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Original article

Coupling of time-resolved synchrotron X-ray diffraction and DSC to elucidate the crystallisation properties and polymorphism of triglycerides in milk fat globules

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Abstract – Crystallisation properties and polymorphism of triacylglycerols (TG) in natural milk fat globules are studied with an apparatus permitting the coupling of time-resolved synchrotron X-ray diffraction (XRD) and high-sensitivity differential scanning calorimetry (DSC) for cooling rates $0.1 < R_{cooling} < 1000$ °C·min^{-1}. We showed that the fat phase of dairy products displays a complex polymorphism. For the first time, six different types of crystals were identified, several of them in coexistence, and their time- and temperature-dependent evolutions were quantitatively monitored. They correspond to lamellar structures with double-chain length (2L: 40.5–48 Å) and triple-chain length (3L: 54–72 Å) organisations of TG. Depending on the cooling rate, at least five crystalline subcell species were observed at wide angles: α and sub-α, two β' and one β. All these crystalline structures coexist with a liquid phase even at low temperatures. Thermal events recorded by DSC were related to the structural information on the organisation of TG obtained by XRD. Moreover, we showed that crystallisation in fat globules induces a higher disorder and a smaller size of crystals than in bulk, i.e. in anhydrous milk fat. Furthermore, XRD permitted the identification of different crystallisation behaviour in natural milk fat globules with different sizes, which could be implicated in the manufacture of dairy products involving tempering periods in the technological process (e.g. butter, ice cream, whipped products). The increased knowledge of milk fat crystallisation in water-dispersed and highly complex food products might have a certain value for technical applications as well as for the development of new processes and dairy products.

crystal / phase transition / triacylglycerol / emulsion droplet / fat globule size

摘要 – 时间分辨的X射线衍射和差示量热扫描法研究乳脂肪球中三酸甘油酯的晶体性质和多晶性。本文采用时间分辨的X射线衍射仪 (XRD) 和高灵敏度的差示量热扫描仪(DSC)在冷却速率为 $0.1 < R_{cooling} < 1000$ °C·min^{-1} 时, 研究了天然乳脂肪球中三酸甘油酯(TG) 晶体特性和多晶性。实验证明乳制品中的脂肪相具有复杂的多晶性。首次鉴定出 6 种不同的晶型, 他们中大多数是共存的, 并且定量地测定了这六种晶型的时间和温度之间的变化关系。他们对应的层状结构是具有双倍链长(2L: 40.5–48 Å) 和三倍链长 (3L: 54–72 Å) 的 TG 组织。

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根据冷却速率, 在一个很宽的范围内至少观察到 5 个亚晶胞结构, 分别为α、亚-α、2个 β′ 和1个 β 型结构。即使在较低的温度下, 这些晶型结构也是与液态相共存的。DSC 的热分析结果与 XRD 观察到的 TG 组织结构特性是一致的。此外, 我们还证明了脂肪球中的脂肪晶体与游离脂肪 (如无水脂肪) 相比, 前者处于高度的无序状态, 并且晶体的尺寸较小。根据 XRD 分析证明了不同大小的天然乳脂肪球具有不同的晶体性质, 这种性质有可能与乳制品 (如奶油、冰淇淋、搅打乳制品) 加工过程中所涉及到老化工艺有直接的关系。了解乳脂肪晶体在水分散的和复杂的食品体系中的结晶特性将有助于解决生产实际问题和开发新的加工技术及新的乳制品。

晶体 / 相转移 / 三酸甘油酯 / 乳状液滴 / 脂肪球大小

Résumé – Couplage de la DSC et de la diffraction des rayons X résolue en temps en utilisant le rayonnement synchrotron pour élucider les propriétés de cristallisation et le polymorphisme des triglycérides dans les globules gras du lait. Les propriétés de cristallisation et le polymorphisme des triglycérides (TG) dans les globules gras naturels du lait sont étudiés avec un appareil permettant le couplage de la diffraction des rayons X (DRX) résolue en temps et de la microcalorimétrie différentielle (DSC), pour des vitesses de refroidissement comprises entre 0,1 et 1000 °C·min^{-1}. Nous avons montré que la phase lipidique des produits laitiers possède un polymorphisme complexe. Pour la première fois, six types différents de cristaux ont été identifiés, plusieurs d'entre eux étant en coexistence, et leurs évolutions ont été caractérisées de manière quantitative, en fonction du temps et de la température. Elles correspondent à des structures lamellaires avec des structures à deux longueurs de chaîne (2L : 40,5–48 Å) et à trois longueurs de chaîne (3L : 54–72 Å). En fonction des vitesses de refroidissement, au moins cinq sous-cellules cristallines ont été identifiées aux grands angles : α, sub-α, deux β′ et β. Toutes ces structures cristallines coexistent avec une phase liquide, même à basse température. Les évènements thermiques enregistrés par DSC ont été reliés aux informations structurales de l'organisation des TG obtenues par DRX. Nous avons montré également que la cristallisation dans les globules gras induit un plus grand désordre et une plus petite taille des cristaux qu'en milieu anhydre. De plus, la DRX a permis d'identifier différents comportements de cristallisation dans les globules gras de différentes tailles, qui pourraient intervenir lors de la fabrication de produits laitiers nécessitant des périodes de tempérage (par exemple le beurre, la crème glacée, les produits foisonnés). L'augmentation des connaissances sur la cristallisation de la matière grasse du lait dans les systèmes dispersés et dans les produits alimentaires complexes contribuera aux innovations technologiques ainsi qu'au développement de nouveaux produits.

cristal / transition de phase / triglycéride / gouttelette d'émulsion / taille des globules gras

1. INTRODUCTION

Triacylglycerols (TG), which are the main constituents of natural fats, determine the physical and thermal properties of high-fat products. Moreover, the fat phase of food products is sometimes partially crystallised at room and storage (4–7 °C) temperatures, as is the case for milk and dairy products.

Milk fat is an essential nutriment consumed in many food products, in which it is frequently found dispersed as small droplets called milk fat globules, e.g. in milk, cream and cheeses. Crystallisation of TG in milk fat globules is of prime importance because it affects many properties such as (i) the rheological properties, (ii) the resistance of fat globules to disruption and then to coalescence, (iii) the susceptibility of globules to churning, (iv) the stability of whipped cream and (v) the consistency and mouth feel of high-fat products. Thus, it is important to understand better the physical properties of fat globules, e.g. their thermal and crystallographic properties, for industrial applications and to improve the quality of food products.

Milk fat globules have a diameter ranging from 0.02 to 15 μm with

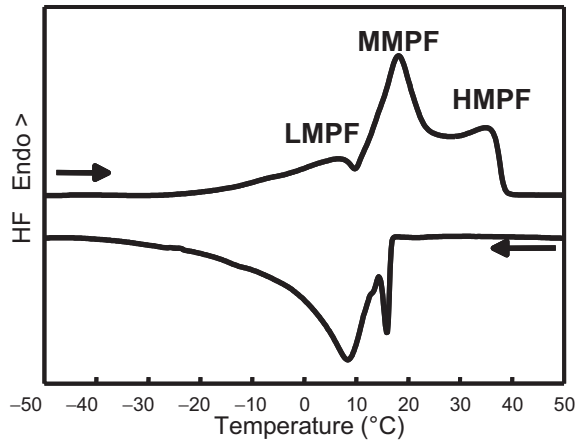

Figure 1. Thermal properties of anhydrous milk fat. Crystallisation and melting curves recorded upon cooling at 2 °C·min^{-1} and subsequent heating at 2 °C·min^{-1} using differential scanning calorimetry (DSC). LMPF: low melting point fraction; MMPF: middle melting point fraction; HMPF: high melting point fraction.

a volume-weighted diameter of about 4 μm [9]. Natural milk fat globules, which are enveloped by a biological membrane (the milk fat globule membrane, MFGM), are mainly composed of TG (~98% of milk lipids), which are esters of fatty acids and glycerol. More than 400 fatty acids have been identified in milk, with a wide diversity of chain lengths, number of unsaturation, branching and position on the glycerol. More than 200 individual molecular species of even-numbered TG have been quantified [4]. Furthermore, it is well known that milk fat composition changes with cow feeding, season and stage of lactation. Thus, milk fat is undoubtedly the most complex fat found in nature. The consequence of this TG and fatty acid composition is that anhydrous milk fat (AMF), which is the fat isolated from butter, has a broad melting range from −40 °C to +40 °C (Fig. 1) and no true melting point, like pure compounds. Therefore, at intermediate temperatures and at equilibrium (e.g. room and fridge temperatures) milk fat is a mixture of crystals and oil. Moreover, this fat-composition-related complexity is dramatically enhanced by the existence of a polymorphism of monotropic type for each TG [22, 25]. As observed for most of the lipids, each TG of milk fat can exhibit several crystalline forms, the occurrence of which strongly depends on its thermal history. Each polymorphic form is characterised by its own melting point. Furthermore, the fact that TG mixtures also exhibit multiple melting points, depending on their composition, renders the overall melting behaviour of milk fat even more complex.

TG polymorphism relates to the ability of molecules to arrange themselves within a crystal lattice in a number of different ways of lateral packing of the fatty acid chains and of longitudinal stacking of molecules in lamellar structures (Fig. 2) [6]. Thus, pure TG and mixtures of TG can adopt several crystalline arrangements. Transitions between these polymorphic forms are mostly irreversible; monotropism implies that they are only possible via a liquid and when leading to the formation of more stable species [25].

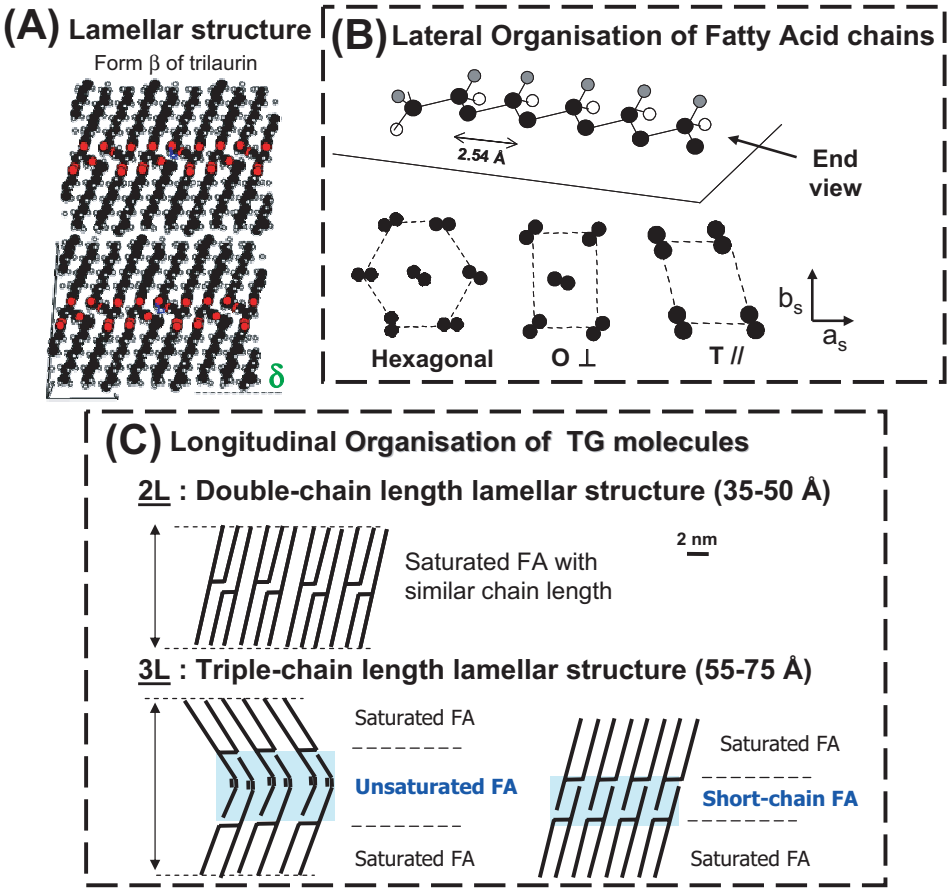

Figure 2. Main types of TG packings. **(A)** Lamellar structure formed by triacylglycerol (TG) molecules in the solid state: example of the β form of trilaurin. **(B)** Top: the stable conformation of the hydrocarbon chains of saturated fatty acid (FA) is a planar zigzag shown here as a 3D view along its main axis; bottom: three main types of chain lateral packings (only carbon atoms are drawn, hydrogen atoms are not drawn): hexagonal, orthorhombic perpendicular (O⊥) and triclinic parallel (T//) subcells to which α, β' and β polymorphic forms correspond. **(C)** Two main types of TG chain longitudinal stacking (fatty acids are drawn as straight lines).

The three main polymorphic forms frequently observed for the lateral packing of fatty acid chains correspond to different subcells that have been described in detail [22, 25]: hexagonal (α form), orthorhombic perpendicular (β' form) and triclinic parallel (β form) (Fig. 2B). The density, enthalpy of fusion, melting point and stability increase in the order α, β' and β, according to the monotropic character of the polymorphism. In the α form, the packing of chains is not very tight and the chains have considerable rotational freedom, whereas in the β form, the chains are very densely packed. TG crystals are made by the stacking of TG molecule layers, the thickness of which depends on the length and unsaturation of the fatty

acid chains, and their angle of tilt with respect to the basal planes formed by the methyl end groups of the TG (Fig. 2C). The longitudinal organisations of TG in lamellar structures are primarily related to the number of chains stacked one on top of the other in the main crystalline cell. For TG, this number frequently takes a value of 2 or 3 and corresponds to the stacking of double (2L) or triple (3L) chain length lamellar structures, leading to distances in the range 35–50 Å and 55–75 Å, respectively [6,25]. Roughly, 3L forms are usually related to low-melting, long-chain monounsaturated and/or mixed long- and short-chain TG, whereas 2L forms are generated mostly by similar long-chain, high-melting, trisaturated TG [25].

Crystallisation in milk fat globules has been investigated by different techniques. Freeze-fracture electron microscopy has made possible the study of fats and food systems such as milk, cream and butter [24,26]. Polarised light microscopy permitted the classification of fat globules into four main types as a function of the crystal habit [14, 28]. These studies gave an insight into the orientation and the size of the crystals at a microscopic level, but little information exists at a molecular level on the organisation of TG molecules in the crystals formed within fat globules. 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 and in emulsions. The authors that studied anhydrous milk fat by DSC observed that it crystallises and melts in several steps (Fig. 1). A typical melting curve of AMF shows three endothermic peaks, corresponding to low melting point (LMP), medium melting point (MMP) and high melting point (HMP) fractions [27]. These peaks correspond to large groups of TG that melt separately and behave like solid solutions. The occurrence of numerous thermal transitions during DSC experiments, the partial overlapping of the melting peaks, their respective enthalpies and temperatures depend strongly on the thermal treatments (e.g. heating and cooling rates, tempering) and on the entire thermal history of the sample [1, 22]. The complex DSC recordings are difficult to interpret. Thus, DSC experiments need to be coupled with other techniques.

XRD is an essential tool for elucidating the molecular packing of molecules in the solid state and polymorphism of pure TG and complex fats, since it provides structural information on the organisation of molecules, and it complements DSC. X-rays are the ideal direct probe to determine the internal structure of crystals, since they provide information on the repetitive patterns of the electron density of the array of atoms. The atoms that constitute the TG molecules inside a perfect crystal are arranged periodically in planes at a repetitive distance, d (Fig. 3). As a beam of X-rays comes to the crystal at an angle, θ, the X-rays of wavelength λ are reflected by the planes at that same angle θ. The optical path length required to produce constructive interference is $n\lambda$, with integral values of n, providing specific crystal symmetry allows it. The equation that determines the angle at which constructive interference occurs is known as Bragg's law: $2d\sin\theta = n\lambda$ [5,25]. The actual diffraction measurement during an experiment is the one formed between the incident beam and the reflected beam, which is denoted by 2θ. It is often convenient to use the scattering vector q instead of the scattering angle 2θ since the former is independent of the X-ray wavelength. They are related as follows: $q = (4\pi/n\lambda)\sin\theta$. Thus, $q = 2\pi/d$.

The two levels of organisation, e.g. the lateral packing of the fatty acid chains and the longitudinal stacking of TG molecules

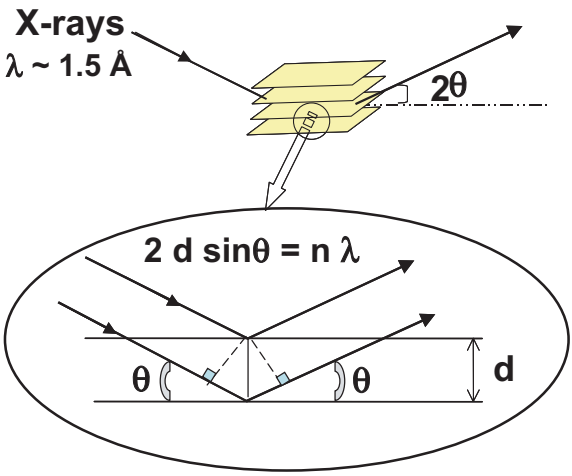

Figure 3. Demonstration of Bragg's law of diffraction: $2d\sin\theta = n\lambda$ (d in Å is the repetitive distance between the planes arranged periodically; θ in degrees is the angle of incidence of X-ray relative to the crystalline plane; λ in Å is the X-ray wavelength; the integral values of n are required to produce constructive interference nλ).

in lamellae formed in the solid state (Fig. 2), are easily identifiable from XRD patterns recorded at wide and small angles, respectively. The thickness of the lamellar structures, which can be measured by XRD at small angles ($0° < \theta < 5°$), corresponds to TG longitudinal stacking called TG long spacings. From the measurement of d (Å) and with knowledge of the fatty acid composition (chain length and unsaturation), it is possible to deduce if the stackings correspond to 2L or 3L organisations (Fig. 2C). The cross-sectional packings of the aliphatic chains are characterised by specific short spacings, independent of chain length and observable at wide angles in the range $8.5° < \theta < 13°$. Short spacings are widely used for identifying the various crystalline subcells characterising the polymorphic forms (Fig. 2B). A single line around 4.15 Å characterises the α form (hexagonal subcell), and a strong line at 4.6 Å among other sharp lines identifies the β form (triclinic parallel subcell), while the β′ form (orthorhombic perpendicular subcell) frequently shows association of two lines, among which one is about 4.2 Å and the other around 3.8 Å.

Recent use of synchrotron radiation, which provides X-ray flux 10^3 to 10^6 times more intense than that generated by usual X-ray sources, permits recordings to be performed within a few seconds or milliseconds. Thus, direct continuous recordings can be performed as a function of time (XRDt) or temperature (XRDT). Moreover, synchrotron radiation permits the study of the organisation of TG in water-dispersed systems such as emulsions and food products and to quantitatively monitor phase changes within emulsion droplets. This is especially interesting to relate the textural and rheological properties of milk fat, and of high-fat food products, to the thermal and crystallographic properties of TG. Therefore, the function of milk fat in many food products cannot be understood without knowing both its composition and physical properties, as well as those of their respective dependencies.

Determination of the thermal and crystallographic properties of TG molecules in the dispersed state, e.g. in milk fat globules, is much more challenging than for fat in bulk, e.g. anhydrous milk fat, since the presence of numerous compounds (e.g. casein micelles, minerals, lactose and water) not involved in the TG organisation will necessarily decrease the intensity of the signal recorded by both DSC and XRD techniques, by a simple dilution effect. Overcoming the difficulties associated with milk fat globule examination requires powerful X-ray sources such as those available at synchrotron laboratories.

The objective of this paper is to provide an overview of the possibilities of investigation offered by the coupling of high-sensitivity DSC and time-resolved synchrotron radiation XRD. Selected examples illustrate the study of the thermal and crystallographic properties of milk fat within the globules, the evolution of organisation of milk fat TG in the solid state and their polymorphism as a function of temperature and time.

2. MATERIALS AND METHODS

2.1. Samples

Concentrated creams (fat content = 400 to 800 $g \cdot kg^{-1}$) were obtained by industrial skimming of whole milk. Anhydrous milk fat (AMF) was extracted from the creams as detailed in Lopez et al. [15]. Natural milk fat globules with different sizes were selected from the same raw whole milk by using a novel microfiltration process [21].

2.2. Methods

2.2.1. Coupled XRD and DSC measurements

Experiments were performed using a technique allowing the coupling of time-resolved synchrotron X-ray diffraction as a function of temperature (XRDT) or time (XRDt) and high-sensitivity differential scanning calorimetry (DSC) in the same apparatus from the same sample. This apparatus, called Microcalix, was recently described in Ollivon et al. [23].

This set-up was used on the D22 and D24 benches of the DCI synchrotron of LURE (Laboratoire pour l'utilisation du rayonnement électromagnétique, Orsay, France). On the D22 bench (λ = 1.5498 Å), the XRD data were recorded simultaneously at small and wide angles with two position-sensitive gas linear detectors (1024 and 512 channels), which were placed about 1 770 mm and 300 mm from the sample, respectively (Fig. 4). On the D24 bench (λ = 1.489 Å), a single detector can be placed at 300 mm or 900 mm (q = 0–0.55 $Å^{-1}$). The small-angle detector allows the characterisation of the longitudinal organisation of TG molecules, whereas the wide-angle detector permits the identification of the chain packing (Fig. 2). The channels of the detectors were calibrated to express the XRD data in scattering vector q ($q = 4\pi \sin\theta/\lambda$; q in $Å^{-1}$, θ in degrees is the angle of incidence of the X-ray relative to the crystalline plane, λ in Å is the X-ray wavelength) [5]. The crystalline 2L β form of high-purity tristearin was used as a reference for the wide-angle channel to scattering vector q calibration of the detector (4.59, 3.85, 3.70 ± 0.01 Å) [22]. Silver behenate, which is characterised by a long spacing of 58.380 ± 0.001 Å, was used to calibrate the small-angle detector [3]. The calorimeter coupled to XRD was calibrated with lauric acid.

XRD data and DSC measurements were synchronously collected versus time by a single microcomputer. A Visual Basic (Microsoft, Redmond, USA) program was specially written to ensure simultaneous display of the small-angle XRD, wide-angle XRD and DSC results during their collection (Fig. 4A).

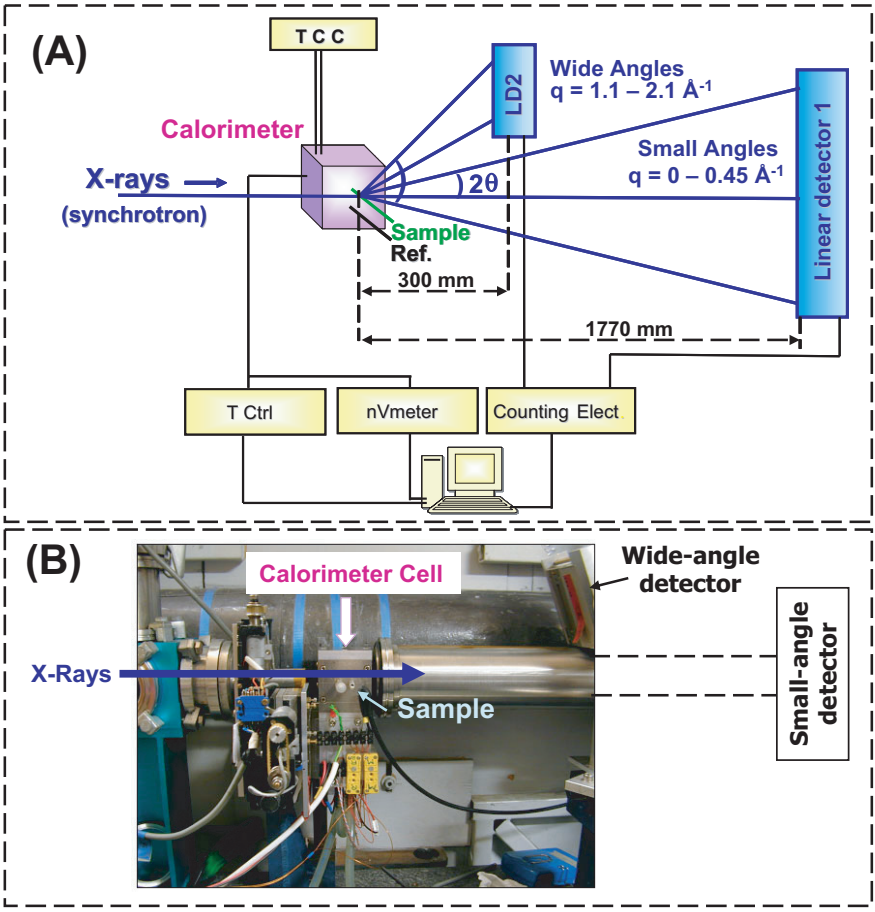

Figure 4. Experimental set-up of the microcalorimeter in the time-resolved synchrotron X-ray diffraction environment. **(A)** Schematic representation: the cell is positioned with the capillary containing the sample perpendicular to the beam in such a way that the diffraction patterns are recorded in the vertical plane by one or two one-dimensional proportional detectors (LD) at small and wide angles. Counting electronic (Counting Elect.), nanovoltmeter (nVmeter) and temperature controller (T Ctrl) are monitored by a single computer. The temperature-controlled cryostat (TCC) is kept at constant temperature (e.g. 6 °C). **(B)** Set-up on the D22 bench of the synchrotron (LURE, Orsay, France).

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. These capillaries were specially designed for X-ray studies since they allow minimum attenuation of the beam and parasitic scattering. Furthermore, they are characterised by poor thermal conductivity and thus guarantee minimum thermal losses. Samples were prepared by loading the capillaries with about 20 µL of cream or melted AMF using a syringe and a thin Teflon capillary.

The samples in the capillary were heated to 60 °C for 5 min to ensure that all crystals and nuclei were melted. Then, the samples were cooled with cooling rates in the range 0.15 °C·min^{-1} ⩽ $R_{cooling}$ ⩽ 1000 °C·min^{-1}. The most rapid $R_{cooling}$ were obtained by rapid introduction of the capillary into the calorimeter pre-cooled to the temperature, e.g. −8 °C or 4 °C. Tempering in isothermal conditions was also performed, e.g. at −8 °C, 4 °C and 20 °C. Then, XRD patterns were recorded as a function of time and upon subsequent cooling or heating (in general at 2 °C·min^{-1}). The samples of concentrated fat globules were not cooled at T < −8 °C to avoid ice formation.

Each XRD pattern recorded as a function of time (crystallisation and melting experiments) was analysed using Peakfit software (Jandel scientific, Erkrath, Germany). XRD peaks were fitted by the Gaussian-Lorentzian (sum) equation, to determine the position of the maximum, maximal intensity and area under each XRD peak [11].

2.2.2. DSC experiments

Thermal analyses were conducted by DSC, using a DSC-7 calorimeter (Perkin Elmer, St Quentin en Yvelines, France) equipped with an Intracooler II and running under Pyris software and a TA Q 1000 calorimeter (TA Instruments, New Castle, DE). About 5 to 10 mg of cream were weighed in a hermetic aluminium pan of 50 μL. An empty pan was used as reference.

2.2.3. Fat globule size measurements

The fat globule size distributions were measured by laser light scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, UK). From the size distribution, the average volume-weighted diameter, $d_{43} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$ (where n_i is the number of fat globules in a size class of diameter d_i), was calculated by the instrument software.

3. RESULTS AND DISCUSSION

The organisation of TG molecules in the solid state (e.g. in fat crystals) was investigated in milk fat globules, by varying the cooling rates. The crystallisation properties of TG in milk fat globules were studied upon slow cooling (0.15 °C·min^{-1}), at intermediate cooling rates (0.5 < $R_c$ooling ≤ 3 °C·min^{-1}) and after quenching (~1000 °C·min^{-1}), to characterise the most unstable crystalline structures and their reorganisation as a function of time.

The examination of TG polymorphism in milk fat globules is especially difficult since (i) both small- and wide-angle XRD should be considered at the same time and compared to determine the evolution of each of the species as a function of time; (ii) the X-rays diffracted by each of the crystalline structures are reduced to the proportion of the fraction considered; (iii) the whole XRD signal is largely absorbed by the surrounding water and its solutes (e.g. casein micelles); and (iv) the peak broadening results from the crystallisation constraints in dispersed systems.

3.1. Crystallisation in milk fat globules during slow cooling: $R_{cooling}$ < 0.5 °C·min^{-1}

The crystals formed in milk fat globules upon slow cooling (0.15 °C·min^{-1}) and their melting behaviour (2 °C·min^{-1}) were considered. For the first time to the authors' knowledge, the XRD experiments performed at small angles showed that

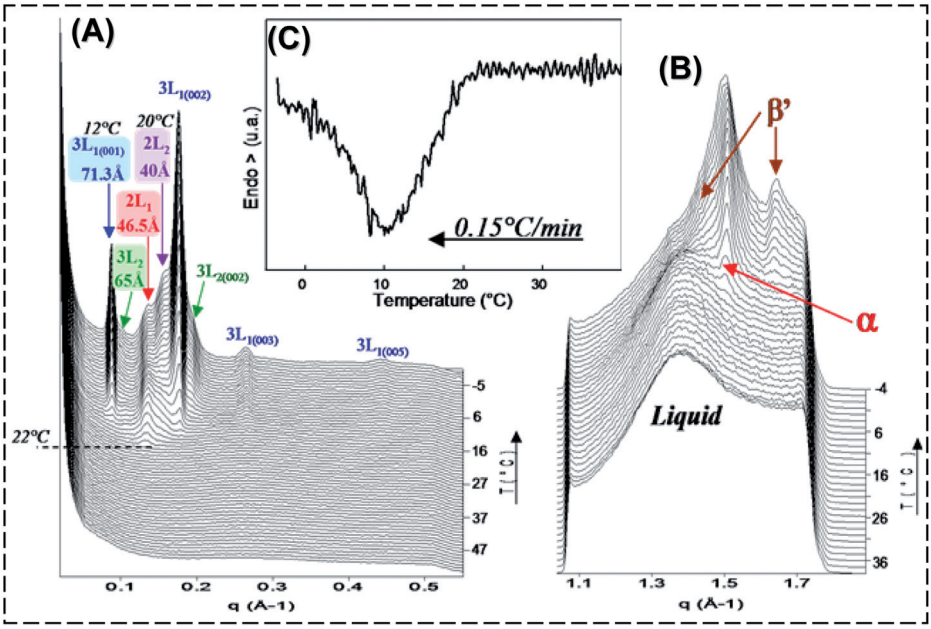

Figure 5. Structural evolution, expressed in scattering vector q ($Å^{-1}$), of triacylglycerols dispersed in milk fat globules during cooling from 60 °C to −8 °C at 0.15 °C·min^{-1}. Three-dimensional plots of the XRD patterns recorded (**A**) at small angles and (**B**) at wide angles. (**C**) Differential scanning calorimetry curve recorded simultaneously.

crystallisation in fat globules occurs successively, producing four types of crystals [11]. The lamellae formed upon slow cooling correspond to two 2L structures (46.5 and 40 Å) and to two 3L structures (71.3 and 65 Å) (Fig. 5A). Nucleation occurs in the α form, then the α + β′ polymorphic forms coexist until the end of the cooling (Fig. 5B). The DSC crystallisation curve recorded simultaneously shows a single exothermic peak (Fig. 5C). The 4 types of crystals start to form within a 10 °C range, from about 22 °C, preventing separation of overlapped peaks by DSC recording. Before the formation of crystals in milk fat globules, characterised by diffraction peaks, both small- and wide-angle XRD patterns show scattering peaks, respectively, centred at 0.28 $Å^{-1}$ (22.44 Å) and 1.38 $Å^{-1}$ (4.55 Å), corresponding to the X-ray signature of TG in their liquid state, as described by Larsson [7].

A quantitative analysis of the XRD data was performed to determine the relative proportion of the 5 different phases, 4 solids and 1 liquid, formed in milk fat globules upon slow cooling and their evolution as a function of temperature. It is generally accepted that the sum of integrals of the whole diffraction peaks of a phase is roughly proportional to its abundance in a material [5]. The total diffracted intensity is not affected by the effect of crystal perfection, crystal size or the polymorphic form, since X-ray scattering is only proportional to the number of atoms present in the organised system. Thus, we determined the areas for each XRD peak recorded at small angles during cooling, using Peakfit software (Fig. 6 left). The relative abundance

of each of the phases was obtained by summing the areas of each of the small-angle XRD peaks corresponding to the same crystalline structure (e.g. the 5 areas of peaks corresponding to the 5 orders of the $3L_1$ structure). Figure 6 right shows the evolution of the relative proportions of the 5 phases as a function of temperature during cooling of milk fat globules at 0.15 °C·min^{-1}. The successive occurrences and growths of the 4 different solid phases at the expense of the liquid or of one of the phases, newly formed but metastable, are clearly evidenced.

After cooling, the samples were heated from −8 °C to 60 °C at 2 °C·min^{-1} to study the melting behaviour of TG in milk fat globules (Fig. 7). Figure 7A shows the XRD patterns recorded at small and wide (insert) angles during heating. The determination of the evolution of maximal intensity of each small-angle XRD peak as a function of temperature (Fig. 6B) permits one to relate the structural and the thermal events (Fig. 6C). The decrease in intensity of the XRD peaks recorded at small angles corresponds to the melting of the corresponding crystalline structures. From about 12 °C, a new 2L β' (40 Å) structure formed during the heating, corresponding to the increase in intensity (Fig. 7B) of the peak observed at small angles (Fig. 7A). At wide angles, the XRD patterns show α → β' → liquid transitions (Fig. 7A). This recrystallisation of TG during heating with a polymorphic transition of monotropic type (α → β') showed that fat crystals formed during slow cooling of milk fat globules were still metastable. The DSC melting curve showed 3 endotherms that correspond to the melting of the crystalline structures, as indicated in Figure 7C. TG dispersed in milk fat globules were in their liquid state for T > 37 °C.

The crystallisation behaviour of AMF in similar conditions was completely different [13], showing the influence of the dispersion state.

3.2. Crystallisation in milk fat globules for cooling rates $0.5 \leqslant R_{cooling} \leqslant 3$ °C·min^{-1}

At intermediate cooling rates, the first crystalline structures formed in milk fat globules are 2L structures ($2L_1$ = 47 Å and $2L_2$ = 42 Å). Then, the successive formation of 5 sharp XRD peaks corresponds to the crystallisation of TG molecules in a 3L (71.2 Å) structure (results not shown in this paper; see [13]). The 3 lamellar structures that are successively formed upon cooling have a hexagonal packing of the fatty acid chains (α form). At −8 °C, the reversible formation of a pseudo-orthorhombic perpendicular packing of the acylglycerol chains, called sub-α, was observed. This α → sub-α transition that occurs at low temperature has also been observed in AMF but is favoured in the dispersed state [16].

The melting behaviour was studied upon heating at 2 °C·min^{-1}. Figure 8A shows the 3D plot of the small- and wide-(insert) angle XRD patterns. Numerous structural rearrangements were observed before final melting of TG molecules in milk fat globules, showing that cooling at intermediate rates leads to the formation of unstable crystalline varieties. Roughly, the melting of the α 3L (71.2 Å) structure is first observed and some TG transform into a new 3L (65 Å) structure that melts rapidly, then the melting of the α 2L (47 and 42 Å) structures is observed. In the 16 °C ⩽ T ⩽ 24 °C domain, XRD patterns show a transition and the formation of a new diffraction line, corresponding to a β' 2L (39 Å) structure, before final melting of TG for T > 40 °C. This β' 2L (39 Å) structure that crystallises during heating is probably formed by the reorganisation of the TG initially incorporated in the other lamellar structures formed during the cooling process. At wide angles, we observed the following transitions upon heating: sub-α ↔ α → β′ → liquid (Fig. 8A,

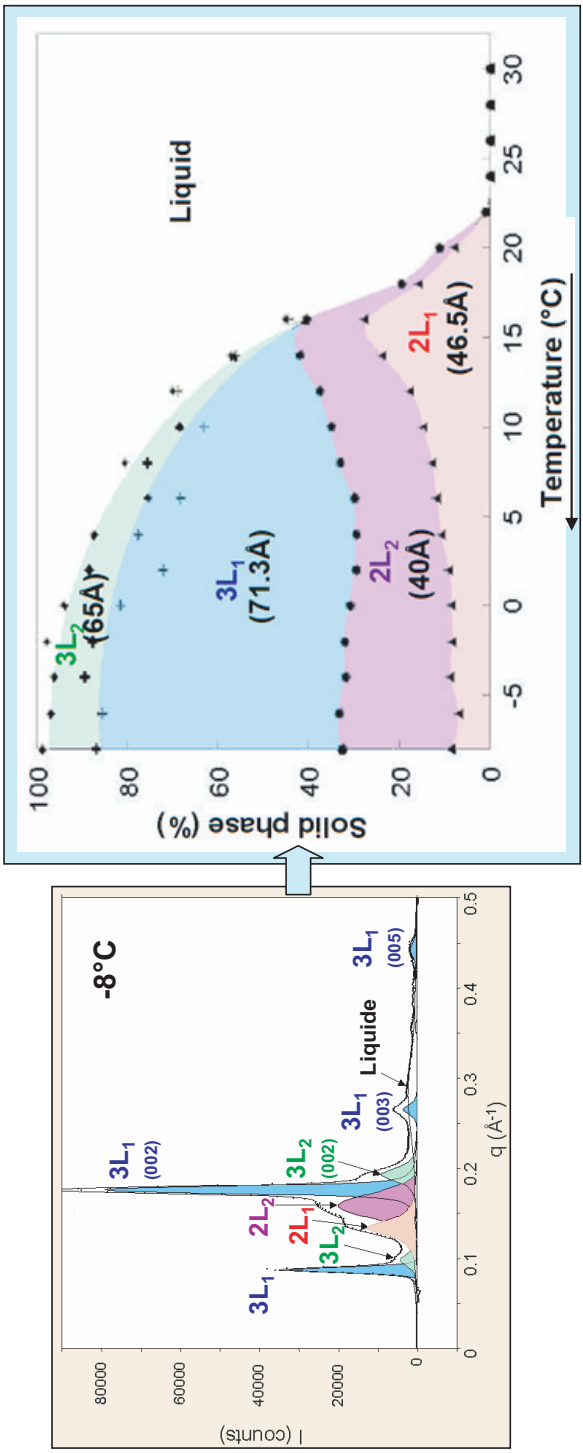

Figure 6. Analysis of X-ray diffraction (XRD) pattern allowing the determination of the relative proportion of the crystalline structures as a function of temperature. Left: example of Peakfit analysis performed on a small-angle XRD pattern recorded at −8 °C at the end of cooling of milk fat globules from 60 °C to −8 °C at 0.15 °C·min^{-1} (Fig. 5). The area under the peaks corresponding to the same lamellar structure have the same colour. Right: decomposition of the small-angle XRD patterns into the four solid phases and the liquid one, as evaluated from peak areas using Peakfit analysis. Phase contents are expressed as a percentage of the total fat.

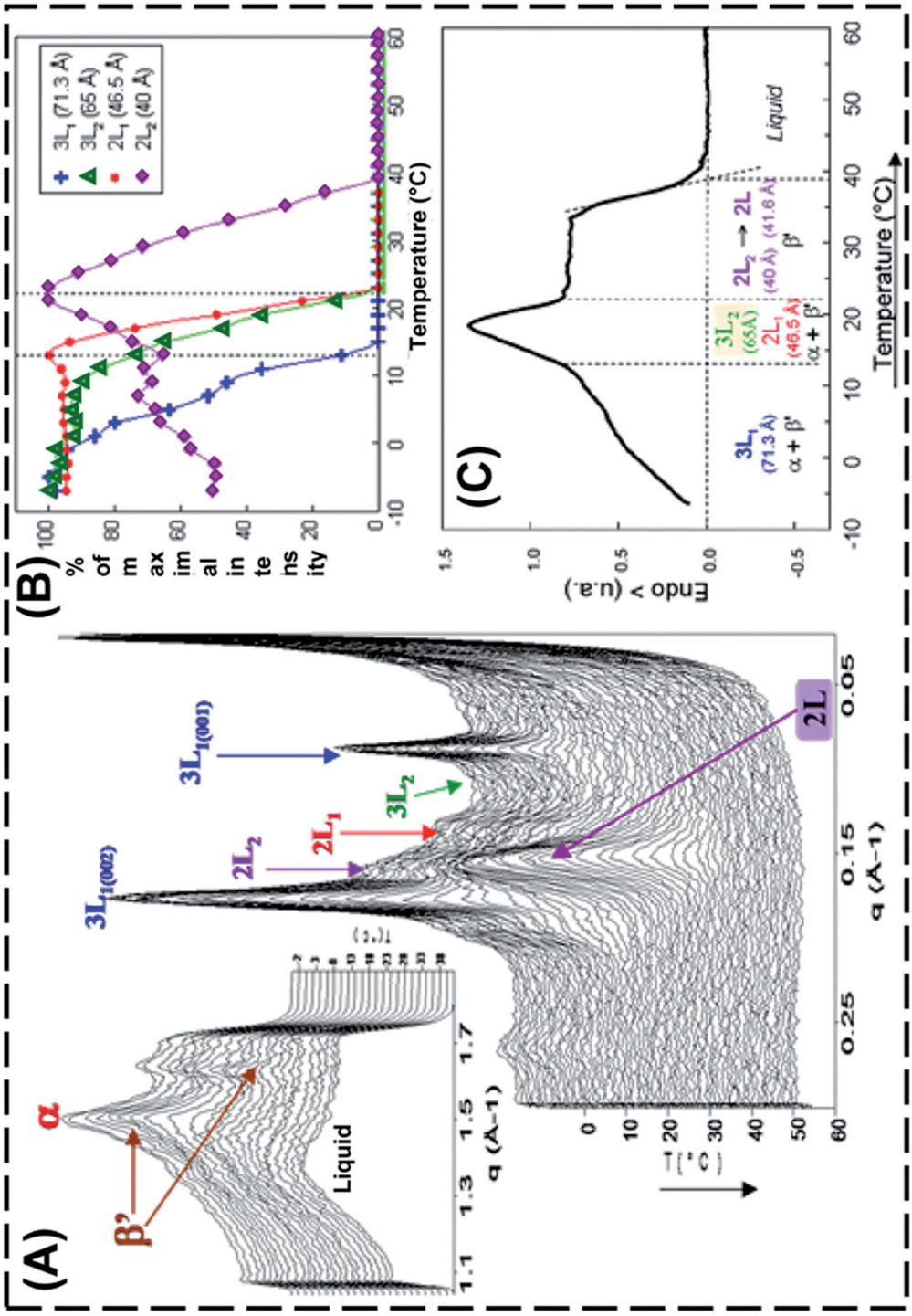

Figure 7. Melting behaviour of triacylglycerols in milk fat globules upon heating at 2 °C·min^{-1} after slow cooling at 0.15 °C·min^{-1}. (**A**) Three-dimensional plots obtained from the X-ray diffraction patterns recorded at small and wide (insert) angles as a function of temperature. (**B**) Evolution of the intensities of the small-angle X-ray diffraction peaks, recorded during the heating (the intensities have been expressed as a percentage of the peak maximum). (**C**) Melting curve recorded simultaneously by differential scanning calorimetry.

insert). The evolution of maximal intensities of each XRD peak recorded at both small and wide angles during heating of cream allowed us to delimit the domains of existence of the crystals and to relate the thermal events recorded by DSC to the structural information recorded by XRD (Fig. 8B) [14].

3.3. Crystals formed after quenching: $R_{cooling} \sim 1000\ °C \cdot min^{-1}$

The most unstable crystalline structures formed by TG molecules in natural milk fat globules and in AMF were investigated after quenching from 60 °C to −8 °C [10,12]. We identified the formation of 2 lamellar structures from the liquid: 3L (70.4 Å) and a transient 2L (47 Å) with a lateral organisation of the fatty acid chains corresponding to the most unstable polymorph, i.e. the α form (hexagonal subcell) (Fig. 9). The acquisition of XRD patterns as a function of time after quenching permitted the observation that the α 2L (47 Å) structure was very unstable since it disappeared during a 20-min isothermal recording.

The crystallographic properties of milk fat globules were also investigated after quenching from 60 °C to 4 °C, which is the temperature of storage of dairy products, and followed as a function of time in isothermal conditions at 4 °C [15]. After such a rapid cooling, the main crystalline structure formed corresponded to an α 3L (70.5 Å). As for quenching at lower temperatures (e.g. −8 °C), the α 2L (47 Å) was also characterised, but it disappeared more rapidly after quenching at 4 °C in comparison with −8 °C.

The rapid cooling of milk fat from 60 °C to low temperatures, e.g. −8 °C or 4 °C, induces a brusque liquid → solid phase transition. The amount of the liquid phase at the temperature of quenching can favour the structural rearrangement of TG molecules. Phase transitions occurred as a function of time, in isothermal conditions, with changes in both the polymorphic forms and the longitudinal organisation of TG molecules. These polymorphic transitions, e.g. $\alpha \rightarrow \beta' \rightarrow \beta$, were related to successive increases in the density of fat globules during storage at 4 °C [15].

Furthermore, we observed that the phase transitions were delayed in fat globules in comparison with AMF [15].

3.4. Crystals formed after tempering in isothermal conditions

The crystals formed in milk fat globules after tempering at different temperatures corresponding to the storage and consumption of dairy products were characterised.

The structures formed after storage for $t > 5$ days at 4 °C were identified (Fig. 10). They correspond to 2 lamellar organisations: mainly a 2L (40.5 Å) and a 3L (54.2 Å) structure (Fig. 10A). The lateral packing of the fatty acid chains corresponds to the coexistence of 4 crystalline packings: one α form, two β′ forms and traces of the β form (Fig. 10B). Similar structures were characterised in milk fat globules and AMF [15]. The absence of evolution of the thickness of the lamellae upon subsequent heating and the absence of recrystallisation were in favour of the polymorphic stability of the crystals formed after this tempering period (results not shown in this paper, see [15]). The endothermic peaks recorded by DSC upon heating were related to the successive melting of the crystalline structures formed during the tempering period: the 3L structure, then the 2L structure [15].

This work also evidenced the formation of crystals during storage of milk fat globules (creams) for $t > 20$ h at room temperature (e.g. T ~ 20 °C): 2L (40.5–41.1 Å) lamellar structure [8]. The melting of this 2L structure corresponds to a broad endothermic peak recorded by DSC [18]. Upon subsequent slow cooling of the fat

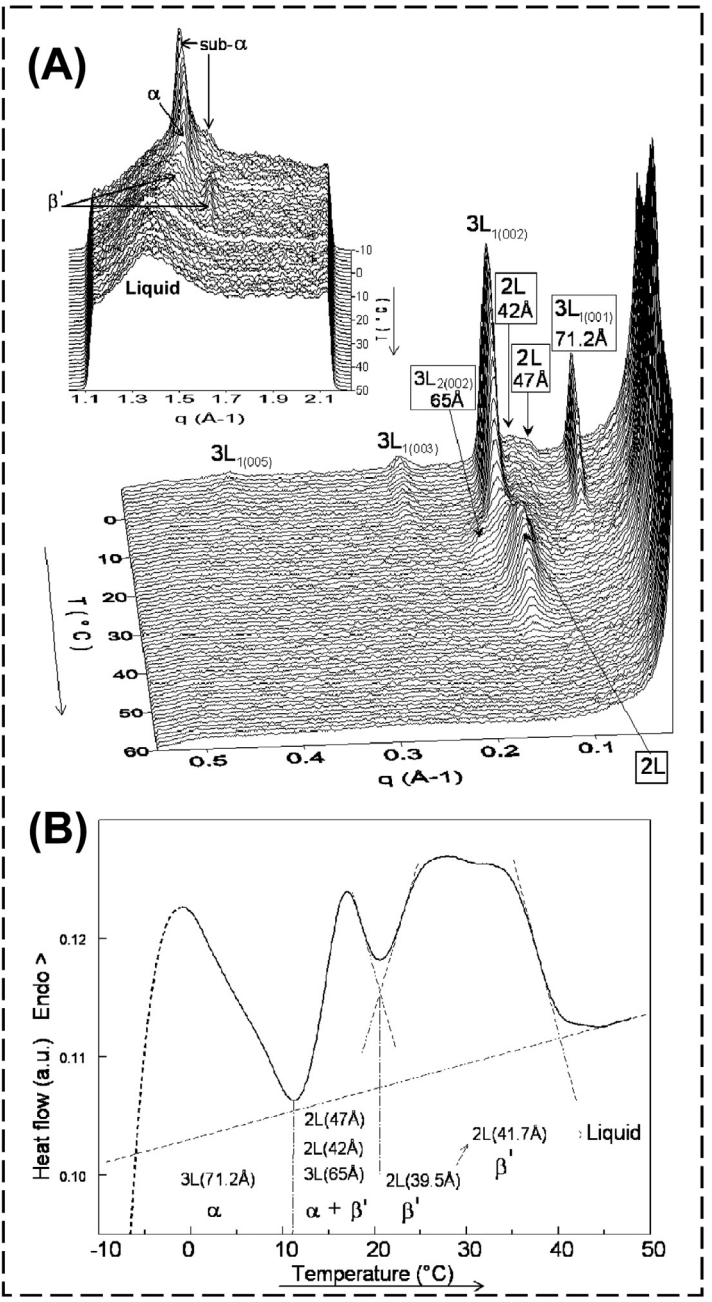

Figure 8. Structural evolution, expressed in scattering vector q ($Å^{-1}$), of triacylglycerols dispersed in milk fat globules during heating at 2 °C·min^{-1} after cooling at 1 °C·min^{-1}. (**A**) Three-dimensional plots of the XRD patterns recorded at small and wide (insert) angles. (**B**) Differential scanning calorimetry curve recorded simultaneously.

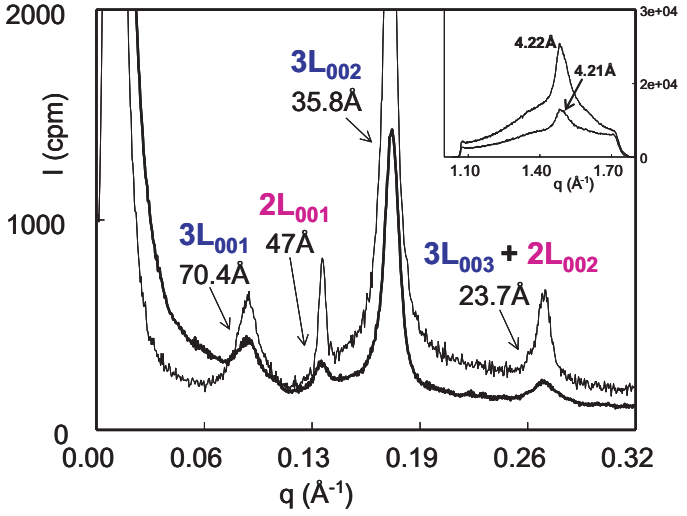

Figure 9. Small- and wide- (insert) angle X-ray diffraction patterns recorded at −8 °C after quenching of cream (thick line) and anhydrous milk fat (thin line) from 60 °C.

globules tempered at 20 °C, the amount of crystals in this 2L form increases and two additional types of 3L structures are formed ($3L_1$ type: 68.5–70.7 Å; $3L_2$ type: 61.2–64.2 Å), the characteristics of which depend on the size of the milk fat globules [20].

The crystalline structures formed after a tempering period, mainly the 2L (40–41 Å) structure, correspond to stable polymorphic species and are characterised by a higher final melting point [18].

3.5. Influence of milk fat globule size on their crystallisation properties

The thermal and crystallographic properties of creams were analysed as a function of temperature and time with the whole droplet size distribution of natural milk fat globules [10,11,14,15]. In this part of the work, we focused on the influence of fat globule size on TG crystallisation and polymorphism.

Natural milk fat globules (i.e. covered by the MFGM) of smaller size (~1–3 μm) and larger size (~5–7 μm) were selected from the same initial milk (fat globules ~4 μm) by using a novel microfiltration process [21]. Figure 11A shows that the DSC crystallisation curves recorded for creams containing natural milk fat globules with different sizes correspond to a single exothermic peak and that the initial temperature of crystallisation (T_{onset}) is delayed for smaller fat globules. Figure 11B shows the decrease in T_{onset} as a function of the decrease in natural milk fat globule size [20]. Lopez et al. [14] prepared emulsions by homogenising milk fat with β-lactoglobulin and using different pressures to obtain significantly different average droplet sizes. The latter authors showed that reducing the size of the emulsion droplets induces a higher supercooling and that the T_{onset} is displaced toward lower temperatures. The delay in crystallisation observed for natural milk fat globules and emulsion droplets was independent of the composition of the milk fat and

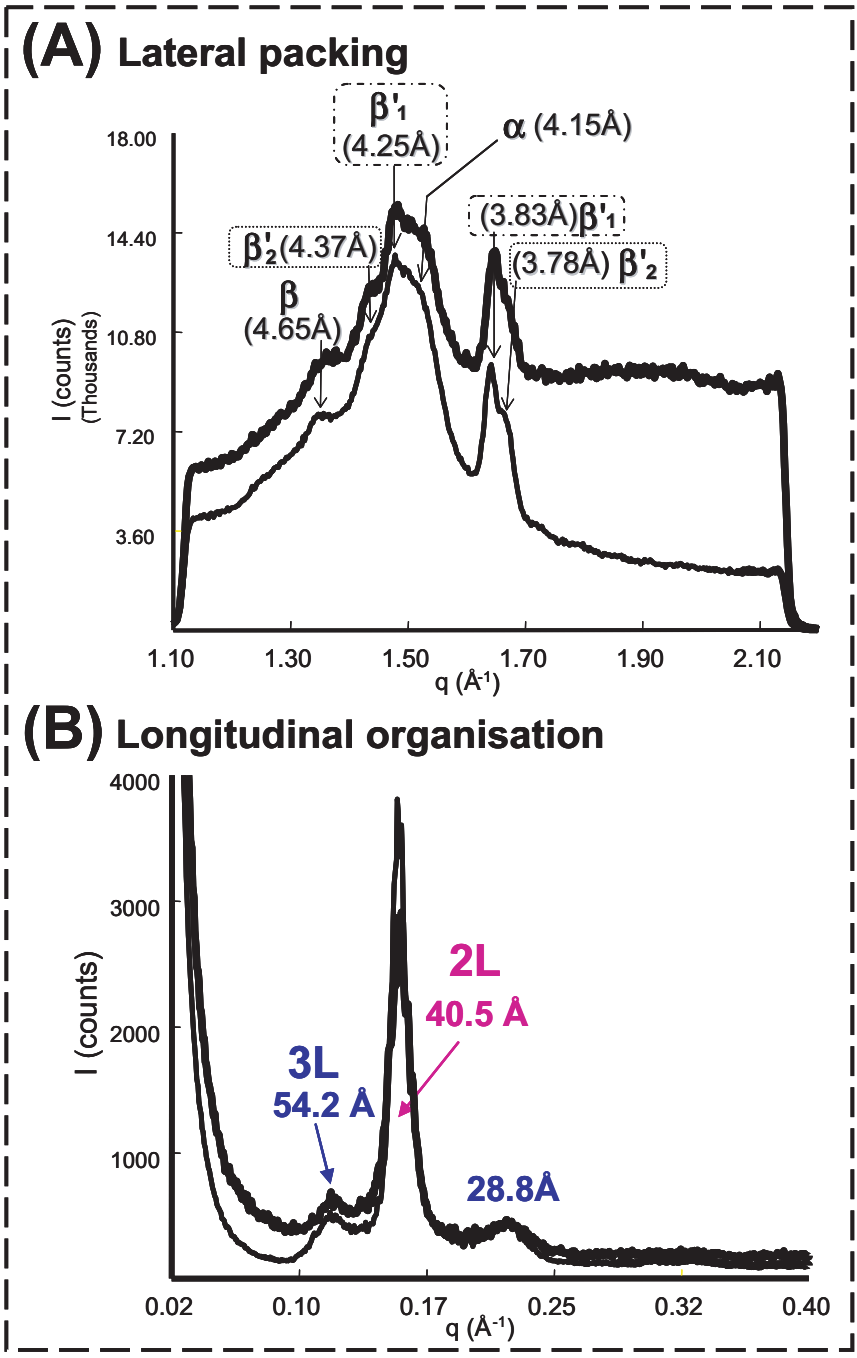

Figure 10. X-ray diffraction patterns recorded at wide angles (**A**) and small angles (**B**) after storage of cream (thick line) and anhydrous milk fat (thin line) for 5 days at 4 °C.

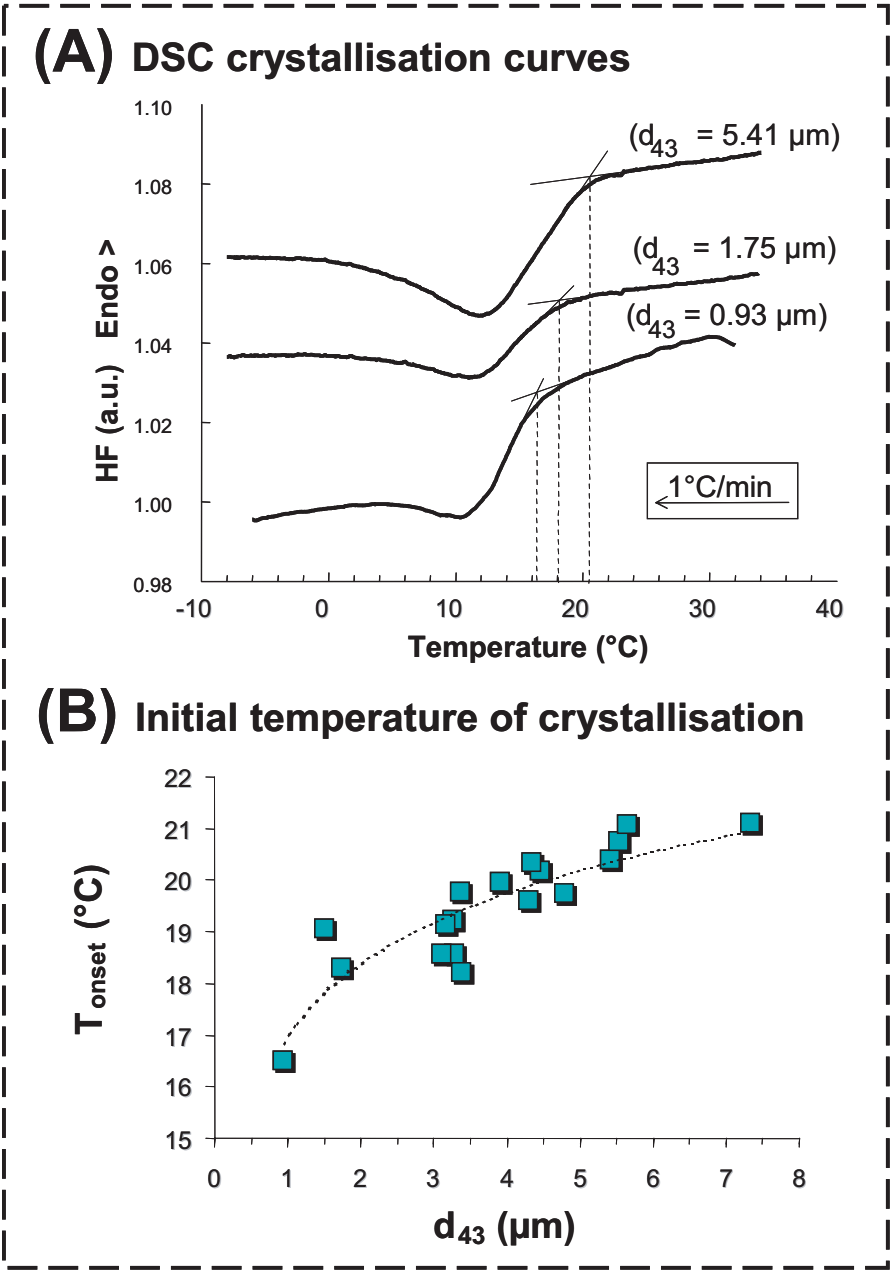

Figure 11. Crystallisation properties of natural milk fat globules as a function of their size. **(A)** Differential scanning calorimetry (DSC) curves recorded upon cooling of milk fat globules at 1 °C·min^{-1}. The size of small fat globules is indicated in the figure. **(B)** Evolution of the initial temperature of crystallisation as a function of natural milk fat globule diameter (d_{43}) calculated from the DSC crystallisation curves.

the surface of droplets (MFGM vs. milk proteins) and was related to the size of dispersed fat particles. This delay in crystallisation can be explained by the theory of heterogeneous nucleation in dispersed systems, e.g. initiated by catalytic impurities. In emulsions such as creams, at least one nucleus must be formed in each droplet to achieve full crystallisation, and the time needed to obtain the nucleus is inversely proportional to globule volume. Consequently, a lower temperature due to a higher supercooling is needed to induce crystallisation in a dispersed system with smaller fat globules since more catalytic impurities are required [29].

Lopez et al. [14] showed that whatever the size of fat globules, crystallisation mainly occurs in the α 3L (72 Å) upon cooling at 1 °C·min^{-1}. However, the decrease in the fat globule size induces (i) the formation of less 2L structures and (ii) a decrease in small-angle XRD peak maximum intensity correlated with an increase in peak width. This was interpreted as resulting from defaults in the organisation of TG molecules in the crystals, either directly due to the curvature of the surface from which crystals are supposed to grow, or indirectly to the faster relaxation that can also induce the formation of crystals of smaller size.

This work showed that there are no major differences in the type of crystalline structures formed by TG, after eliminating thermal history. However, the level of organisation of TG in the crystals and/or the size of the crystals can differ as a function of the size of the fat globules. As a summary, the smaller the fat globule size, the larger the supercooling, the faster the crystals' growth and the larger the crystal disorganisation inside the globules.

Differences in the crystallisation properties were observed after tempering milk fat globules of different sizes at 20 °C and subsequent cooling at 0.5 °C·min^{-1} to −8 °C (Fig. 12). The smaller fat globules contained much more 3L structures and less 2L structures than larger ones [20]. Moreover, the crystals formed in the larger fat globules (d_{43} = 7.15 μm) melted upon heating but did not evolve from a polymorphic point of view, whereas the crystals formed in the smaller fat globules (d_{43} = 0.93 μm) were metastable and reorganised to form more stable species before final melting of TG. These differences could have interesting applications in the manufacture of dairy products in which tempering periods are involved in the technological process (e.g. butter, whipped cream, ice cream).

3.6. Compound crystals are formed in milk fat globules

The studies performed on milk fat globule crystallisation showed that it occurs successively, producing 1, 2, 3 or 4 different varieties depending on the cooling rate and that only small-angle XRD experiments permit the characterisation of these different types of crystals [10, 11, 14, 15]. We showed that upon cooling the first longitudinal organisations of TG molecules dispersed in fat globules correspond to 2L lamellar structures with long spacings of 40–42 and 46–48 Å. These organisations may correspond to crystallisation of high-melting point TG, with saturated and similar chain length fatty acids (e.g. PPS, PPP, MPP; P: palmitic acid, S: stearic acid, M: myristic acid). Then, crystallisation of 3L structures occurs: $3L_1$ = 70–72 Å and $3L_2$ = 65 Å. They may correspond to crystallisation of TG molecules with unsaturated fatty acids, or to TG with fatty acid chains of different lengths (e.g. BuPP, BuPO, PPO; Bu: butyric acid, O: oleic acid). Recently, Lopez et al. [19] showed that the 2L structures are formed by the stearin fraction, whereas the 3L structures are formed by the olein fraction, after dry

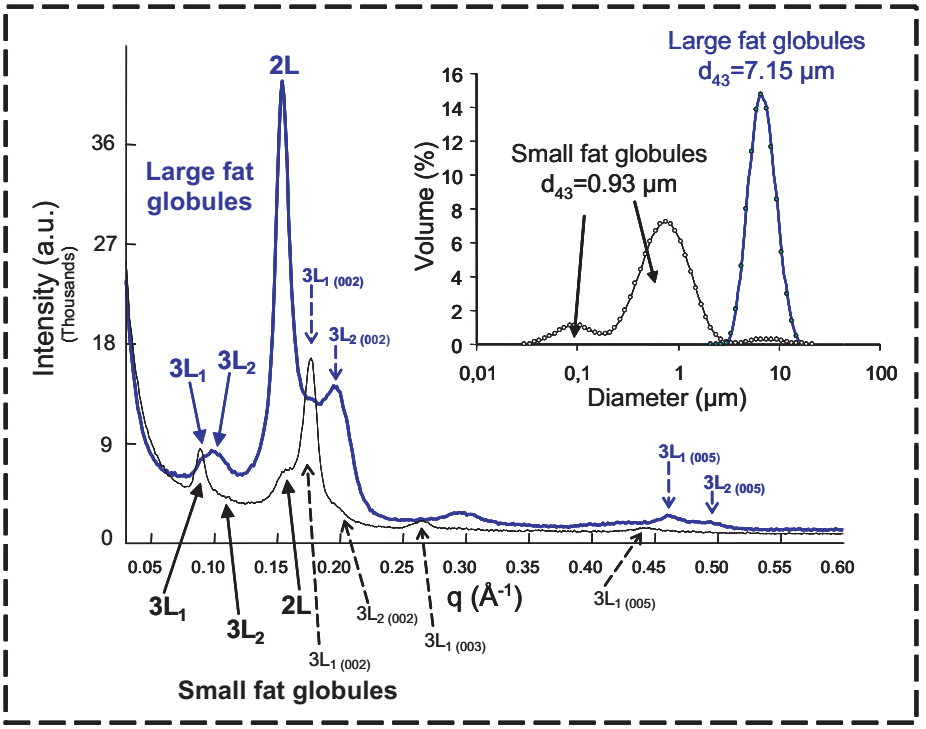

Figure 12. Comparison of the small-angle X-ray diffraction patterns recorded at −8 °C with small and large natural milk fat globules that were initially stabilised at 20 °C for 24 h and subsequently cooled at 0.5 °C·min^{-1} down to −8 °C. Insert shows the size distribution of milk fat globules.

fractionation from the same initial batch of milk fat, performed at 21 °C.

The comparison of the small number of crystal types formed (4 longitudinal organisations and 5 polymorphic forms identified) with the large number of TG molecules present in milk fat (200 TG species were identified) provides evidence that each of the varieties corresponds in fact to compound crystals. The formation of compound crystals, also called mixed crystals as they contain different TG molecular species in the crystal lattice, is not surprising in a natural fat with a wide compositional range of TG molecules, such as milk fat.

4. CONCLUSION

The use of high-flux X-ray sources (synchrotron) permitted the study of the polymorphism and phase transitions displayed as a function of temperature and time by the complex mixture of TG dispersed in milk fat globules. XRD experiments performed at both small and wide angles allowed the identification on a molecular scale of the TG longitudinal stacking and the lateral packing of fatty acid chains in lamellar structures, respectively. Thanks to the resolution obtained with the various set-ups used in these studies, the small-angle XRD analysis performed for the first time on milk fat in the dispersed

state was essential for the identification of the longitudinal organisation of TG molecules in the lamellar structures and their time- and temperature-induced transitions. Earlier studies concerning milk fat polymorphism were based on the description of XRD peaks corresponding to the lateral packing of TG, and permitted only the identification of the crystalline subcells. Furthermore, we showed that different types of crystals (1 to 4) and several polymorphic forms (1 to 5) coexist in milk fat globules at low temperatures, together with a liquid phase. The coupling of XRD with DSC recordings permitted us to relate the structural transitions to the thermal behaviour of milk fat globules. These tools allowed, for the first time to our knowledge, the identification and the characterisation of crystalline structures within water-dispersed and highly complex, natural or processed food products as well as their transitions (several studies on cheese and ice cream are under way). Moreover, the comparison of the crystallisation properties in the dispersed state and in bulk provides information on the influence of the interface on crystallisation and its composition (Lopez et al., in preparation). This work was performed on bovine milk fat globules but similar work is under way for other species: dromedary [17], goat [2], ewe and buffalo.

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