

Physical Changes of β -Sitosterol Crystals in Oily Suspensions During Heating

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Anna von Bonsdorff-Nikander,¹ Satu Lievonon,² Leena Christiansen,¹ Milja Karjalainen,¹ Jukka Rantanen,^{1,3} and Jouko Yliruusi^{1,3}

¹Division of Pharmaceutical Technology, Faculty of Pharmacy, University of Helsinki, Finland

²Division of Food Technology, Department of Food Technology, University of Helsinki, Finland

³Viikki Drug Discovery Technology Center (DDTC), Faculty of Pharmacy, University of Helsinki, Finland

ABSTRACT

The aim of this research was to describe the thermal behavior of β -sitosterol crystals in oil-suspensions with a focus on the role of water during heating. The suspensions were prepared by recrystallization in order to achieve a microcrystalline particle size. The structural changes together with the mechanical properties of the suspensions during heating were studied by using variable temperature X-ray powder diffractometry (VT-XRPD), differential scanning calorimetry (DSC), and dynamic mechanical analysis (DMA). Hydrated β -sitosterol crystals in an oil-suspension dehydrated, despite the composition of the suspensions, at low temperatures. At high β -sitosterol concentration, the monohydrate crystal form changed partially to a hemihydrated form, and when only a small amount of water was initially incorporated, the hemihydrate crystal form dehydrated to a mostly anhydrate crystal form. The released water, which was immiscible in the surrounding oil, caused the recrystallization of hydrated β -sitosterol during cooling. This procedure indicated a reversible dehydration process. Structural and thermal analysis of β -sitosterol crystals in suspensions, together with mechanical analysis made it possible to understand various physical changes during heating.

KEYWORDS: β -sitosterol, suspension, pseudopolymorphs, dehydration.

INTRODUCTION

Phytosterols are naturally occurring components of plants and are used as cholesterol-lowering agents owing to the inhibition of cholesterol absorption in the small intestine. The use of phytosterols has faced several problems because of the unpleasant gritty texture and the poor solubility in oil and water.^{1,2} To avoid the gritty sensation in the mouth a microcrystalline particle size is desirable. This is achieved

by recrystallization, as microcrystalline-sized phytosterols are suspended into dietary fats and oils.^{3,4} This type of system allows incorporation of up to 30% of phytosterol into food products without any chemical reactions or additives. Incorporation of the suspension into medicinal or food products (eg, capsules or dietary spreads) always includes heating, and thus the knowledge of the thermal changes is of importance.

The most common phytosterol, β -sitosterol, has previously been observed to exist in 3 different crystal forms in solid state: anhydrate, hemihydrate, and monohydrate.³ In the presence of water, β -sitosterol recrystallizes as needle-shaped hemihydrate or monohydrate crystals. The monohydrated crystal form is unstable and tends to lose hydrated water even at room temperature.⁵ Christiansen et al³ showed that the dehydration of β -sitosterol monohydrate is a 2-step process. At temperatures below 60°C, approximately half of the water from crystallization left the structure and a hemihydrated form was observed. Further heating, up to 90°C, completed the dehydration process resulting in anhydrate, platy-like, β -sitosterol crystals.

Dehydration that occurs at low temperatures is characteristic of channel hydrate dehydration. The explanation for the ease with which β -sitosterol dehydrates could be the migration of water molecules along the tunnels.³

The method describing how to produce a microcrystalline β -sitosterol suspension in oil has previously been described.^{3,4} The crystallization of β -sitosterol in oil in the presence of water resulted in a formation of microcrystalline, hydrated β -sitosterol crystals. The difference in bioavailability is often related to the different dissolution rate of sparingly soluble drugs. According to the Noyes-Whitney equation, dissolution is directly proportional to the effect of the surface area, and hence the reduction of the particle size may affect the dissolution rate and thus influence drug absorption.

Crystal form and habit of the hydrated crystals were dependent on the water content of the suspension. The physical stability of the suspension has been evaluated. The suspensions were stored for 4 months without any changes to the size and habit.⁶

Corresponding Author: Anna von Bonsdorff-Nikander, Division of Pharmaceutical Technology, Faculty of Pharmacy, PO Box 56 (Biocenter 2), FIN-00014 University of Helsinki. Tel: +358 9 191 59 161; Fax: +358 9 191 59 144. E-mail: anna.vonbonsdorff@helsinki.fi

The present study describes the thermal behavior of β -sitosterol crystals in oil-suspensions. This study also deals with the role of different β -sitosterol concentrations on the suspensions during heating. Additionally, the role of water has been evaluated by using compositions both with and without water being added. In the presence of water, the solubility of a sterol in oil decreases. This is due to the formation of monohydrated β -sitosterol, which is less soluble in oil.^{3,7,8}

MATERIALS AND METHODS

Sample Preparation

β -sitosterol, containing 75.5% β -sitosterol (13.0% β -sitostanol) was purchased from Calbiochem, Biosciences Inc (La Jolla, CA) and medium-chain triglycerides (MCT)-oil from SHS International Ltd (Liverpool, UK). The MCT-oil is a mixture of triglycerides containing mainly caprylic and capric acids.

The suspensions were prepared by heating β -sitosterol and MCT-oil in a vessel while stirring. β -sitosterol was dissolved at $\sim 100^\circ\text{C}$ and a clear solution was formed.^{3,4} After cooling the solution to 90°C , purified, ion-exchanged water of the same temperature was added. The vessel, in which the suspension was made, was immediately immersed in ice and the suspension was stirred until it had reached room temperature ($+25^\circ\text{C}$).

Four different compositions were made; all of these were prepared in triplicate. The suspensions contained either 5%, 17%, or 30% β -sitosterol. One of the suspensions was prepared without water and the other contained 13% water. The remaining part of the composition was MCT-oil. After preparation, the samples were kept at room temperature for half an hour. The samples were then stored in airtight plastic containers at $+4^\circ\text{C}$. The stability of the suspensions was studied earlier.⁶ In that study the suspensions were stored over a period of 4 months, at $+4^\circ\text{C}$, with no significant changes in size or habit of β -sitosterol crystals.

Variable Temperature X-ray Powder Diffractometry

The crystal structure of the β -sitosterol suspensions were analyzed on the same day as the preparation, by a variable temperature X-ray powder diffractometry (VT-XRPD). All the measurements were made with a theta-theta diffractometer (Bruker AXS, D8 Advance, Karlsruhe, Germany) in a symmetrical reflection mode with Cu K_α radiation (1.54 \AA) using Göbel mirror bent gradient multilayer optics. The scattered intensities were measured with a scintillation counter. The angular range was from 3° to 30° using a step size of 0.05° and the measuring time was 1 s/step. Each

sample was measured at several temperatures between 25°C and 60°C at ambient humidity.

Estimation of an Average Crystallite Size

The average crystallite size (t) in the suspensions was estimated by using the Scherrer formula from the width of the highest reflections⁹:

$$t = 0.9 / (B \cos \theta),$$

where λ is the wavelength of the radiation and θ is half of the scattering angle. B was calculated using the formula

$$B = (b_n^2 - b_l^2)^{1/2},$$

where b_n is the full width at half maximum (FWHM) of the diffraction reflection of the sample and b_l is the instrumental broadening (0.03°), which is estimated as an FWHM of a Si reflection of a standard sample. By using the Scherrer equation, other sources of diffraction broadening such as changes in lattice strain were neglected. A change from one pseudopolymorphic form to another as well as a change in lattice strain makes it difficult to estimate how much the latter contributes to the diffraction broadening. Additionally, the lattice strain tends to cause an underestimation of the actual crystallite size.¹⁰

Differential Scanning Calorimetry Measurements

A differential scanning calorimeter (DSC) (Mettler DSC30 with TC15 TA processor, Mettler-Toledo AG, Greifensee, Switzerland) was used to determine the behavior of the suspension with different sterol concentrations during heating. The analyses were made by STAR^e Thermal Analysis System, Version 3.1 (Mettler-Toledo-AG). The DSC was calibrated using the melting temperature of n-pentane, n-hexane, mercury, ion exchanged water, gallium, and indium. In addition, the melting point of indium was checked once during the determinations.

Each sample was first heated from 25°C to 60°C , at a heating rate of 5°C min^{-1} . To get more information about the reversibility of the dehydration process, these determinations were followed by DSC measurements in which the samples were heated up to 60°C , 5°C min^{-1} , then cooled down to 25°C and again heated up to 60°C , 5°C min^{-1} . The samples (10-30 mg) were hermetically sealed in $40 \mu\text{L}$ aluminum pans. An empty aluminum pan was used as a reference sample.

Dynamic Mechanical Analysis

Dynamic-mechanical analyzer (DMA 242, Netzsch-Gerätebau GmbH, Selb, Germany) was used to observe the storage

modulus, loss modulus, and $\tan \delta$ as a function of temperature. The DMA was equipped with a DMA 242 measuring unit, a cooling gas controller, a DMA 242, and TASC 414/3 controller, and Netzsch DMA 242 software Version 1.5. The instrument was used with a disk-bending sample holder, which has been previously described more precisely by Laaksonen and Roos.¹¹ The sample holder consisted of 3 stainless steel layers with circular holes of 30 and 26 mm in diameter. The layers were separated by 2 0.05 mm thin PET plastic films and the sample was located between these 2 films. During the analysis, sinusoidally varying stress was applied to the material observed as a function of temperature. The samples were analyzed, the following day of preparation, at frequencies of 0.1, 0.5, 1, 2.5, and 5 Hz, and the amplitude was 7.5 μm . The samples were heated at a heating rate of 2°C min^{-1} from 30°C to 60°C . These analyses were used to obtain the storage modulus, G' , which refers to the ability of a material to store energy and is related to the elasticity of the material.¹² Low G' values indicate liquid-like materials, when the ability to store energy is practically lost, whereas high G' values indicate elastic materials.

Hot Stage Microscopy

Thermal events were observed on a hot stage (Mettler Toledo FP 82H Hot Stage with a Mettler Toledo FP90 Central Processor) under light microscope (HSM) (Leica DMLB, Leica Mikroskopie und System GmbH, Wetzlar, Germany). A small amount of the suspension was placed on a glass slide, covered with a coverslip and heated at 5°C/min in the temperature range of 25°C to 70°C (magnification $\times 20$). The thermal observations were made at ambient humidity and temperature.

Karl Fischer Analysis

As one suspension was prepared with no added water, the amount of the water in the starting material had to be taken into account when analyzing the results. The water content of the β -sitosterol powder was determined as received by a Karl Fischer titrimetry (Mettler DL35, Mettler-Toledo). Hydranal-Titrant 2 (Sigma, St Louis, MO) was used as the titer and Hydranal Solvent CM (Sigma) as the solvent.

All the measurements were performed in at least duplicate except for the HSM analysis, which was performed only once. The analyzing methods that were used were proven to be reliable and repeatable.

RESULTS AND DISCUSSION

Visual Characterization of β -Sitosterol Crystals During Heating

Due to the added water, the crystals were initially needle-like, which is typical for hydrated β -sitosterol crystals. The

suspension that was prepared with no added water contained both needle-shaped and platy particles. In a previous study, the median β -sitosterol crystal length in suspensions prepared in similar manner (5% water) was measured.⁶ The median crystal length in suspensions decreased from 40 μm to 19 μm as the sterol concentration was increased from 5% to 17% due to higher degree of saturation during crystallization.

Thermal changes in the suspension containing 17% (wt/wt) β -sitosterol are presented visually in Figure 1. As in the other suspensions containing water, the major changes were seen in the temperature range of 50°C to 70°C . At this point the small crystals dissolved and the larger crystals grew at the expense of the smaller ones (Ostwald ripening). The somewhat darker color of the crystals at 50°C , and the clearly darker color of the larger crystals at 60°C and 70°C , is often related to dehydration.¹³ The same kind of darkening of the crystals was not observed in the suspension without added water. Despite the dehydration, the crystal habit remained unchanged during heating. Thus the morphology of the crystals has not been taken into consideration when discussing the factors affecting mechanical properties. The HSM also showed that despite some crystal growth during heating no crystal aggregation was observed. The somewhat high viscosity of the suspensions prevents phase separation and aggregation.^{3,6}

Crystal Structure of β -Sitosterol in Oily Suspension

The XRPD patterns of all the suspensions included reflections of crystalline β -sitosterol and an amorphous background from the oil (Figure 2). The X-ray patterns at 25°C initially showed that the suspensions that included added water (13%, wt/wt), consisted mainly of monohydrated crystal form (Figure 2B to 2 days). Karl Fischer determinations showed that β -sitosterol starting material contained 0.7% water. Additionally, as the suspensions were prepared at an ambient humidity some of the moisture could have been absorbed during the preparation. Hence the suspension with no added water (17% β -sitosterol) caused a diffraction pattern, at 25°C , corresponding to a hydrated β -sitosterol crystal structure (Figure 2A). During heating, approximately between 40°C and 45°C , the crystals dehydrated and the initially mainly hemihydrated crystal structure changed to an anhydrate crystal form. The dehydration was best observed as the reflection at 18.8 \AA ($4.7^\circ 2\theta$), which corresponds to the hemihydrate form, switched to a reflection at 17.6 \AA ($5.0^\circ 2\theta$). This reflection is in turn typical of an anhydrate β -sitosterol crystal form.³ The reflections at 11.7, 8.80, 5.87, and 5.23 \AA (7.5° , 10.0° , 15.1° , and $16.9^\circ 2\theta$) have also been identified as characteristic reflections for anhydrate form of β -sitosterol.

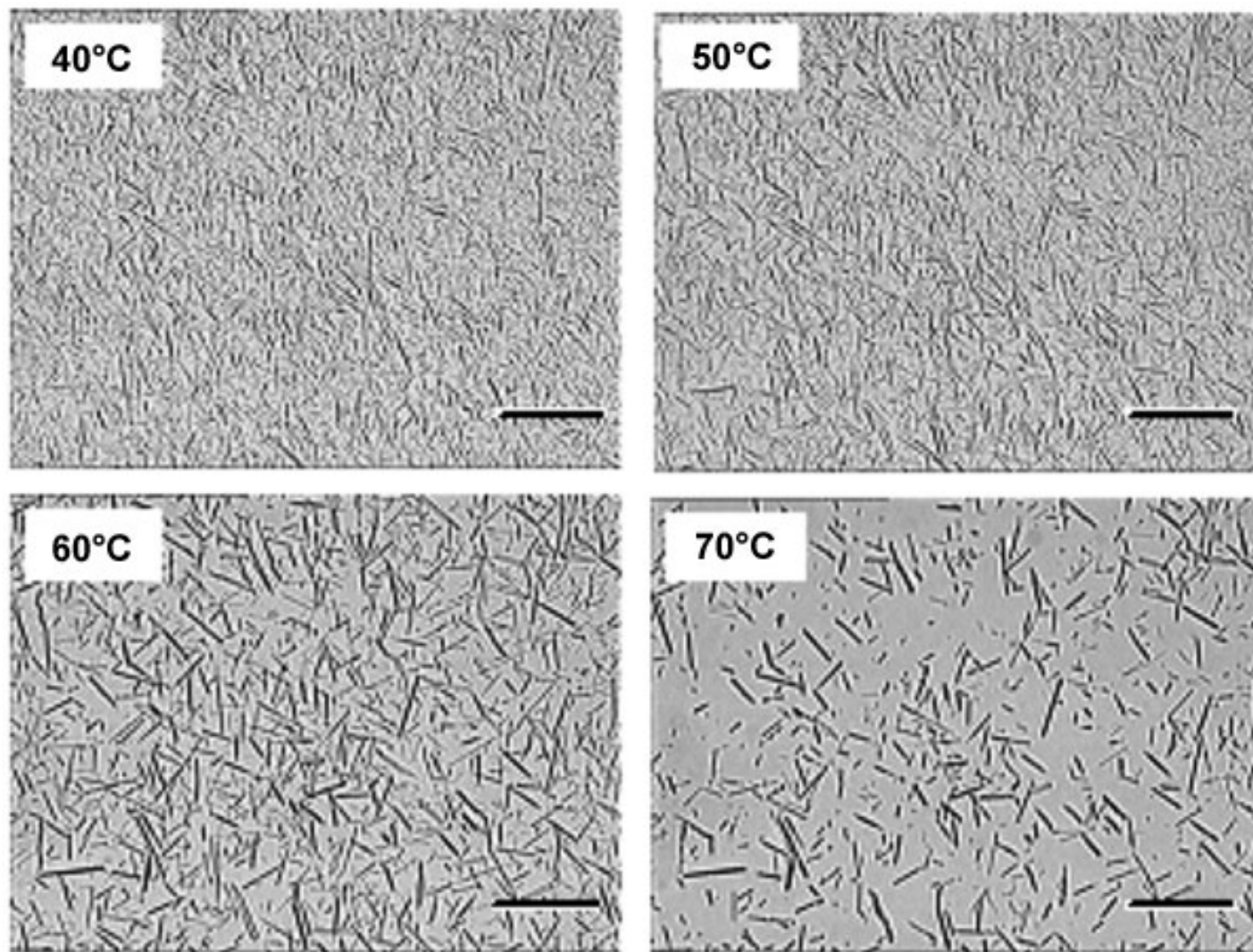


Figure 1. Hot stage microscopy image, taken at different temperatures, of β -sitosterol suspension containing 17% β -sitosterol, 13% water, and 70% MCT-oil. A small amount of the suspension was heated at 5°C/min in the temperature range of 25°C to 100°C (magnification $\times 20$). Bar represents 100 μm .

The suspension containing only 5% β -sitosterol produced an almost amorphous diffraction pattern (from the oil, see Figure 2E), due to the relatively small amount of crystalline β -sitosterol. At room temperature, the solubility of β -sitosterol in MCT-oil, when water is present, is 1.5% to 2.0% (wt/wt).³ Depending on the drug and the excipients the detection limit for x-ray measurements can be as high as 10%.⁶ The XRPD patterns presenting changes in crystal structure during heating remained very amorphous, except for a few detectable reflections between $\sim 15^\circ$ and 20° (2θ), corresponding a Bragg distance between 6 and 4 Å, during the entire heating process.

As expected, with increasing amount of β -sitosterol, the intensities of the reflections increased. The initial (at 25°C) x-ray pattern of the suspensions containing either 17% or 30% β -sitosterol (Figure 2C and 2 days) corresponded mainly to the monohydrated β -sitosterol crystal form. Yet,

the results indicated that when β -sitosterol concentration was 17%, the dissolution of smaller crystals and the growth of larger crystals, during heating, might have affected the preferred orientation of the crystals. This can be seen as a growth of some reflection intensities during heating. When the β -sitosterol concentration in the suspension increases, the crystal size distribution becomes smaller and more homogeneous due to a higher rate of supersaturation during recrystallization.^{3,6} The more equal crystal size distribution reduces the effects of Ostwald ripening and when the β -sitosterol concentration was as high as 30%, the change from one pseudopolymorphic form to another was observed. With increasing temperature the 3 distinguishable reflections between 17° and 20° changed to a group of more irregular reflections, typical for the hemihydrated crystal form. The double reflection at 4.7° and 5.0° however indicated that both hemihydrated and monohydrated forms were still present at 60°C. The presence of some overlapping

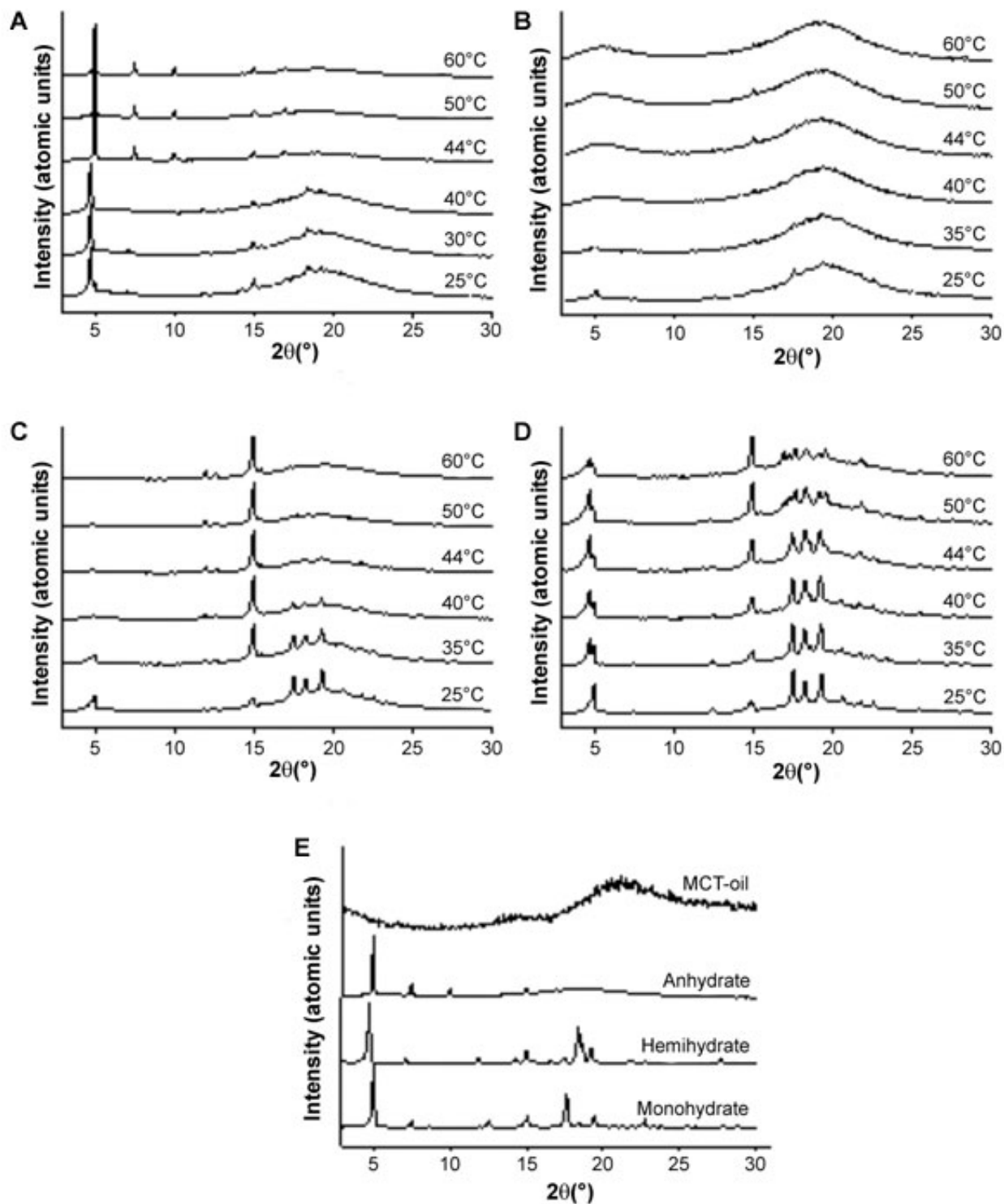


Figure 2. X-ray diffraction patterns of β -sitosterol suspensions containing different amount of sterol (A) 17:0:83 (β -sitosterol: no added water: MCT-oil), (B) 05:13:82 (β -sitosterol: water: MCT-oil), (C) 17:13:70, and (D) 30:13:57. VT-XRPD patterns were obtained at selected temperatures. The X-ray patterns of solid anhydrate, hemihydrate, and monohydrate sterol crystal forms (E) have been presented earlier by Christiansen et al.³

reflections in hemi- and monohydrated forms (monohydrate: 5.90, 5.03, 4.81, and 4.56 Å corresponding to 15.0°, 17.6°, 18.4°, and 19.4° 2θ ; hemihydrate: 5.90, 5.05, 4.81, and 4.61 Å corresponding to 15.0°, 17.5°, 18.4°, and 19.2° 2θ)

makes it more difficult to observe the differences between these 2 crystal structures.¹⁴ In this study, the crystals have a needle-like morphology, which are mostly prone to preferred orientation. Preferred orientation can cause changes

in the intensity of the observed reflections.^{9,15} The occurrence of the preferred orientation is also likely in the case of anhydrous crystals, because of a platy-like habit. As one of the goals for this study was the identification of the different crystal structures, our predominant interest was the position of the x-ray reflections. The difference in the sample environment between DSC and XRPD might be responsible for the minor difference at what temperature the dehydration started¹⁴ (see section 3.4 and Figure 3). The DSC measurements were made in sealed pans, while the x-ray measurements were made in an open sample holder. In addition, the sample volume was notably higher in the VT-XRPD measurements.

Average Crystallite Size

The Scherrer equation can be used to estimate the average crystallite size of the samples. These estimations made with Equations (1) and (2) are shown as a function of temperature in Figure 4. In suspensions containing 17% and 30% β -sitosterol and in the suspension with no added water all the clearly detected XRPD reflections have been used in the estimation of the average crystallite size. The only reflection that could have been utilized in the estimation of the amorphous XRPD-pattern of the suspension containing 5% β -sitosterol is the reflection at 15°. Due to the lack of accuracy, this suspension has been left out of the results.

Every solid particle includes a variable amount of crystallites with different shapes, depending (eg, on the crystallization method).¹⁶ A small crystallite size refers to crystal defects with (eg, wrongly situated atoms, or atom vacancies).

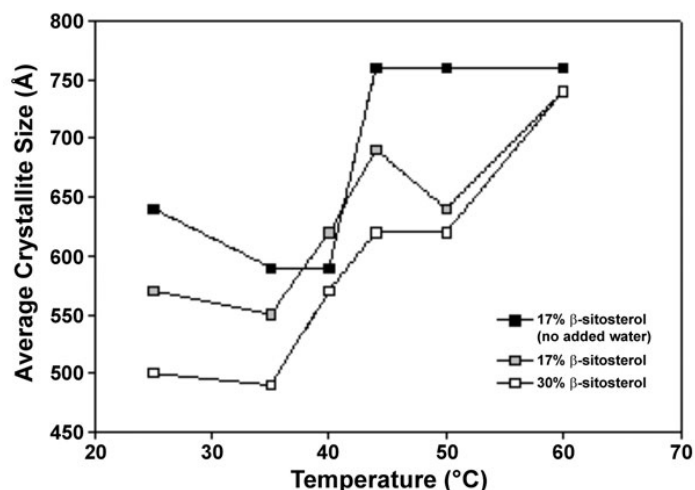


Figure 3. DSC thermograms of (A) β -sitosterol suspensions containing different amounts of sterol (13% water, the rest MCT-oil), one sample with no added water. The samples were heated from 25°C to 60°C. (B) The suspension containing 17% β -sitosterol (17:13:70) was heated from 25°C to 60°C then cooled and reheated to 60°C.

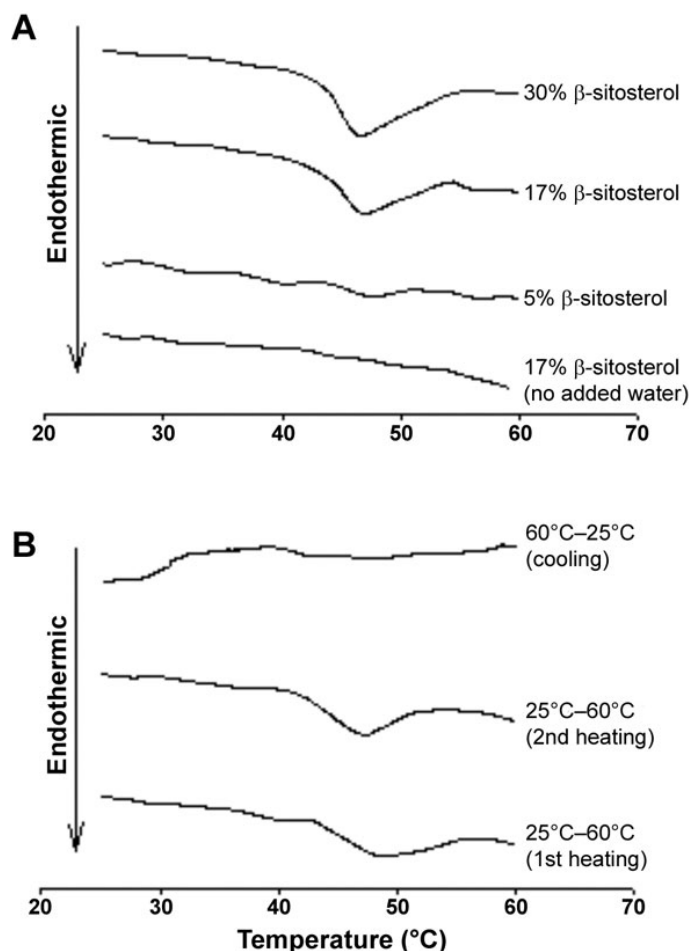


Figure 4. The average crystallite size in the suspension as a function of temperature. The suspensions contained either 17% or 30% of β -sitosterol (13% water, the rest MCT-oil), one suspension with no added water.

The results show that the average crystallite size is dependent on the concentration of β -sitosterol and is decreasing with increasing concentration. This can be explained by increased supersaturation during crystallization, which increases the nucleation at the expense of crystal growth and faster crystallization causing crystal defects. The increase in the average crystallite size as a function of temperature (at temperatures above 35°C) indicated that during heating dissolution of smaller crystallites and probably also recrystallization into more perfect and larger crystallites occurred. In addition, in the presence of water, when the solubility of β -sitosterol in oil decreases, the recrystallization process is rapid, resulting in more crystal defects.

Thermal Behavior of β -Sitosterol Crystals

The DSC thermograms of the oil-suspensions containing either 17% or 30% β -sitosterol and 13% water showed a broad endotherm between 40°C and 60°C. The β -sitosterol monohydrate structure appeared to be loosely associated

with the water of crystallization and the dehydration of β -sitosterol monohydrate to a partly hemihydrated form occurring below 60°C in accordance to previous studies.^{3,8} The diffusion of water molecules lying next to each other along the channels is the explanation for the ease with which the monohydrate β -sitosterol dehydrates into hemihydrate crystal form.^{3,17} In this study, the DSC results strengthen this assumption as the broad endotherm or distinguishable endotherm is typical for water molecules that are weakly bonded to the other molecule.¹⁸

There were no detectable thermal changes in the thermograms of the suspensions containing 5% β -sitosterol (13% water) between 25°C and 60°C due to the rapid dissolution of the crystals already at low temperatures (Figure 3A).

DSC is a particularly useful technique in the study of hydrates with dehydration steps at low temperatures.¹⁹⁻²¹ Yet, the sensitivity of the current DSC was not high enough to detect an observable dehydration endotherm when measuring the suspension with no added water. However, in this case, based on X-ray diffraction results (see *Crystal Structure of β -Sitosterol in Oily Suspension*) a loss of water occurred and the formation of predominantly anhydrate crystals took place.

In order to acquire more information about the reversibility of the dehydration process, the heating of the suspension was followed by cooling, after which the suspension was reheated (Figure 3B). Both heating curves showed broad endotherms from ~40°C to 55°C. The DSC results indicated that the dehydration process was reversible. The released water molecules were available for β -sitosterol crystals owing to the immiscibility of water with the oil phase. During cooling, the recrystallization of hydrated β -sitosterol crystals occurred. The slight difference in the onset temperature between the first and the second heating might be due to the different orientation of the crystals. Recrystallization to either monohydrate or hemihydrate β -sitosterol crystals on the other hand causes the dissimilarity between the endothermic area in the first and second heating. The DSC measurements were performed in hermetically sealed pans and thus the measuring environment was isolated and the water did not evaporate during heating.

Mechanical Properties of the Suspensions

In the present study, DMA indicated changes in the elasticity during heating (Figure 5). An increase in elasticity was observed with an increase of the solid material. The suspension with no added water was more liquid-like than the other suspensions as the solubility of the sterol in oil was increased.^{7,8} Similar results were obtained earlier when the viscosity of the suspensions was measured with a cone and plate rheometer.³

The elasticity of all the suspensions started to decrease as the temperature rose just above 30°C and continued to decrease as the temperature approached 40°C. At this stage the decrease of the storage modulus (G') was observed. This was due to the dehydration process which led to a less elastic suspension. The released water stayed as a separate phase acting as a lubricant between the solid particles and the oil, making the suspension more liquid-like. The dehydration process was more evident with the growing β -sitosterol concentration. At temperatures below 40°C some dissolution of the smallest β -sitosterol crystals might also have caused a decrease in G' . The appearance of the diminished intensities in the reflections, below 40°C, in the VT-XRPD patterns may also indicate dissolved β -sitosterol crystals (Figure 2A-D).

The subsequent increase of G' , as the temperature climbed beyond 40°C, can be explained by the occurrence of recrystallization. At that point the suspension became more elastic. At higher temperatures, the suspensions became once again more liquid-like, since even the larger crystals started to dissolve (see also Figure 1).

An exception to this occurrence was the suspension containing 5% β -sitosterol. Due to its initially low viscosity and high water content, compared with the β -sitosterol concentration, the sample started to move substantially between the sample holders at higher temperatures. This problem

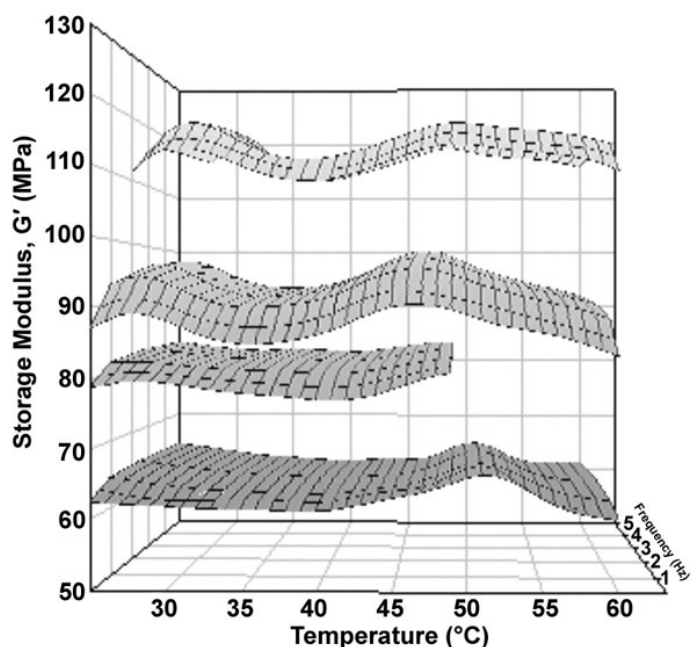


Figure 5. The storage modulus (G') of β -sitosterol suspensions containing different amount of sterol: (A) 17:0:83 (β -sitosterol: no added water: MCT-oil), (B) 05:13:82 (β -sitosterol: water: MCT-oil), (C) 17:13:70, and (D) 30:13:57. The measurement of suspension containing 5% β -sitosterol was technically impossible beyond 50°C.

has been previously noted by Meyvis et al,²² as a thin water layer between the suspension and the sample holder acting as a lubricant. This led to an unreliable result, which is the reason why the results are shown only up to 50°C. By DSC it was impossible to notice any thermal changes during heating when studying the thermal behavior of the suspensions containing 5% β -sitosterol (Figure 3B) or the suspension with no added water (Figure 3A). Due to the sensitivity of the DMA technique, it was possible to see the different phases even in these suspensions during heating.

CONCLUSIONS

In an oil-suspension, hydrated β -sitosterol crystals dehydrated at low temperatures despite the composition of the suspensions. The ease with which the β -sitosterol dehydrated might be explained by diffusion of water molecules along channel hydrates. Yet, the results suggested that hydrated β -sitosterol was recrystallized during cooling, indicating a reversible dehydration process. Structural and thermal analysis of β -sitosterol crystals in oil-suspensions, together with mechanical analysis made it possible to understand various physical changes during heating.

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