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Crystalline structures formed in cream and anhydrous milk fat at 4 °C

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Abstract – Crystallization and polymorphic evolutions of triacylglycerols (TG), at 4 °C, in cream and anhydrous milk fat (AMF) samples were studied using high-sensitivity differential scanning calorimetry (DSC), time-resolved synchrotron X-ray diffraction (XRD) and density measurement, and compared as a function of time after quenching from 60 to 4 °C. The evolution of the two crystalline structures initially formed in both samples, corresponding to a metastable 2L (47 Å) and a 3L (70.5 Å) lamellar organization with hexagonal chain packings (α form), were monitored in isothermal conditions at 4 °C as a function of time t. For t > 15 min, short spacings showed the formation of a β' packing of the chains in coexistence with the α form. In AMF, the occurrence of some β form was also recorded. For long spacings, a 2L (39 Å) structure was formed in cream with the 3L (70.5 Å) structure still being preponderant after conditioning for 30 min at 4 °C. In AMF samples, a TG polymorphic transition 3L (70.5 Å) → 2L (39 Å) + 3L (66 Å), correlated with an exothermic peak recorded simultaneously by DSC, was clearly visible. At t > 100 h conditioning at 4 °C, the same crystalline structures were found in cream and AMF with the coexistence of at least a 2L (40.5 Å) and a 3L (54.2 Å) longitudinal organization, associated with α, β'_2, β'_1 and β packings of the chains. The melting behaviors of cream and AMF were monitored on heating at 2 °C·min^{-1} by coupled XRD as a function of temperature and DSC experiments. The absence of further structural evolution and polymorphic transition until final melting demonstrates the relative stability of the structure formed. The evolution of cream density after quenching to 4 °C revealed two transitions, the amplitudes, rates and duration of which coincided with DSC and XRD experiments. All data are in agreement in demonstrating that two transitions, a fast and a slow one, occur in dairy products after fast cooling to 4 °C.

Crystallization / polymorphism / triacylglycerol / X-ray diffraction / DSC

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Résumé – **Structures cristallines formées dans la crème et la matière grasse de lait anhydre à 4 °C.** La cristallisation et les évolutions polymorphiques des triacylglycérols (TG) sont étudiées dans des échantillons de crème et de matière grasse de lait anhydre (MGLA) après un refroidissement rapide de 60 à 4 °C par microcalorimétrie différentielle (MCD), diffraction des rayons X (DRX) utilisant le rayonnement synchrotron et mesures de densités, puis comparées en fonction du temps à 4 °C. Les deux structures cristallines initialement formées dans la crème et la MGLA correspondent à des organisations lamellaires à deux longueurs de chaîne (2L) d'épaisseur 47 Å et à trois longueurs de chaîne (3L) d'épaisseur 70,5 Å (grandes distances), associées à la formation d'une sous-cellule hexagonale aussi appelée forme α (petites distances). Les évolutions de ces structures cristallines sont étudiées à une température constante de 4 °C en fonction du temps de stockage t. Pour t > 15 min, les petites distances montrent la formation d'une organisation des chaînes du type β' en coexistence avec la forme α. Dans l'échantillon de MGLA, des traces de forme β sont également détectées. Aux grandes distances, une structure 2L (39 Å) se forme dans l'échantillon de crème bien que l'organisation 3L (70,5 Å) reste toujours prépondérante après 30 min. Dans l'échantillon de MGLA, une transition polymorphique 3L (70,5 Å) → 2L (39 Å) + 3L (66 Å), corrélée avec un pic exothermique enregistré simultanément par MCD, est clairement visible. Pour t > 100 h, les mêmes structures cristallines sont observées dans les échantillons de crème et de MGLA avec, au moins, la coexistence des organisations longitudinales 2L (40,5 Å) et 3L (54,2 Å) associées avec des petites distances de types α, β'_2, β'_1 et β. Les comportements de fusion des échantillons de crème et de MGLA sont ensuite étudiés au cours d'un chauffage à 2 °C·min^{-1}, par couplage de DRX en fonction de la température et de MCD. L'absence d'évolution de l'épaisseur des structures lamellaires et de transition est en faveur de leur stabilité polymorphique. L'évolution de la densité de la crème après un refroidissement rapide à 4 °C révèle deux transitions dont l'amplitude, la vitesse et la durée coïncident avec les résultats de MCD et de DRX. Toutes les données sont en accord pour démontrer que, au moins, deux transitions, une rapide et une lente, sont enregistrées dans les produits laitiers après leur refroidissement rapide à 4 °C.

Cristallisation / polymorphisme / triacylglycérol / diffraction des rayons X / calorimétrie

1. INTRODUCTION

Milk fat is dispersed within globules stabilized by a complex membrane [9, 21]. In fresh milk, the size-distribution of fat globules ranges from about 0.2 to 10 μm with an average diameter of 4 μm [21]. Triacylglycerols (TG), which represent about 97% of the total fat, are constituted by fatty acids varying in chain length and unsaturation [4, 8]. The complex TG composition induces a melting range which spans from about –40 to 40 °C. As a consequence, at intermediate temperatures, milk fat is partially crystallized and corresponds to a mixture of crystals and oil.

Crystallization of milk fat affects many properties such as (i) its rheological properties, (ii) the susceptibility of globules to churning, (iii) the resistance of the globules to disruption and (iv) the consistency and mouth feel of high-fat products. Thus, it is important to understand better the physical properties of cream for industrial applications. It is also of interest to compare crystallization of TG within fat globules with those in bulk milk fat.

Differential scanning calorimetry (DSC) studies have given an insight into the thermodynamics of milk fat and milk fat fraction transitions in bulk [14, 20, 28] and in emulsions [29]. The authors that studied anhydrous milk fat (AMF) by DSC have long observed that it crystallizes and melts in several steps. A typical melting curve of AMF often shows three endothermic peaks, corresponding to low, medium and high melting fractions [20, 28].

X-ray diffraction (XRD) is by far the most powerful method to determine the crystal structure of fats [13]. TG have the ability to crystallize in different polymorphic forms, of which only one is completely stable in given conditions. This polymorphism being

mainly monotropic, forms are metastable and the transitions are irreversible. The different polymorphic forms have different compositions, different crystal lattices and different melting points which increase with increasing stability [23, 26]. The longitudinal organizations of TG in lamellar structures usually correspond to double (2L) or triple (3L) chain length structures with thicknesses of 40–50 Å and 55–70 Å, respectively. According to the Bragg law: $2.d.\sin(\theta) = n.\lambda$ (where d in angstroms is the repetition distance between two reticular planes, θ is the angle of incidence of X-ray relative to the crystalline plane and λ is the X-ray wavelength) [6], these stackings of the TG molecules, called "long spacings", are studied by XRD at small angles ($0^o < \theta < 5^o$). The cross-sectional organizations of the aliphatic chains, called "short spacings", correspond to packings in hexagonal (α), orthorhombic (β') or triclinic (β) sub-cells in their increasing order of stability. These packings are studied by XRD at wide angles ($8.5^o < \theta < 13^o$). The α polymorphic form, characterized by a single diffraction peak at 4.15 Å, shows rotational disorder along the hydrocarbon chain axes. β' form is characterized by two diffraction peaks at 3.8 and 4.2 Å. β form, which is the most stable packing, is characterized by a peak at 4.6 Å among other sharp peaks [13, 26].

Over the last decade, some improvements of the diffraction techniques, mainly due to the use of the high X-ray flux generated by synchrotron sources, allowed new insight into the domain of lipid structure determination. Either excellent XRD peak resolution at small angles, mainly obtained by increasing sample-to-detector distance, or rapid recordings of XRD patterns as a function of time or temperature are now allowed when not both together. It has also been shown that when the fat phase is not too diluted, it is possible to follow polymorphic evolutions within the dispersed droplets of an emulsion [15]. The technique used allowed one to follow simultaneously fast polymorphic evolution at subzero temperatures in cream samples at both small and wide angles, simultaneously with DSC experiments.

As for many foods, many dairy products need to be stored at low temperatures, to ensure that deterioration, whatever the origin, biological, chemical and/or physical, is limited if not stopped. For anhydrous products and water in oil emulsions (W/O), such as butter, this temperature can be negative (when expressed in ^{o}C), allowing long storage periods. However, most of the dairy products are oil in water emulsions (O/W), e.g. milk and cream, for which negative temperatures are forbidden since ice formation should be imperatively avoided. Due to the temperature gradients in storage rooms, a slightly positive value is imposed, which is frequently chosen around 4 ^{o}C even for domestic purposes. At this temperature about 70% of the fat phase is solid [14], depending on the fat composition and the thermal history of the product. For fractionated milk fats the solid proportion can range from about 0 to 100%. Although this crystallization of the fat phase is more clearly evidenced in the W/O than in O/W emulsions, since in the former case it directly affects the continuous phase, it also influences properties of the latter.

The object of the present research was to describe what physically happens in the fat phase of dairy products as a function of time when they are placed and kept at the conservation temperature of 4 ^{o}C. According to [21], phase transitions have been observed in isothermal conditions in milk fat fractions, but have never been proved in milk fat. However, they have been suspected by observations made by differential thermal analysis [21]. The coupling of XRD recorded at both small and wide angles and high-sensitivity DSC is used in this work to follow in situ the fat crystallization and polymorphic evolutions at 4 ^{o}C within the milk fat globules. The structural and thermal behavior of TG dispersed within fat

globules is compared to that of anhydrous milk fat extracted from cream in order to determine the influence of the dispersed state onto these evolutions during long storage at 4 °C.

2. MATERIALS AND METHODS

Concentrated creams (fat content = 40% wt/wt) obtained by industrial skimming of fresh whole milk collected in Brittany in July 2000, were purchased at the dairy plant (Laiterie du Val d'Ancenis, Ancenis, France). Anhydrous milk fat (AMF) was extracted from 10 g of cream using the following procedure: first, isopropanol (8 mL) (Carlo Erba Reagenti, Val-de-Rueil, France) was added to the cream, the mixture was vortexed, then hexane (12 mL) (Carlo Erba Reagenti, Val-de-Rueil, France) was added. The resulting mixture was vortexed again and centrifuged for 5 min at 500 *g*. The upper organic phase was separated and added to a second organic phase obtained by a second extraction of the lower phase using 12 mL of hexane. The pooled fractions were solvent evaporated under vacuum until they reached a constant weight. Fresh whole milk employed for density measurement was obtained directly from Viltain farm (Jouy-en-Josas, France) in May 2001 and used a few hours after collection. Cream concentrations (40 and 62%) were measured after solvent extraction.

2.1. Globule size-distribution measurements

One mL of the concentrated cream was dispersed in 9 mL of a sodium dodecyl sulfate (SDS) solution (SDS 1%, NaCl 80 mmol·L^{-1}, pH 6.7) to ensure complete defloculation of the fat globules. The globule size-distribution was measured at room temperature by laser light scattering using a Malvern Mastersizer (Malvern Instruments, Malvern, UK). The system was equipped with a lens of 45 mm focal length and the manufacturer's presentation code 0505 was selected to take into account the refractive index of milk fat. The volume/surface average diameter of globules ($d_{32} = \Sigma n_i d_i^3 / \Sigma n_i d_i^2$, where n_i is the number of droplets with diameter d_i) is 2.97 μm and the fat surface area ($A = 6\,\Phi / d_{32}$, where Φ is the volume fraction of fat) found is 2.02 m^2·mL^{-1} fat, with a relative standard deviation of the droplet diameter of 1.38 μm.

2.2. XRD/DSC measurements

Coupled X-ray diffraction (XRD) recorded at both small and wide angles and high-sensitivity differential scanning calorimetry (DSC) set-up was installed successively on both D22 and D24 benches of the DCI synchrotron of LURE (Laboratoire pour l'Utilisation du Rayonnement Électromagnétique, Orsay, France). This set-up has already been described [11, 16]. Briefly, on the D22 bench, two linear detectors allow the simultaneous recording of small ($q = 0 – 0.45$ Å^{-1}) and wide ($q = 1.1 – 2.1$ Å^{-1}) angle XRD data with sample to detector distances of 177 and 30 cm, respectively. Both XRD data and DSC signals are simultaneously collected from the same sample with a single computer in order to avoid any time or temperature shift in the data collection. On the D24 bench, a single linear detector was used at small angles ($q = 0 – 0.55$ Å^{-1}) with a sample-to-detector distance of 90 cm. The channels of the detectors are calibrated to express the XRD data in the scattering vector q with $q = 4\pi \cdot \sin(\theta) / \lambda = 2.\pi / d$; q in angstroms^{-1}, θ in degrees is the angle of incidence of the X-ray relative to the crystalline plane, λ is the X-ray wavelength, d in angstroms is the repetition distance between two planes. The calibration of the detectors was made at wide angles with high-purity tristearin [22] complemented at small angles with silver behenate [2] standards, as previously described [17].

The calorimeter coupled to X-ray diffraction was calibrated with lauric acid [3].

The samples of creams and AMF to be analyzed by coupled XRD and DSC techniques were loaded into thin glass Lindeman capillaries (GLAS Muller, Berlin, Germany) as described in [17]. The samples were heated at 60 °C for 10 min to ensure all crystals are melted. The capillaries were rapidly introduced into the calorimeter pre-cooled to 4 °C, and immediately analyzed in isothermal conditions. XRD patterns were recorded as a function of time (i) for 30 min with frames of 30 s on the D24 bench, and (ii) for 45 or 60 min with frames of 60 s on the D22 bench. With reference to the meaning of "immediately" used above, there are about 15–20 s between the sample introduction into the calorimeter and the beginning of the recording of the first pattern, because of security requirements on synchrotron benches. After quenching at 4 °C and identification of the crystalline structures formed in isothermal conditions, the samples of cream and AMF were placed in the refrigerator at 4 °C. During storage at 4 °C, the capillaries were regularly turned upside down to reduce to a minimum creaming of the fat globules in the cream samples. After storage at 4 °C for a time t, crystalline structures formed in samples of cream and AMF were studied by recording (i) an XRD pattern of 300 s on the D24 bench after t = 60 h, and (ii) an XRD pattern of 600 s on the D22 bench after t = 105 h (sample of AMF) or t = 135 h (sample of cream). Then, the samples were characterized by coupled XRD as a function of temperature (XRDT) and DSC techniques during their heating from 4 to 50 °C at 2 °C·min^{-1}.

XRD patterns recorded as a function of time or temperature were analyzed using PEAKFIT software (SPSS Science Software, Erkrath, Germany). The diffraction peaks were fitted with the Gaussian-Lorentzian sum (amplitude) equation as previously described [17].

2.3. Density measurements

The densities of creams separated from fresh whole milk by centrifugation at 35 ± 5 °C at 360 *g* for 10 min were measured at 4 ± 0.5 °C using a high precision digital densimeter (model 01D, SODEV Inc., Sherbrooke, Quebec, Canada) [24]. The temperature of the densimeter was stabilized for 2 weeks before the experiment using a combination of a cryostat (Bioblock Scientific, Vanves, France) and a temperature buffer corresponding to circulation of the cooling liquid in 6 L of water contained in a Dewar. The densimeter was standardized using air (1.274×10^{-3} g·mL^{-1}) and water (0.999973 g·mL^{-1}) at 4 °C [7]. The principle of density measurement is based on the properties of a mechanical oscillator, made of a U-shaped tube, the resonance period (τ) of which is a function of the density (D) of the fluid that it contains, according to the relationship.

$$D = A + K \cdot \tau^2$$

where A (–5.268352) and K (738136.73) are constants of the system determined with the standards.

Samples melted at 60 °C were quenched by direct introduction of the creams into the densimeter tube (0.2 mL). Frequency was collected with a 8 s period using a HP 53 131A frequency meter (10 digits, Hewlett-Packard, Les Ulis, France), also stabilized for 2 weeks, using Benchlink data collection software.

3. RESULTS AND DISCUSSION

The thermal and structural consequences of rapid quenching of cream and AMF from 60 °C to 4 °C were compared in order to study the influence of (i) the dispersion in small droplets, and (ii) the fat globule interface, on the crystalline structures formed by milk fat TG. The polymorphic

evolutions of both products at 4 °C were monitored as a function of time for 30 to 60 min after quenching. Then, the consequences of a long storage, more than 4 d at 4 °C, were examined in light of the isothermal polymorphic evolution of TG in milk fat products. Finally, the samples of cream and AMF were heated at 2 °C·min^{-1} in order to study the TG melting behaviors.

3.1. Isothermal evolution at 4 °C after quenching

XRD was monitored at both small and wide angles as a function of time on cream and AMF samples quenched from 60 to 4 °C together with isothermal microcalorimetry recordings.

Figures 1 a and b show the time evolutions of both small- and wide- (inserts) angle XRD patterns of cream and AMF, respectively, during 30 to 60 min conditioning at 4 °C after quenching. While small-angle patterns inform on longitudinal stacking of TG molecules (long spacings), generally in double or triple layers (noted 2L or 3L) by giving their thickness and organization, the wide-angle patterns yield the lateral packing (short spacings) of chains in these layers and the sub-cells they adopt. Table I summarizes the long and short spacings observed at 4 °C for both samples. Evolutions of XRD patterns with time allow the determination of the phase transitions occurring in the sample. For instance, in both cream and AMF samples the first diffraction peak formed at wide angles at q = 1.52 Å^{-1} (4.13 Å), on the top of a broad peak centered at q = 1.43 Å^{-1} (4.4 Å) corresponding to the lateral packing of TG chains in the liquid state [12], is the unstable polymorphic variety α (Figs. 1a, 1b, inserts). Obviously, this crystalline α form coexists with some liquid phase. It is worth

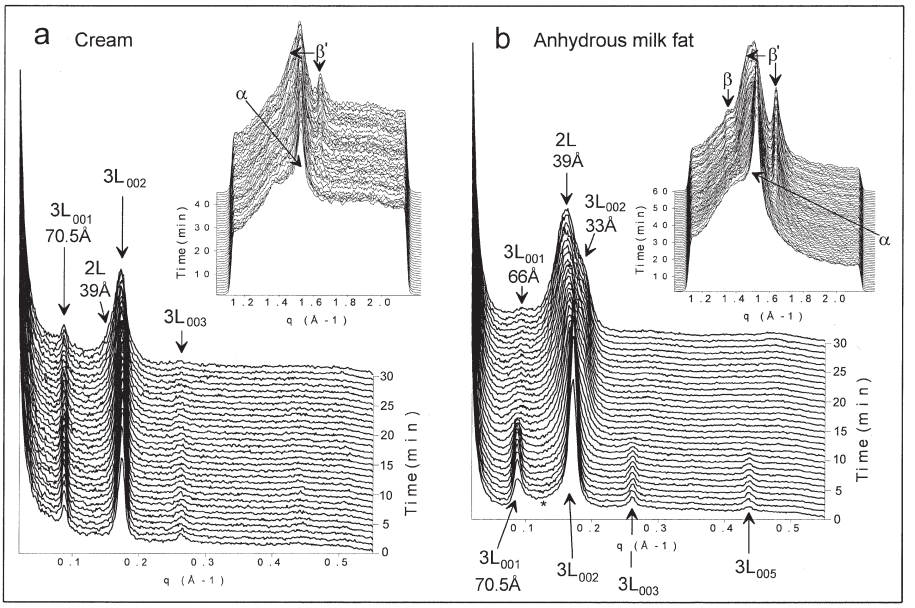

Figure 1. 3D plots of the isothermal evolutions of small- and wide- (inserts) angle X-ray diffraction patterns recorded at 4 °C after rapid quenching from 60 °C of (**a**) cream and (**b**) anhydrous milk fat samples. The crystalline structures deduced from data analysis are directly indicated in the figure; (*) indicates the presence of X-ray scattering.

Table I. Long and short spacings observed by X-ray diffraction (XRD) after different conditioning times at 4 °C following the quenching of cream and anhydrous milk fat samples, as determined by peak fit analysis. Major peaks are printed in bold characters.

Time after quenching at 4 °C	Cream				Anhydrous milk fat			
	Long spacings (Å)	Stacking Type	Short spacings (Å)	Sub-cell	Long spacings (Å)	Stacking Type	Short spacings (Å)	Sub-cell
1 min[b]	70.6 36.2 23.8	$3L_{001}$ $3L_{002}$ $3L_{003}$	4.13	α	70.5 36.5 24	$3L_{001}$ $3L_{002}$ $3L_{003}$	4.13	α
30 min[b]	**71.5** 39.5 **36.5** **24**	$3L_{001}$ 2L $3L_{002}$ $3L_{003}$	4.14 3.84 4.28	α β’	66 **39** 33	$3L_{001}$ 2L $3L_{002}$	4.17 3.84 4.26 4.65	α β’ β
60 h	60.1 **55.3** 48 **40.1** 34 **29.4**	$3L_{001}$ 3L ? $2L_1$ 2L $3L_{002}$	-[a]		60.9 **55** 47.7 **41.3** **39.1** 34.4 **29** 20.6 19.3 13.7	$3L_{1(001)}$ $3L_2$? $2L_1$ $2L_2$ $2L_3$ $3L_{1(002)}$ $2L_{1(002)}$ $2L_{2(002)}$ $2L_{1(003)}$	-[a]	
>100 h[c]	**54.3** 47.4 **40.2** **28.4**	3L ? $2L_1$ $3L_{002}$	4.15 3.78 4.25 3.83 4.37 4.65	α β’ β’ β	59.4 **54.2** 48 **40.5** **38.3** 32.8 **28.6** 20.5 19	$3L_{1(001)}$ $3L_2$? $2L_1$ $2L_2$ 2L $3L_{1(002)}$ $2L_{1(002)}$ $2L_{2(002)}$	4.15 3.78 4.25 3.83 4.37 4.65	α β’ β’ β

2L = double-chain length structure; 3L = triple-chain length structure; [a] small-angle XRD not determined at t = 60 h since recorded on D24 bench, [b] small and wide angle XRD originate from D24 and D22 benches, respectively; [c] both recorded on D22 bench, after 105 h for AMF and 135 h for cream.

noting, for the cream sample, the important level of the baseline which corresponds to X-ray scattering by the aqueous phase (Fig. 1a, insert). At small angles, sharp diffraction peaks are immediately recorded after quenching. These peaks correspond to a 3L stacking of TG molecules (d = 70.5 Å) in the α form, as previously observed for quenching from 60 to –8 °C of cream and AMF samples [15, 16]. Four of the six orders of diffraction observable in the scattering vector q domain analyzed (Miller index hkl, with h = k = 0 and l = 1, 2, 3, 5 ; noted $3L_{001}$ for the first order of a triple chain length packing) are clearly visible in both cream and AMF patterns at q = 0.0891 $Å^{-1}$,

0.1721 Å^{-1}, 0.2618 Å^{-1} and 0.4363 Å^{-1}. In addition, it is worth noting that in some experiments in which the XRD recording was started within the 20 s after sample quenching, the unstable 2L (47 Å) variety, the rapid vanishing of which was already reported [15, 16], was observed (data not shown). At 4 °C it only remains for less than 1 min, while the 3L variety develops (Fig. 2). The higher liquid content expected at 4 °C compared to –8 °C [15] favors the 2L (47 Å) $\rightarrow$3L (70.5 Å) transition. In the AMF sample (Fig. 1b), the existence of a broad X-ray scattering between the two first orders of the 3L (70.5 Å) structure should also be reported. This scattering, which vanishes at the main $\alpha \rightarrow \beta$' transition (Fig. 1b, insert), was attributed to the existence of some stacking disorder as already observed in biological membranes [1].

After a 15 to 20 min delay, wide-angle XRD patterns recorded for both cream and AMF samples (Figs. 1a, 1b, inserts) show the appearance of two new diffraction peaks at about q = 1.636 Å^{-1} (3.84 Å) and q = 1.4736 Å^{-1} (4.26 Å). Both peaks were analyzed as the formation of β' packing of the chains. In AMF (Fig. 1b, insert), the formation after about 30 min of a peak at q = 1.35 Å^{-1} (4.65 Å) is interpreted as some occurrence of the β form, which generally is the most stable TG polymorphic packing. While only weak differences are recorded in wide-angle XRD, evolutions at small angles are different for cream and AMF samples. In AMF (Fig. 1b), after 12 min conditioning at 4 °C, the 3L (70.5 Å) structure observed with sharp peaks completely vanished, giving rise to a 2L (39 Å) structure characterized by a diffraction peak at q = 0.1611 Å^{-1}, and a 3L (66 Å) structure characterized by two diffraction peaks at q = 0.0952 Å^{-1} ($3L_{001}$) and q = 0.1904 Å^{-1} ($3L_{002}$), the peak widths of which are much larger than that of the 3L (70.5 Å) α form. Broad upper-order diffraction peaks are also observed at larger scattering vectors q (Fig. 1b). However, while the TG polymorphic transition 3L (70.5 Å) $\alpha \rightarrow$2L (39 Å) + 3L (66 Å) is complete and clearly visible in the small-angle domain for AMF, this is not the case for the cream sample. In cream (Fig. 1a), after about 20 min, the $3L_{002}$ peak broadening is due to the formation of a 2L (39 Å) structure. This observation is in good agreement with the formation at wide angles (Fig. 1a, insert) of the β' form and the coexistence of α and β' forms after this period of time. However, after conditioning for 30 min at 4 °C, the 3L (70.5 Å) structure is still preponderant.

Figure 2 a shows the time-evolutions of maximal intensities of XRD peaks recorded at small angles for an AMF sample in isothermal conditions at 4 °C (Fig. 1b). Data analysis clearly shows that all peaks corresponding to the 3L (70.5 Å) structure formed within the first two minutes (see below) vanished during the 7 to 11 min period following the quenching, while two new varieties, 2L (39 Å) and 3L (66 Å), developed almost simultaneously (Fig. 2a). This analysis clearly evidences that a polymorphic transition takes place in AMF, in isothermal conditions, after a few minutes following the quenching to 4 °C. The existence of such a transition is attested by the simultaneous DSC recording of a broad exothermic peak between 5 and 30 min, with a maximum around 12 min (Fig. 2b). Such exothermic transitions have already been observed at 10 °C and above during isothermal conditioning following AMF sample quenchings [5]. The exothermic character of the peak and the fact that it corresponds at least partly to a $\alpha \rightarrow \beta$' conversion (Figs. 1a, 1b, inserts) confirms the monotropic character of the transition. Such a transition, which is necessarily irreversible, affects only part of the molecules. The TG species which are crystalline before and after the transition are not necessarily exactly the same. Moreover, it is possible that the solid fat content (SFC) is not constant. This, and the difficulty of

determining the exact baseline, prevented us from calculating specific enthalpies for the transition. The isothermal reaction can be decomposed as follows:

– From small-angle XRD: 3L (70.5 Å) + liquid 1 → 2L (39 Å) + 3L (66 Å) + liquid 2;

– from wide-angle XRD: α + liquid 1 → β' + traces of α + traces of β + liquid 2.

While it is clear that before the transition, the 3L form is α and displays a period likely corresponding to 70.5 Å extended long chains, it was not possible univocally to determine the polymorphic correspondence after the transition. However, some elements could be taken into account. (i) The decrease of the 3L structure thickness from 70.5 to 66 Å likely corresponds to a tilt

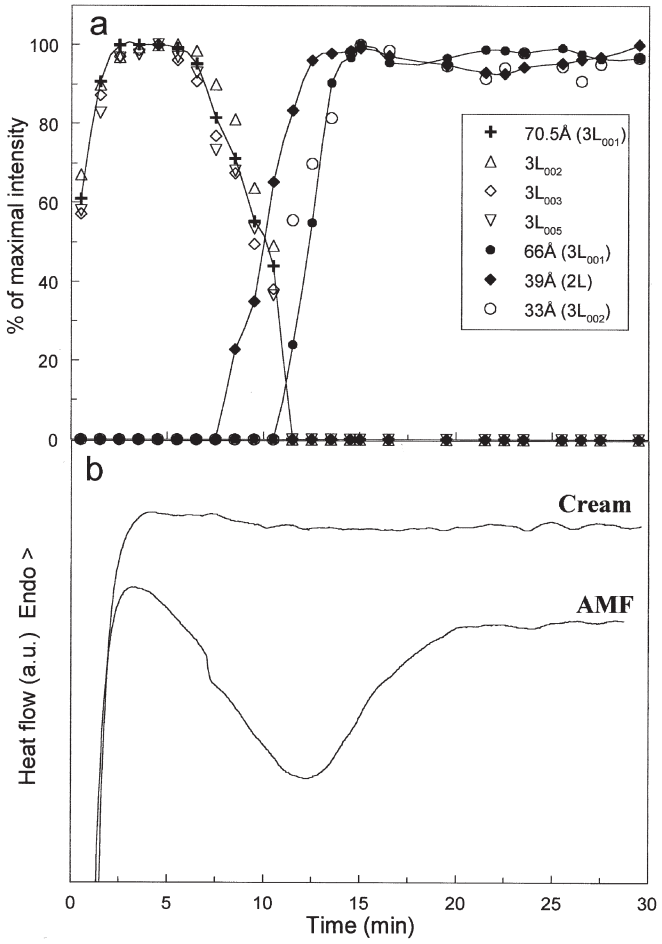

Figure 2. Structural evolution of milk fat in isothermal conditions at 4 °C after rapid quenching from 60 °C. (**a**) Time-evolution of the intensities, taken at the peak maximum and normalized to 100%, of the X-ray diffraction peaks observed at small angles for an anhydrous milk fat sample (Fig. 1). (**b**) DSC recordings of cream and AMF samples obtained simultaneously with XRD experiments shown in Figure 1. The signal jumps observed on the left side of the DSC recordings correspond to the equilibration of the microcalorimeter after sample introduction.

of the TG chains in the lamellar structure. Such a change occurs at an $\alpha \rightarrow \beta'$ transition. (ii) The period of 39 Å is only attributable to a tilted 2L stacking, as already discussed in [27]. A 2L packing without tilted chains as in the α form made from milk fat TG would have resulted in a d-value of around 45 Å. (iii) The residual α observed after the transition could originate from a molecular segregation taking place after the first recrystallization, likely that of the 2L (39 Å) β' structure from Figure 2, leading to a supersaturation in molecules unable to crystallize in this last packing and forced to recrystallize into 3L. The observation of a 3L α structure in AMF after slow cooling at 0.1 $^{o}C \cdot min^{-1}$ may indicate that the lateral packing of the above species is of the α type [18]. On the other hand, taking into account the rather high period observed (66 Å), it may also correspond to the coexistence of $\alpha + \beta'$ packings, not all the layers being crystallized in the same sub-cell.

As a summary, the rapid cooling of dairy products from 60 to 4 ^{o}C induces a first liquid $\rightarrow$ solid TG phase transition with the formation of a metastable 2L (47 Å) organization and a 3L α structure of period d = 70.5 Å characterized by sharp peaks at small angles. In isothermal conditions at 4 ^{o}C, this crystalline organization further completely transforms as a function of time into 2L (39 Å) and 3L (66 Å) lamellar structures through an $\alpha \rightarrow \alpha + \beta' + \beta$ secondary exothermic transition. This secondary process is faster in AMF (within 30 min) than in cream (≥1h). In this respect, the fact that the polymorphic evolution is delayed in the dispersed system shows that (i) dispersion state plays a role in the transition process, and (ii) comparing this transition with primary crystallization, which is nucleation dependent, the polymorphic transition is likely also dependent on the nucleation rate of the more stable variety. The delay in the transition is explained by a lack of stable nuclei in each of the fat globules at 4 ^{o}C after 30 min compared to AMF.

The enlargement of the XRD peaks observed at small angles upon transition are likely partly compensated by a reduction of line width at wide angles. However, globally, the process being exothermic, it should correspond to a decrease of the mean molecular volume and/or an increase of the TG amount crystallized or both (see below, density measurement).

3.2. Polymorphic evolution during conditioning of milk fat at 4 ^{o}C

Further structural evolutions of TG, in the bulk phase of AMF or dispersed in the milk fat globules of cream, were followed by XRD after different periods of time during the storage of the samples at 4 ^{o}C. Figure 3 shows the XRD patterns recorded at 4 ^{o}C on the D22 bench of LURE simultaneously at small and wide angles, for AMF and cream samples after about 4 and 6 d of storage at 4 ^{o}C, respectively. Each XRD pattern corresponds to a duration of data acquisition of 600 s. XRD patterns recorded for cream and AMF samples are very similar at both small (Fig. 3a) and wide (Fig. 3b) angles. Patterns recorded at small angles display three diffraction peaks at about $q = 0.119$ Å^{-1}, $q = 0.155$ Å^{-1} and $q = 0.218$ Å^{-1}. These peaks may correspond to the coexistence of two lamellar structures, a 3L (of which two orders are observed, see discussion below) and a 2L, of about 54–57 Å and 40.5 Å, respectively (Fig. 3a). At wide angles (Fig. 3b), XRD patterns show diffraction peaks at 4.15 Å, 3.78 Å, 3.83 Å, 4.25 Å, 4.37 Å and 4.65 Å (Tab. I). These peaks correspond to the coexistence of likely four crystalline packings of the acylglycerol chains: one α, two β' and one β, as indicated in Figure 3b.

The important differences in XRD patterns recorded at 4 ^{o}C at both small and

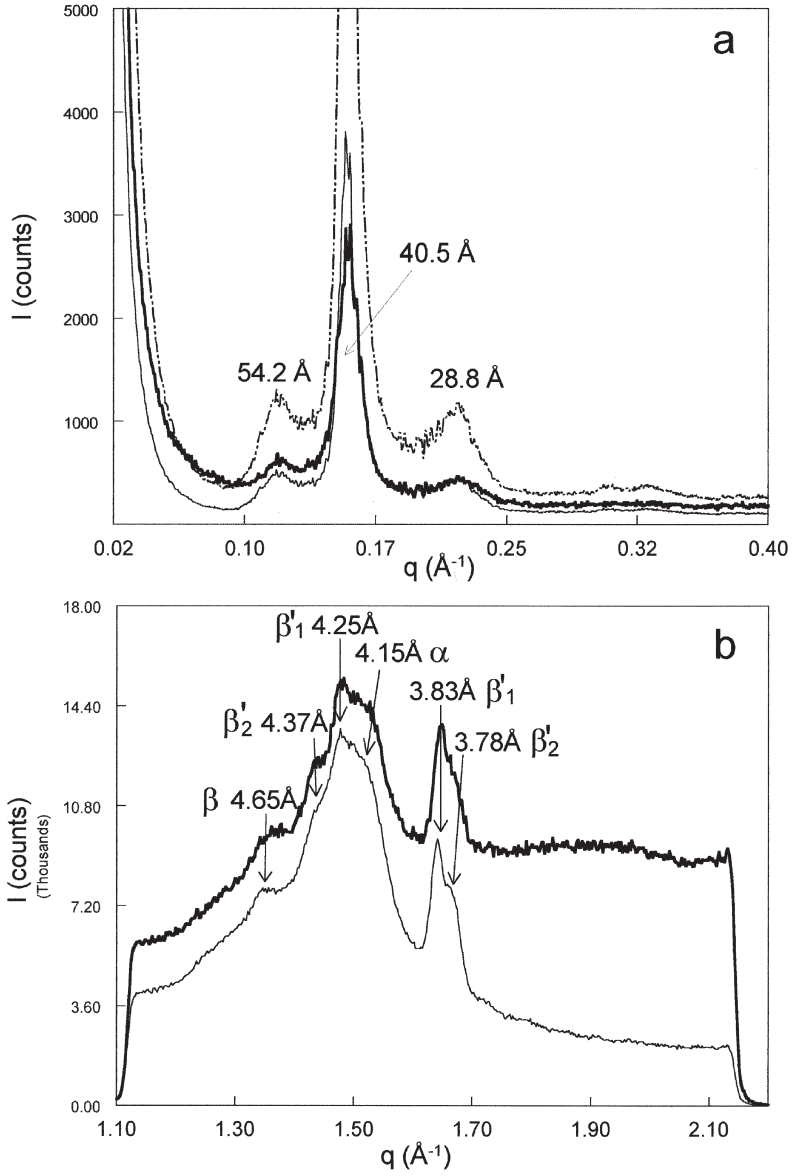

Figure 3. X-ray diffraction (XRD) patterns recorded simultaneously at 4 °C after storage of cream and anhydrous milk fat (AMF) samples at this temperature for 135 and 105 h, respectively, following a rapid quenching from 60 °C. (**a**) small-angle XRD patterns of AMF 100% (— – – —), AMF (same pattern but reduced to 40%) (thin line) and cream 40% fat (thick line), (**b**) wide-angle XRD patterns of AMF 40% (thin line) and cream 40% fat (thick line). The crystalline structures deduced from data analysis are directly indicated in the figure.

wide angles for t = 30 min, (Fig. 1) and t > 100 h in cream and AMF samples (Fig. 3) demonstrate (i) the existence of an isothermal polymorphic evolution in both samples for t > 30 min, and that (ii) the crystalline varieties formed at 4 °C are not stable in a period of time of 30 min after quenching and in fact correspond to varieties of intermediate stability.

The above rough analysis of small-angle XRD data reported the coexistence of two crystalline phases (Fig. 3). However, it appears that the ratio of the long spacings corresponding to first and second orders of the 3L variety is about 1.88 (54.2 Å / 28.8 Å) and thus differing from the expected value (2.0). The discrepancy in the ratio is attributable either to the broadening of the two 3L peaks and their overlapping with the main 2L peak or to the existence of more species (see below). The asymmetry observed for the main 2L peak of AMF is in favor of the latter hypothesis [10]. On the other hand, in the present experimental conditions it was not possible to make a univocal attribution of these four sub-cells identified at wide angles to the two varieties observed at small angles. These points will be further discussed below after considering the melting of the various structures.

Density measurement was used to monitor the structural evolution within the milk fat globules following the quenching of cream samples at 4 °C. Figure 4 shows one of the two evolutions of density of the creams obtained by concentration from fresh whole milk as recorded for 15 h. After an initial sharp jump, due to sample temperature equilibration and ending at point A, the duration of which did not exceed a few minutes, two regimes are easily delimited.

The first jump, noted AB, the whole duration and amplitude of which were about 100 min and 7×10^{-3} g·mL^{-1}, respectively, was identified as corresponding to the $\alpha \rightarrow \beta$' transition while the second, noted

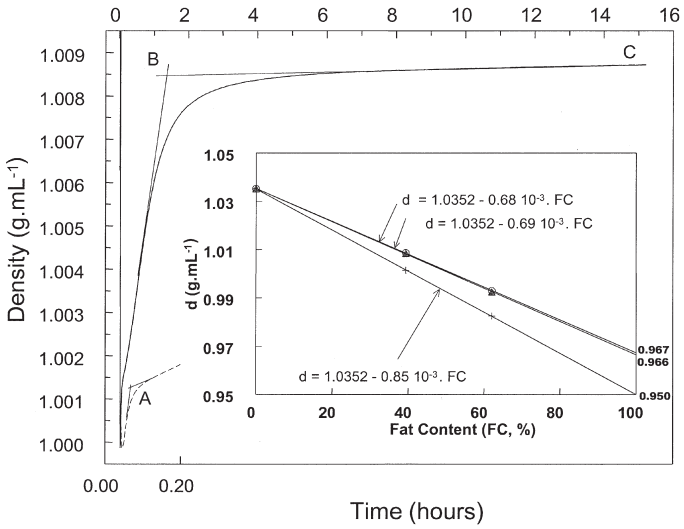

Figure 4. Evolution of cream (40% fat) density as obtained by densitometry monitoring. The density is monitored for 15 h following quenching of cream obtained by direct introduction of the sample heated at 60 °C into the densimeter loop precooled at 4 °C (top x scale). The curve corresponding to the first minutes (0.2 h) following introduction is zoomed (bottom x scale) to show the location of the breakpoint A. The insert shows linear plots of the density measurements at breakpoints A and B, asymptote point C and extrapolation to 100% fat.

Table II. Consequences of the polymorphic evolution of TG within the fat globule upon chain packing and density.

State of fat globule	Liquid	α	β' and β
CH_2 volume (Å^3)	29.5–30[a]	25–26[a]	23.4–23.6[a]
Fat globule density	0.926[b]	0.950[c]	0.966–0.967[c]

[a] volume occupied by a methylene group from [26], [b] value calculated by extrapolation of data from [25], [c] this work.

BC, was attributed to further polymorphic changes (β' and β formation), as discussed above. The main density evolution observed is explained by (i) a reduction of the sub-cell volume when changing from α to β' (the decrease in volume occupied by a methylene group is about 8% when changing from about 25.5 Å^3 (hexagonal sub-cell, α) to 23.5 Å^3 (orthorhombic perpendicular sub-cell, β'); (ii) a partial compensation by larger disorder at small angles attributed to less compact packing between layers; and (iii) a possible increase in the amount of TG crystallized, as discussed above.

The second evolution of the density, from B to C, corresponds to a slow increase, the slope of which is about 1/200 of that of the density jump observed during the first transition (evolution is about 4×10^{-4} g·mL^{-1} in 12 hours compared to 0.0065 g·mL^{-1} in 1 h).

Figure 4, insert, shows a set of linear plots of the densities as measured at breakpoints A and B and at that of the asymptote to the density curve after 16 h, as a function of fat concentration. For each of the three plots (i) the serum density measured at 4 °C $d_{ser} = 1.0352 \pm 0.0001$ (g·mL^{-1}) is added as an independent measurement, and (ii) the equation of density variation obtained by linear fitting is given. The extrapolation of these fits to 100% fat give the fat globule density in the various polymorphic varieties observed. These values are reported in Table II for comparison with that of "liquid" fat globules calculated from extrapolation to 4 °C of liquid fat densities, measured at $30 \leq T \leq 80$ °C [25]. The fat globule densities obtained by extrapolation to 100% fat (0.950 for α, 0.966 for β' and 0.967 for a mixture β + β') were compared with the volumes occupied by methylene groups found in the literature and that are related to the chain compactedness. The density jumps are roughly proportional to the changes of sub-cell compactedness. The increase in compactedness that accompanies the liquid → α jump is about twice that observed for α → β' or β transition. These density data confirm our interpretation of the results. Densities of milk and cream at 4 °C after various periods of time, whatever their concentrations, can be deduced from equations.

3.3. Heating of cream and AMF samples at 2 °C·min^{-1}

The samples of cream and AMF conditioned at 4 °C for 135 and 105 h, respectively, were heated from 4 to 50 °C at 2 °C·min^{-1} and characterized simultaneously by XRD as a function of temperature (XRDT) and DSC experiments. The XRDT patterns recorded at both small and wide angles during heating of the cream and AMF samples are presented in Figures 5 a and b, respectively. XRD patterns were recorded for 60 s during the increase in temperature at 2 °C·min^{-1}. Thus, each XRD pattern, characterized by its mean temperature, reflects the averaged structural events occurring during these 60 s.

In both cream and AMF samples, the diffraction peaks recorded at small angles at 54.2 and 28.8 Å simultaneously decrease in intensity in the $4 \leq T \leq 21.5$ °C domain

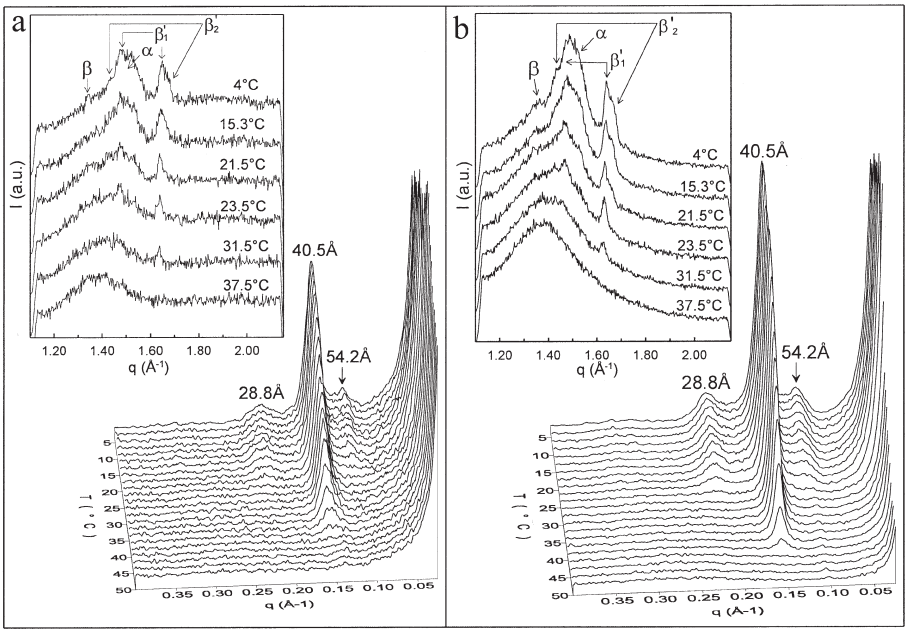

Figure 5. Evolutions of the small- and wide- (insert) angle X-ray diffraction patterns recorded during heating of (**a**) cream and (**b**) anhydrous milk fat at 2 °C·min^{-1} from 4 to 50 °C following 135 and 105 h storage at 4 °C, respectively.

until their vanishing (Figs. 5a, 5b). The melting of this 3L structure is associated with the decrease in intensity of X-ray scattering at very small angles ($q < 0.07$ Å^{-1}) clearly recorded for the AMF sample (Fig. 5b). From 23.5 °C, only the 2L (40.5 Å) crystalline structure is recorded at small angles and only peaks corresponding to the β'_1 polymorphic form (3.85 and 4.28 Å) are recorded at wide angles. These results mean that the peaks centered at 54.2 and 28.8 Å were related to the α, β'_2 and β polymorphic packings recorded at wide angles. Then, as a function of the temperature increase, the β'_1 2L (40.5 Å) structure progressively melts until its vanishing. Milk fat dispersed within fat globules or in bulk, submitted to the same thermal treatments, shows the same structural evolution as a function of temperature.

In principle, the monitoring of the melting of the various structures formed allows one to discriminate between the coexisting varieties. The superimposition of the intensity plots permits the determination of the coincidence in occurrence and vanishing of peaks even when they are in different angle domains (e.g. small and wide angles) [17]. The same method of data analysis was applied to this study.

The evolutions of maximal intensities of diffraction peaks recorded at small angles for the cream and AMF samples were plotted on the same graph to allow comparisons (Figs. 6a, 6b). The DSC melting curve recorded simultaneously during heating of the samples at 2 °C·min^{-1} is presented in Figure 6c to relate the structural and thermal events recorded by coupled XRDT and DSC techniques. The melting of the 3L structure (54.2 and 28.8 Å peaks) formed in both samples is correlated with the endotherms recorded in both DSC curves up to about 25 °C. Above this temperature

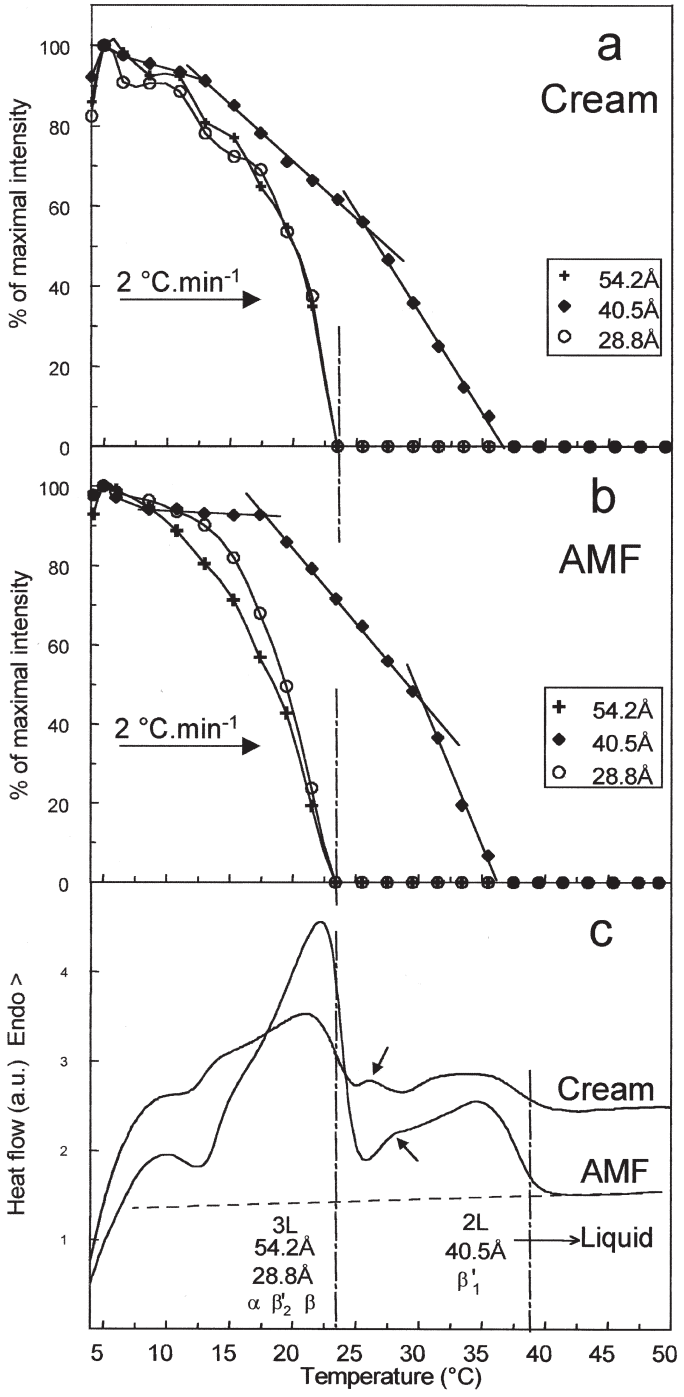

Figure 6. Evolution of maximal intensities of the peaks recorded by X-ray diffraction at small angles during heating at 2 °C·min^{-1} of (**a**) cream and (**b**) anhydrous milk fat (AMF). (**c**) DSC melting curves recorded simultaneously for cream and AMF samples as indicated in the figure.

the endotherms correspond to the melting of the 2L (40.5 Å) structure. Figures 6 a and b show that there are mainly two steps in the decrease in intensity of the 2L (40.5 Å) structure. In cream (Fig. 6a), the intensity linearly decreases first in the $4 \leq T \leq 25.5$ °C domain with a slope of –2.83%/°C, then in the $25.5 \leq T \leq 37$ °C domain the slope is –4.98%/°C; the temperature of the inflexion point between the two domains was calculated at 25.7 °C. In AMF (Fig. 6b), the intensity of the 40.5 Å peak decreases from 17.4 to 29.5 °C with a slope of –3.70%/°C and in the $31.5 \leq T \leq 37$ °C domain, the slope of the decrease in intensity is –7.42%/°C; the inflexion temperature is 29.9 °C. In both samples, the first domain of decrease in intensity of the 40.5 Å peak is related to an endotherm recorded simultaneously by DSC. The second temperature-delimited domains correspond to the final melting of the 2L structure. The linear fits of the two regimes for both Figures 6 a and 6 b are justified a posteriori by the shift of the DSC intermediate melting peak (indicated by arrows).

The study of the long spacings associated with the 2L structure during its melting showed an increase from 40.5 to 41 Å in AMF and an increase from 40.5 to 41.5 Å in cream. These increases in the thicknesses of the lamellar structures may correspond to the selective melting of fatty acids with shorter chains [14].

While the same crystalline structures are formed by TG dispersed within milk fat globules or in bulk in AMF, the small-angle XRD peaks recorded for cream show

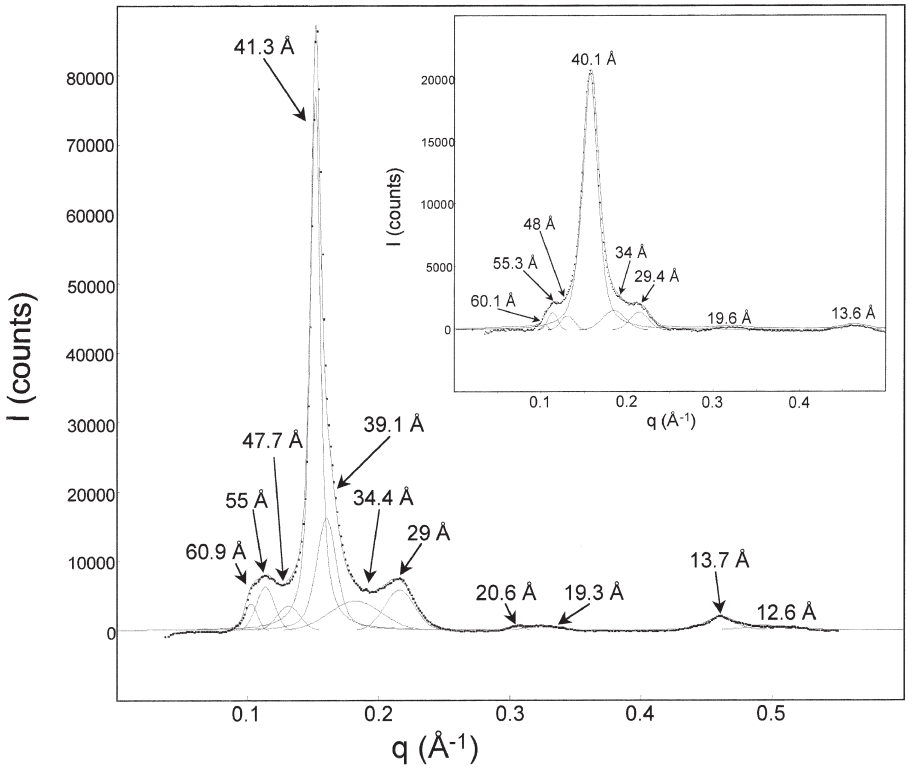

Figure 7. Peak fit analysis of small-angle X-ray diffraction patterns recorded at 4 °C for anhydrous milk fat and cream (insert) after 60 h storage at this temperature.

broadening in comparison with those of AMF. Indeed, the ratio of the middle-height widths of the 2L structure formed by TG in cream and AMF samples is 1.69 ± 0.23. This result may be due to a disorder in TG stacking or to the formation of crystals of smaller sizes within fat globules of cream due to the interface curvature.

The ten patterns recorded at small angles on heating between 4 and 22 °C allowed the analysis of the evolution of the ratio R = 54.2 Å / 28.8 Å discussed above, as a function of temperature. This ratio R = 1.888 ± 0.011 can be considered as constant and different from the expected value (2.0). As all patterns can be considered as independent measurements, it confirms the necessity of an alternative analysis to the above results by an appropriate decomposition of the peaks recorded at small angles.

A more careful analysis of small-angle XRD data has been undertaken by minimization of the residual. Figure 7 shows the decomposition in Gaussian-Lorentzian peaks of the small-angle XRD patterns recorded for AMF and cream (insert). Results of this decomposition in a minimum number of peaks to fit the XRD patterns are given for both products at t = 60, 105 and 135 storage hours after quenching in Table I. The attribution of diffraction peaks to specific longitudinal stackings was made, taking into account the respective intensities of the various peaks and their orders. The two more intense peaks with periods of about 40 Å (39–41 Å depending on the time after quenching) correspond necessarily to 2L packings for which the second order is less intense than the third one. Reciprocally, the peaks at lower q values are necessarily 3L and display a second order more intense than the first one. However, this decomposition is only tentative, further analysis with a better angular resolution and a higher X-ray flux, which would be needed for its confirmation, is under way.

Indeed, the thermal and structural changes observed in this work for long storage at 4 °C of cream samples represent further evolutions of the fat polymorphism when compared to those observed on cooling at 0.15 °C·min^{-1} [17]. The density changes and the X-ray diffraction data show that the present system continues to evolve even after hours or days of storage at 4 °C. It is worth noting that parallel evolutions have been observed in commercial cream and butter.

The morphological evolutions of the fat crystals obtained in fat globules of a fresh whole milk and in bulk AMF at 4 °C by fast quenching in conditions similar to that described above were monitored for 24 h under the objective of a polarized light microscope equipped with a digital camera and a temperature-controlled stage, the setup of which is described in Lopez et al. [19]. No detectable morphological evolution in size, shape, orientation and/or location of the crystals was recorded. Therefore, the reorganizations observed at a molecular level are apparently not accompanied by rearrangements at the supramolecular level. However, evolutions of DSC recordings as well as rheological properties of AMF have been observed for at least one month [14].

4. CONCLUSION

Water-rich foods are generally stored at about 4 °C to minimize all types of unwanted evolution. In this study, we have studied the consequences of the choice of this temperature for dairy products. As in many foods, the fat phase of these products is partially crystallized at this temperature and displays a complex polymorphism which is time- and temperature- dependent. The evolutions of crystalline structures formed in fat globules of cream and anhydrous milk fat were examined and compared as a function of time at 4 °C using

DSC and time-resolved synchrotron X-ray diffraction. Several isothermal polymorphic transitions were observed. These transitions that occur in the solid phase are favored and accelerated by the relatively high amount of liquid phase in milk at 4 °C (about 30%, [14]). Quenching of cream and AMF yields an initial 3L α(70.5 Å) crystalline structure that rapidly transforms, in AMF within 30 min and more slowly in cream, into 2L (39 Å) and 3L (66 Å) β' phases. These secondary crystals evolve towards even more stable forms in the following hours (likely within one day). The patterns recorded after about 4 or 6 d evidence the coexistence of several sub-cells (likely $\alpha + 2\beta' + \beta$) related to a major 2L structure with a thickness of 40.5 Å. This major longitudinal stacking of TG molecules is accompanied by some other long spacings less clearly identified. The absence of evolution of the characteristics of these phases on heating is in favor of their polymorphic stability. The two-step evolution of cream specific density observed at 4 °C perfectly agrees with the above interpretations. Thus, density measurements potentially appear to be alternative technique for the characterization of dairy products.

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