprastructures of fat in the protein network (**Figure 2**) but significantly ($P < 0.01$) different oiling off properties (**Table 1**). The greater oiling off measured for experimental cheeses was attributed (i) to their larger FDM 49.96 ± 1.76% (w/w) vs 46.46 ± 0.42% (w/w) for LFO cheeses and (ii) to the lower proteolysis on LFO cheese, e.g., the larger content of intact para-caseins which limit the exudation of fat. However, the larger FDM of the experimental cheeses compared to HFO cheeses did not lead to greater oiling off, since the inclusions of fat were smaller.

The oiling off of cheeses may be favorable to their quality or be considered as a default when it is in excess. Some oiling off of the cheeses during baking is desirable since free oil leads to the formation of a hydrophobic surface-oil layer on the cheese and limits dehydration through a reduction of moisture evaporation and the occurrence of associated defects, such as crusting and impaired flow (*38*). However, the origin of the oiling off is not so clear. Oiling off may result from (i) the amount of fat, (ii) the composition of fat, (iii) the suprastructure of fat in the cheese matrix (globular, nonglobular) and the size of fat inclusions, (iv) the interaction between fat and the protein network, and (v) the characteristics of the casein matrix (*7, 37*). From these experiments, we deduced that the oiling off of the cheeses, which corresponds to the macroscopic properties of fat, were favored (i) by a larger FDM content, (ii) by the presence of larger inclusions of fat, and (iii) by the properties of the protein network in relation with proteolysis. For a similar FDM, these results are in accordance with the direct correlation between the size of fat inclusions within the casein matrix and functionality, particularly free oil formation and meltability which has been reported (*39*).

Hence, CLSM is a useful tool to investigate the organization of fat in the cheese matrix and explain the functional properties such as free oil formation. Moreover, the effect of the size of fat inclusions on free oil formation is thought to be related to the emulsion stability of the fat globules and the fat/water interfacial surface layer (*40*). Thus, it is important to investigate the composition of the fat/protein interface in situ in food products.

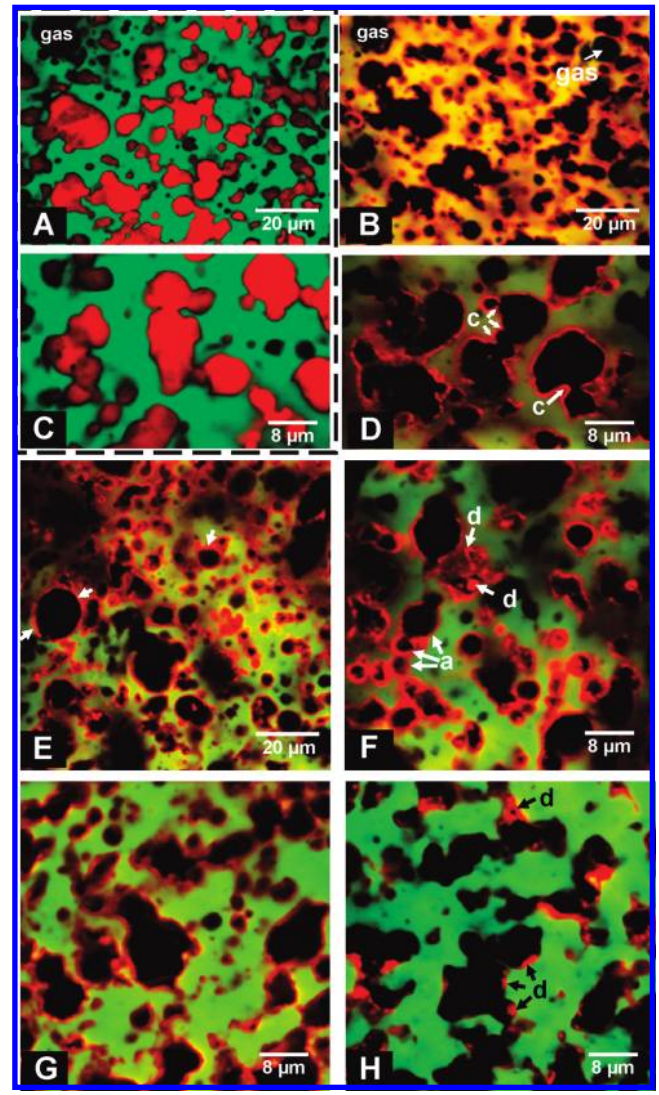

Figure 3. Confocal laser scanning micrographs of Emmental cheeses. The dotted box corresponds to the labeling of triglycerides and proteins: the protein network is labeled with acridine orange, while triglycerides are labeled with Nile red (**A**, **C**). In the other micrographs, the protein network is labeled with acridine orange and the phospholipids are labeled with rhodamine−phosphatidylethanolamine (Rh-PE; **B**, **D**, **E**, **F**, **G**, **H**); the black areas correspond to the triglycerides. (**A**−**D**) experimental cheese, (**E**, **F**, **H**) high free oil cheese, (**G**) low free oil cheese. Letters indicated in the macrographs: (a) aggregate of fat globules; (c) connectivity between fat globules; (d) domains rich in the exogenous phospholipid Rh-PE. The arrows in panel **E** indicate higher intensity of fluorescence of Rh-PE.

Localization of Phospholipids in the Cheese Matrix. To further elucidate the organization of fat in the cheese matrix, the localization of phospholipids was investigated by CLSM using rhodamine-labeled phosphatidylethanolamine (Rh-PE). **Figure 3** shows both the organization of the TG (neutral lipids) and the localization of phospholipids (polar lipids) in the cheese matrix. Focusing on the localization of milk phospholipids after the disruption of the MFGM, no significant differences were observed for the industrial and the experimental cheeses. Parts **A** and **C** of **Figure 3**, show the organization of neutral lipids (labeled with Nile red) and proteins. In the other micrographs

(**Figure 3B**,**D**,**E**−**H**), the red color generated from the emission of fluorescence of Rh-PE was used as a tracer to locate milk phospholipids in the cheeses. As a result of CLSM experiments, milk phospholipids were assumed to be mainly located around fat inclusions which are composed of TG. The irregular shape of the fat inclusions resulted from the partial fusion of fat globules (i.e., partial coalescence at low temperature), in which connectivity between fat particles remains, as indicated in **Figure 3D**. This connectivity may correspond to the fusion and the reorganization of the MFGM at the surface of fat particles which permits the opening of a pore that connects the TG of the different entities. The aggregation of fat globules, in which they conserve their individual integrity materialized by the presence of the MFGM, was also observed, as indicated in **Figure 3F**.

Heterogeneities in the intensity of Rh-PE fluorescence (Figure **3E**) and domains rich in the exogenous phospholipid were observed around fat inclusions (**Figure 3F**). Changes in the fluorescence properties and quantum yield of Rh-PE may be related to different local concentrations in the exogenous phospholipid, or to a sensitivity of the dye to its environment (hydrophobicity of the phospholipid surface, pH near the headgroup of the phospholipids). The domains rich in Rh-PE may correspond to local differences in the composition and structure of the MFGM. It may be possible that Rh-PE only integrated fragments of the MFGM among the other milk phospholipids, i.e., PE, PI, PC, PS. Brown and London (*41*) reported that the different packing ability of sphingolipids and phospholipids probably leads to phase separation in the membrane. Then, the domains of the MFGM which are rich in sphingomyelin (main sphingolipid with long-chain saturated fatty acids; ∼30% of milk polar lipids) may have provided the integration of the exogenous phospholipid Rh-PE, since they readily pack tightly together. These observations raise the question of the existence of rafts in the MFGM, i.e., domains rich in sphingomyelin, cholesterol, and proteins (*41*). The domains may also correspond to aggregates of phospholipids, for example, vesicles, or to fragments of the MFGM (**Figure 3H**). The surface of fat that was not covered by phospholipids may be composed of milk proteins and peptides resulting from proteolysis of Emmental cheeses. Such a study increased the knowledge of the composition of the fat/protein interface by measuring the fluorescence of Rh-PE, and then contributed to elucidate the nature of the interaction between fat and proteins in situ in food products. A schematic representation of the TG/protein interface mainly composed of phospholipids from the MFGM is proposed in **Figure 5**.

The localization of phospholipids at the TG−casein interface may have functional and sensory consequences. The composition of the fat−protein interface and the interactions between the interface and the casein network may contribute to the oiling off properties of the cheeses. Surface-active components such as phospholipids may contribute (i) to the stability of the inclusions of fat in the cheese matrix by preventing coalescence and separation of the liquid fat at high temperature (e.g., in melted cheese) and (ii) to the rheological and functional properties of cheese (e.g., stretching and meltability). Moreover, the polar character of the headgroup of phospholipids and of the other components of the MFGM which have a high water-binding ability may favor the retention of the serum phase around fat inclusions, in which the enzymatic activities may be enhanced (*6*). The presence of phospholipids originating from the MFGM around fat inclusions may also affect the sensory properties of cheese, since it is a potential source of oxidized flavor (*42*).

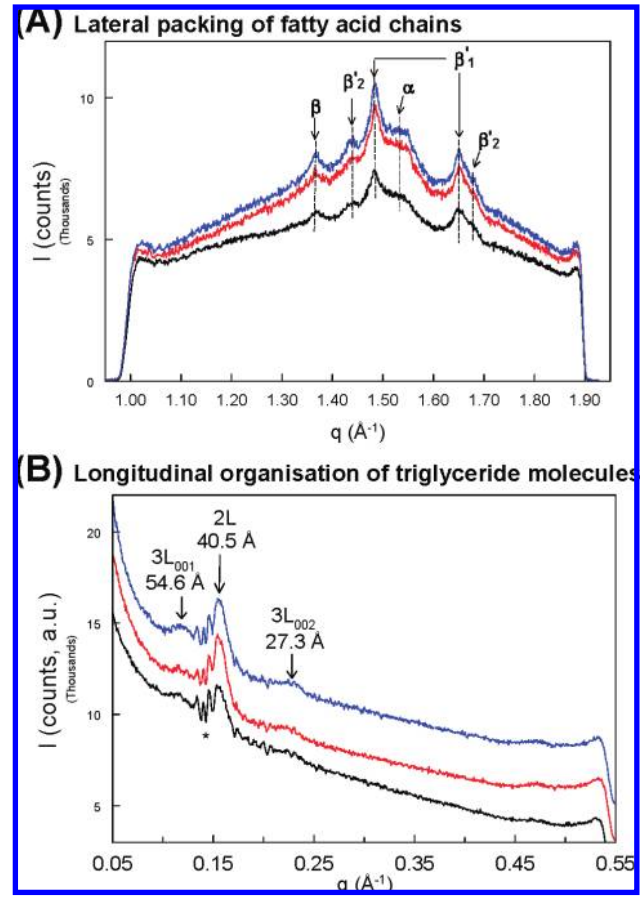

Figure 4. Organization of triglycerides in Emmental cheeses, characterized at a molecular level. X-ray diffraction (XRD) patterns recorded at 4 °C at wide angles (**A**) and small angles (**B**). The polymorphic forms and stackings of triglycerides are indicated in the figure: 2L = double chain length structure; 3L = triple chain length structure. Blue color, high free oil cheese; red color, low free oil cheese; black color, experimental cheese. (*) problem with the linear detector at about 0.14 $Å^{-1}$.

Quantification of the Solid Fat Phase at 4 °C. Table 2 shows the enthalpy of melting of cheese fat ($\Delta H_{partial}$ cheese fat) and of fat extracted from the corresponding ripened cheeses (ΔH_{total} fat). The amount of fat which was crystallized in the industrial and experimental cheeses at 4 °C has been quantified using DSC, with the protocol previously developed in Lopez et al. (*22*). The values of $\Delta H_{partial}$ cheese fat were significantly ($P < 0.05$) different between cheeses and in the range ∼54–59 $J \cdot g^{-1}$. The differences in $\Delta H_{partial}$ cheese fat may be explained (i) by the differences in the suprastructure of fat in Emmental cheeses, (ii) by the types of crystals formed in cheeses due to fatty acid composition and the thermal history, and (iii) by the composition of fat (TG, diacylglycerols, monoacylglycerols, free fatty acids, . . .) in the cheeses. The values of ΔH_{total} fat were also significantly ($P < 0.05$) different and in the range ∼76–80 $J \cdot g^{-1}$. Differences in ΔH_{total} fat may be explained (i) by the composition of fat, i.e., TG molecules and (ii) by the lipolysis of fat in cheese, i.e., different amounts of partial glycerides and free fatty acids.

The calculated solid fat content (SFC) was not significantly ($P > 0.05$) different between the cheeses. This result shows that the SFC is mainly related to the composition of fat and not to the organization of fat in the cheeses (size of fat inclusions).

Table 2. Enthalpy of Melting and Calculated Solid Fat Content in Emmental Cheeses at 4 °C Determined Using Differential Scanning Calorimetry (DSC)[a]

samples of Emmental cheese	thermal parameters: cheese fat $\Delta H_{partial}$[b,c] ($J \cdot g^{-1}$ cheese)	cheese fat $\Delta H_{partial}$[b,c] ($J \cdot g^{-1}$ fat)	AMF ΔH_{total}[b,c] ($J \cdot g^{-1}$ fat)	calcd solid fat content[c] at 4 °C (%)
LFO cheese	16.85 a ± 0.47	59.13 a ± 1.65	80.78 a ± 2.27	73.20 a ± 4.10
HFO cheese	15.80 a ± 0.59	56.42 ab ± 2.10	79.05 ab ± 3.09	71.37 a ± 5.44
exptl cheese	16.98 a ± 0.93	53.89 b ± 2.95	76.68 b ± 2.87	70.28 a ± 6.47

[a] The partial enthalpy of melting of cheese fat ($\Delta H_{partial}$) was determined between 4 °C and the final temperature of melting (T_{offset}) on heating of cheese at $dT/dt = 2$ °C/min. The total enthalpy of melting (ΔH_{total}) was determined with the fat extracted from the corresponding cheese on heating at $dT/dt = 2$ °C/min from −40 °C to T_{offset}. Abbreviations: ΔH = enthalpy of melting; AMF = anhydrous milk fat; LFO = low free oil; HFO = high free oil. [b] Presented values are means ± standard deviations. For the industrial cheeses, three replicate experiments were performed with three independent manufactures of cheeses ($n = 9$; 3 different cheeses × 3 measurements for each cheese). For the experimental cheeses, two measurements were performed for each of the 3 samples corresponding to 1 manufacture ($n = 12$; 2 experimental cheeses × 3 samples × 2 measurements). [c] Values in the same column with the same letter were not significantly different according to the LSD test ($\alpha < 0.05$).

The mean SFC calculated at 4 °C for industrial and experimental Emmental cheeses was 71.6 ± 4.9%. This value is larger than the SFC previously determined for Emmental cheeses (*22*). Lavigne (*17*) reported that about 70% of milk fat is solid at 4 °C, with variations which depend on the composition of fat and the thermal history of the product. A low proportion of liquid fat (~20% total) was reported in cheddar cheese with a ripening temperature of 7 °C (*43*). The high solid fat content in dairy products raises the question of the hydrolysis of TG molecules by the lipolytic digestive enzymes, as discussed in ref (*1*).

The results obtained in this study showed that the different suprastructures of fat (**Figure 2**) and the different physicochemical properties of the cheeses (**Table 1**) did not affect the SFC at 4 °C. This result is surprising as it has been shown that small fat globules have a higher surfusion and start to crystallize at a lower temperature compared to higher fat globules, which may decrease the SFC at a given temperature (*19, 23*). Moreover, the different oiling off properties of the cheeses did not originate from different SFC, which may have contributed to the destabilization of fat inclusions through the action of the fat crystals. These results support the major effect of the suprastructure of fat on free oil formation.

Organization of Triglycerides at a Molecular Level in the Solid Fat Phase. Both the long and short spacings of the lamellar structures formed by TG molecules dispersed in the fat inclusions of Emmental cheeses were characterized by XRD at small and wide angles, respectively (**Figure 4**). Similar XRD peaks were recorded for LFO cheeses, HFO cheeses, and experimental cheeses. **Figure 4A** shows the wide-angle XRD patterns recorded at 4 °C, which yield the lateral packing (short spacings) of the fatty acid chains in the characteristic subcells they adopted. The peak at 1.53 $Å^{-1}$ (4.11 Å) corresponded to the α form (hexagonal subcell), which is the most unstable polymorphic variety formed by TG molecules. Several XRD peaks were attributed to the formation of orthorhombic perpendicular packings (β′ form). More precisely, the peaks at 1.483 $Å^{-1}$ (4.24 Å) and 1.649 $Å^{-1}$ (3.81 Å) were related to the β'_1 form and the peaks recorded at 1.439 $Å^{-1}$ (4.37 Å) and 1.677 $Å^{-1}$ (3.75 Å) were related to the β'_2 form. The peak at 1.37 $Å^{-1}$ (4.59 Å) was characteristic of crystallization of the fatty acid chains in the β form, which is the most stable polymorphic form of TG molecules. **Figure 4B** shows the XRD patterns recorded simultaneously at small angles, which inform on the longitudinal organization (long spacings) of TG molecules in Emmental cheeses. A major XRD peak and two peaks of lower intensity were recorded at 4 °C. The major peak, spanning from 0.14 to 0.17 $Å^{-1}$ and centered at 0.155 $Å^{-1}$ (40.5 Å) corresponded to crystallization of TG molecules in a double chain length structure (2L). The peak spanning from 0.103 to 0.13 $Å^{-1}$ and centered at 0.115 $Å^{-1}$ (54.6 Å) corresponded to the first order of a triple chain length structure ($3L_{001}$). The peak spanning from 0.205 to 0.24 $Å^{-1}$ and centered at 0.23 $Å^{-1}$ (27.32 Å) was attributed to the second order of the 3L structure, and noted $3L_{002}$. XRD experiments performed at small angles showed that two different longitudinal organizations of the lamellar structures incorporated the TG molecules that were solid at 4 °C.

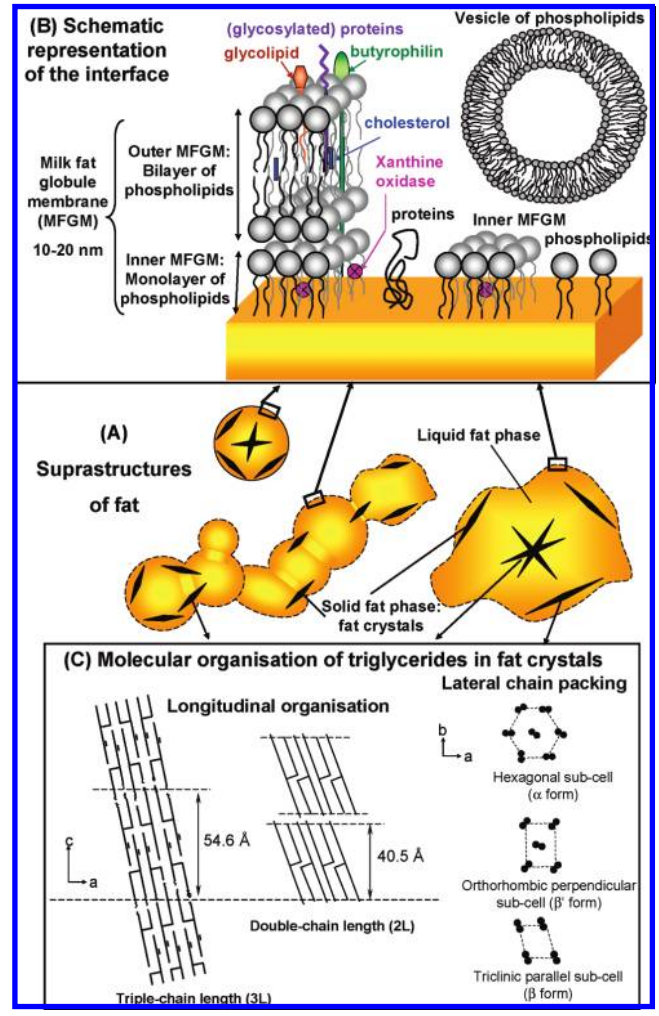

Figure 5. Schematic representation of the different levels of the organization of fat in Emmental cheese: (**A**) suprastructures of fat, (**B**) schematic representation of the interface, and (**C**) molecular organization of triglycerides in fat crystals.

For the first time to the authors' knowledge, the organization of TG molecules in the solid fat phase has been elucidated in a complex food product such as Emmental cheese. The comparison of XRD patterns showed that similar crystalline

structures were formed in the different cheeses: two types of lamellar organizations corresponding to 2L (40.5 Å) and 3L (54.6 Å) structures, associated with four types of lateral packing of the chains, corresponding to the subcells α, β'_1, β'_2, and β (**Figure 4**). Thus, the suprastructure of fat in the cheeses, and more precisely the size of the fat inclusions, did not affect the type of crystals which were formed by TG molecules. Previous studies showed that the size of fat globules affects the organization of TG at a molecular level (*19, 23*). Similar crystalline structures were characterized after quenching of fat globules and anhydrous milk fat from 60 to 4 °C and storage in isothermal conditions for $t > 100$ h at 4 °C (*26*). Soderberg et al. (*44*) identified the formation of a 2L (40 Å) structure in cream using XRD. The coexistence of at least four types of crystals in the solid fat phase in addition to the liquid fat phase resulted from the extreme diversity of the fatty acids in terms of chain length and unsaturation and their position onto the glycerol. Regarding more particularly the longitudinal organization of TG molecules (**Figure 4B**), the 3L lamellar structures are related to the long-chain monounsaturated and/or mixed long- and short-chain TG, whereas the 2L structures are formed mainly by similar long-chain trisaturated TG. A schematic representation of the molecular organization of TG molecules characterized by XRD at 4 °C in the Emmental cheeses (**Figure 4**) is proposed **Figure 5C**.

The improvement of food quality and the development of new products with interesting functional and nutritional properties cannot be achieved without a good knowledge of the role played by the lipids, e.g., both triglycerides and phospholipids.

The characterization of the microstructure and properties of fat in food products requires the utilization of combined techniques allowing the investigation at different scales, from the microscopic to the molecular level. This study showed that the combination of techniques such as CLSM, DSC, and XRD allows (i) the characterization of the suprastructure of fat with the localization of phospholipids mainly around fat inclusions, (ii) to reveal the mechanisms which are mainly responsible for oiling off, (iii) the quantification of the solid fat phase in complex food products: 71.6 ± 4.9% at 4 °C which does not depend on the suprastructure of fat and (iv) the elucidation of the organization of TG at a molecular level: 2L (40.5 Å) and 3L (54.6 Å) with 4 polymorphic forms (α, two β' and β). For a similar fatty acid composition, the solid fat content and the lamellar structures formed by triglycerides mainly result from the thermal history of the dairy products, and more particularly on the duration of storage at 4 °C.

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LITERATURE CITED

(1) Lopez, C.; Briard-Bion, V. The composition, supramolecular organisation and thermal properties of milk fat: a new challenge fort he quality of food products. *Lait* **2007**, *87*, 317–336.

(2) McPherson, A. V.; Kitchen, B. J. Reviews of the progress of dairy science: The bovine milk fat globule membrane - Its formation, composition, structure and behaviour in milk and dairy products. *J. Dairy Res.* **1983**, *50*, 107–133.

(3) Keenan, T. W.; Dylewski, D. P. Intracellular origin of milk lipid globules and the nature and structure of the milk lipid globule membrane. In *Advanced Dairy Chemistry*; Fox, P. F., Ed.; Chapman and Hall: London, UK, 1995; *Vol. 2: Lipids*, pp 89–130.

(4) Lopez, C. Focus on the supramolecular organisation of fat in dairy products. *Reprod., Nutr., Dev.* **2005**, *45*, 497–511.

(5) Blonk, J. C. G.; van Aalst, H. Confocal scanning light microscopy in food research. *Food Res. Int.* **1993**, *26*, 297–311.

(6) Lopez, C.; Maillard, M.-B.; Briard-Bion, V.; Camier, B.; Hannon, J. A. Lipolysis during ripening of Emmental cheese considering organization of fat and preferential localization of bacteria. *J. Agric. Food Chem.* **2006**, *54*, 5855–5867.

(7) Guinee, T. P.; Auty, M. A. E.; Fenelon, M. A. The effect of fat content on the rheology, microstructure and heat-induced functional characteristics of Cheddar cheese. *Int. Dairy J.* **2000**, *10*, 277–288.

(8) Rowney, M.; Roupas, P.; Hickey, M.; Everett, D. W. Milkfat structure and free oil in Mozzarella cheese. *Aust. J. Dairy Technol.* **1998**, *53*, 110.

(9) Gunasekaran, S.; Ding, K. Three-dimensional characteristics of fat globules in cheddar cheese. *J. Dairy Sci.* **1999**, *82*, 1890–1896.

(10) Lopez, C.; Camier, B.; Gassi, J.-Y. Development of the milk fat microstructure during the manufacture and ripening of Emmental cheese observed by confocal laser scannning microscopy. *Int. Dairy J.* **2007**, *17*, 235–247.

(11) Gresti, J.; Bugaut, M.; Maniongui, C.; Bezard, J. Composition of molecular species of triacyllglycerols in bovine milk fat. *J. Dairy Sci.* **1993**, *76*, 1850–1869.

(12) Jensen, R. G.; Newburg, D. S. Bovine milk lipids. In *Handbook of milk composition*; Jensen, R. G., Eds.; Academic Press, Inc.: New York, 1995; pp 543–575.

(13) Small, D. M. The physical chemistry of lipids. From alkanes to phospholipids. In *Handbook of Lipid research*> Hanahan, D. J., Ed.; Plenum Press: New York, 1986; pp 347–382.

(14) Ollivon, M.; Perron, R. Propriétés physiques des corps gras. In *Manuel des corps gras*; Karleskind, A., Wolff J. P., Guttman J. F., Eds.; Lavoisier: Paris, France, 1992; pp 433–442.

(15) Hagemann, J. W. Thermal behaviour and polymorphism of acylglycerils. In *Crystallisation and polymorphism of fats and fatty acids*; Garti, N., Sato, K., Eds.; Marcel Dekker: New York, 1988; pp 9–95.

(16) Timms, R. E. The phase behaviour and polymorphism of milk fat, milk fat fractions, and fully hardened milk fat. *Aust. J. Dairy Technol.* **1980**, *35*, 47–53.

(17) Lavigne, F. Polymorphisme et transitions de phases des triglycerides. Applications aux propriétés thermiques et structurales de la matière grasse laitière anhydre et ses fractions. Ph.D. Thesis, Universite Paris VII, Paris XI and ENSIA, France, 1995.

(18) ten Grotenhuis, E.; van Aken, G. A.; van Malssen, K. F.; Schenck, H. Polymorphism of milk fat studied by differential scanning calorimetry and real-time X-ray powder diffraction. *J. Am. Oil Chem. Soc.* **1999**, *76*, 1031–1039.

(19) Lopez, C.; Bourgaux, C.; Lesieur, P.; Bernadou, S.; Keller, G.; Ollivon, M. Thermal and structural behavior of milk fat. 3. Influence of cooling rate and droplet size on cream crystallization. *J. Colloid Interface Sci.* **2002**, *254*, 64–78.

(20) Famelart, M.-H.; Le Graet, Y.; Michel, F.; Richoux, R.; Riaublanc, A. Evaluation des méthodes d'appréciation des propriétés fonctionnelles des fromages d'Emmental de l'ouest de la France. *Lait* **2002**, *82*, 225–245.

(21) Michalski, M. C.; Camier, B.; Briard, V.; Leconte, N.; Gassi, J. Y.; Goudédranche, H.; Michel, F.; Fauquant, J. The size of native milk fat globules affects physico-chemical and functional properties of Emmental cheese. *Lait* **2004**, *85*, 343–358.

(22) Lopez, C.; Briard-Bion, V.; Camier, B.; Gassi, J.-Y. Milkfat thermal properties and solid fat content determined during the manufacture and ripening of emmental cheese. A differential scanning calorimetry study. *J. Dairy Sci.* **2006**, *89*, 2894–2910.

(23) Michalski, M. C.; Ollivon, M.; Briard, V.; Leconte, N.; Lopez, C. Native fat globules of different sizes selected from raw milk:

thermal and structural behavior. *Chem. Phys. Lipids* **2004**, *132*, 247–261.
(24) Lopez, C.; Lesieur, P.; Bourgaux, C.; Ollivon, M. Thermal and structural behavior of anhydrous milk fat: 3. Influence of cooling rate. *J. Dairy Sci.* **2005**, *88*, 511–526.
(25) Lopez, C.; Bourgaux, C.; Lesieur, P.; Riaublanc, A.; Ollivon, M. Milk fat and primary fractions obtained by dry fractionation. 1. Chemical composition and crystallisation properties. *Chem. Phys. Lipids* **2006**, *144*, 17–33.
(26) Lopez, C.; Bourgaux, C.; Lesieur, P.; Ollivon, M. Crystalline structures formed in cream and anhydrous milk fat at 4°C. *Lait* **2002**, *82*, 317–335.
(27) Lopez, C.; Karray, N.; Lesieur, P.; Ollivon, M. Crystallisation and melting properties of dromedary milk fat globules studied by X-ray diffraction and differential scanning calorimetry. Comparison with anhydrous dromedary milk fat. *Eur. J. Lipid Sci. Technol.* **2005**, *107*, 673–683.
(28) IDF/FIL, Lait, crème et lait concentré non sucré, détermination de la matière sèche, standard 21B. International Dairy Federation, Brussels, Belgium, 1987.
(29) Beaucher, E. Extraction et dosage de la matière grasse sue des emmentals de composition et de taille différentes en globules gras: comparaison de trois méthodes de mesure [Extraction and proportioning of the fat content on Emmentals of different composition and size in fatty globules: comparison of three methods of measurement]. *Sci. Aliments* **2006**, *26*, 349–354, in French.
(30) IDF/FIL, Détermination de la teneur en azote, standard 20B, International Dairy Federation, Brussels, Belgium, 1993.
(31) Gripon, J. C.; Desmazeaud, M.; La Bars, D.; Bergère, J. L. Etude du rôle des micro-organismes et de leurs enzymes dans la maturation des fromages. II. Influence de la présure commerciale. *Lait* **1975**, *55*, 502–516.
(32) Richoux, R.; Roset, G.; Famelart, M. H.; Kerjean, J. R. Diversité de quelques propriétés fonctionnelles à chaud de l'Emmental français. *Lait* **2001**, *81*, 547–559.
(33) 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.
(34) Ollivon, M.; Keller, G.; Bourgaux, C.; Kalnin, D.; Villeneuve, P.; Lesieur, P. DSC and high resolution X-ray diffraction coupling. *J. Therm. Anal. Cal.* **2006**, *85*, 219–224.
(35) IDF/FIL, Catalogue of cheeses, document 141, International Dairy Federation, Brussels, Belgium, 1981.
(36) Michalski, M.-C.; Camier, B.; Gassi, J.-Y.; Briard-Bion, V.; Leconte, N.; Famelart, M.-H.; Lopez, C. Functionality of smaller vs control native fat globules in Emmental cheeses manufactures with adapted technologies. *Food Res. Int.* **2007**, *40*, 191–202.
(37) Rowney, M. K.; Hickey, M. W.; Roupas, P.; Everett, D. W. The effect of homogenisation and milk fat fractions on the functionality of mozzarella cheese. *J. Dairy Sci.* **2003**, *86*, 712–718.
(38) Rudan, M. A.; Barbano, D. M. A dynamic model for melting and browning of Mozzarella cheese during pizza baking. *Aust. J. Dairy Technol.* **1998**, *53*, 95–97.
(39) Kindstedt, P. S. Effect of manufacturing factors, composition and proteolysis on the functional characteristics of mozzarella cheese. *Crit. Rev. Food Sci.* **1993**, *33*, 167–187.
(40) Oberg, C. J.; McManus, W.; McMahon, D. J. Microstructure of mozzarella cheese during manufacture. *Food Struct.* **1993**, *12*, 251–258.
(41) Brown, D. A.; London, E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* **2000**, *275*, 17221–17224.
(42) Law, B. A.; Sharpe, M. E.; Chapman, H. R.; Reiter, B. Relationship of milk fat globule membrane material to flavour development in Cheddar cheese. *J. Dairy Sci.* **1973**, *56*, 716–723.
(43) Norris, G. E.; Gray, I. K.; Dolby, R. M. Seasonal variations in the composition and thermal properties of New Zealand milk fat. *J. Dairy Res.* **1973**, *40*, 311–321.
(44) Söderberg, I.; Hernqvist, L.; Buchheim, W. Milk fat crystallisation in natural milk fat globules. *Milchwissenschaft* **1989**, *44*, 403–406.

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