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ORIGINAL PAPER

Effect of High Intensity Ultrasound on Crystallization Behavior of Anhydrous Milk Fat

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Abstract The need to eliminate *trans*-fatty acids from foods' formulation resulted in the exploration of new lipid sources and alternative processing conditions that will improve the physicochemical characteristics and nutritional qualities of lipid-based foods. In general, the physicochemical characteristics of lipid networks depend on the microstructure and crystallization behavior of the system. The objective of this work was to use high intensity ultrasound (HIU) as an additional processing condition to alter the crystallization behavior of a lipid model system (anhydrous milk fat). Results show that HIU application not only decreases the induction time of crystallization (faster crystallization) at a constant crystallization temperature, but also generates smaller crystals. In addition, higher viscosities are obtained when samples are crystallized after HIU application. The degree of supercooling, ultrasound application settings and a combination of both parameters influence the degree of ultrasound effect on the crystallization behavior.

Keywords High intensity ultrasound · Crystallization · Microstructure · Milk fat · Polarized light microscopy · Induction times

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Introduction

Eating habits have changed dramatically during the last decade. Consumers have become more aware of the quality of the foods that they consume. In particular, foods low in *trans*- and palmitic fatty acids have been in great demand. As a consequence, food companies face the challenge to reformulate their products with healthier lipids while maintaining their quality. When using lipids low in palmitic and *trans*-fatty acids in foods' formulations, softer materials are usually obtained. To obtain the right functionality in a lipid network, both the chemical composition and the processing conditions need be changed [1–3]. However, sometimes these are not sufficient to obtain the desired physicochemical characteristics and functionality that will meet consumers' expectations.

High intensity ultrasound (HIU) can be used as an additional processing tool to modify the crystallization behavior of different systems (sonocrystallization) and therefore obtain the desired physicochemical characteristics in the food. Ultrasonics is a discipline that studies sound waves at frequencies above those within the hearing range of the average person (frequencies above 16 kHz). Ultrasonic propagation is based on stress waves that are transmitted from one molecule to the other by direct and intimate contact between them. Ultrasonic applications can be divided in two categories: low and high intensity applications. Low-intensity applications are those wherein the primary purpose is transmitting the energy through a medium. These waves are used to obtain characteristics of the medium without disrupting it [4, 5]. Typical low-intensity applications are non-destructive testing of materials, medical diagnosis and livestock judging [6]. On the other hand, high-intensity applications are used when the purpose is to produce an effect on a system, or its contents

[7]. A substantial amount of information regarding the use of sonocrystallization can be found in the literature. Pharmaceutical [8–12], chemical [13–21] and food applications [22, 23] are the most common. Specifically for food science applications, Dr. Povey's group has extensively studied the effect of HIU in sucrose solutions. They showed that HIU can modify the primary and secondary nucleation of ice [24, 25]. In addition, Sato et al. [26] showed that HIU can promote the formation of a stable polymorphic form in lipid systems. They studied the effect of HIU on polymorphic behavior of tripalmitin and coca butter. The same group studied the different polymorphic forms obtained when trilaurin, tricaprin, trimyristin were crystallized under the influence of HIU [27, 28]. In addition to these scientific publications, several patents have been issued on the use of HIU in lipid fractionation [29] and chocolate manufacture. Special attention has been paid to the capability of HIU to promote a stable polymorphism [30, 31] in cocoa butter for confectionery applications. The generation of crystalline materials for pharmaceutical uses has also been addressed in a patent by McCausland [32].

Even though there is a vast amount of information that demonstrates the effect of HIU on the crystallization behavior of different molecules, most of the research was either related to aqueous systems or have been focused on confectionery applications. No research has been done to demonstrate the effect of HIU on the crystallization behavior and microstructure of bulk lipids.

The objective of this work was to evaluate the effect of HIU on the crystallization behavior and microstructure of anhydrous milk fat (AMF) with the ultimate goal of using HIU as a novel processing condition to improve the quality of foods.

Materials and Methods

Starting Materials

AMF was used as a model lipid to perform the crystallization experiments due to its low content of palmitic and *trans*-fatty acids. AMF was a donation from KRAFT.

Crystallization Experiments

AMF was crystallized at a fast cooling rate (10 °C/min) at different crystallization temperatures (T_c = 22, 24, 26, 28 and 30 °C). Samples were heated to 80 °C to allow complete melting of the triacylglycerides and placed in a double walled thermostatized crystallization cell (Fig. 1a). To increase the heat transfer between the external circulating water and the AMF, samples were stirred using a magnetic stirrer (200 rpm). Sample temperature was monitored as a function of time (Fig. 1b). When the sample reached T_c, the agitation was stopped and HIU was applied. The sample was kept at T_c for 90 min and the crystallization progress was followed using a polarized light microscope (PLM).

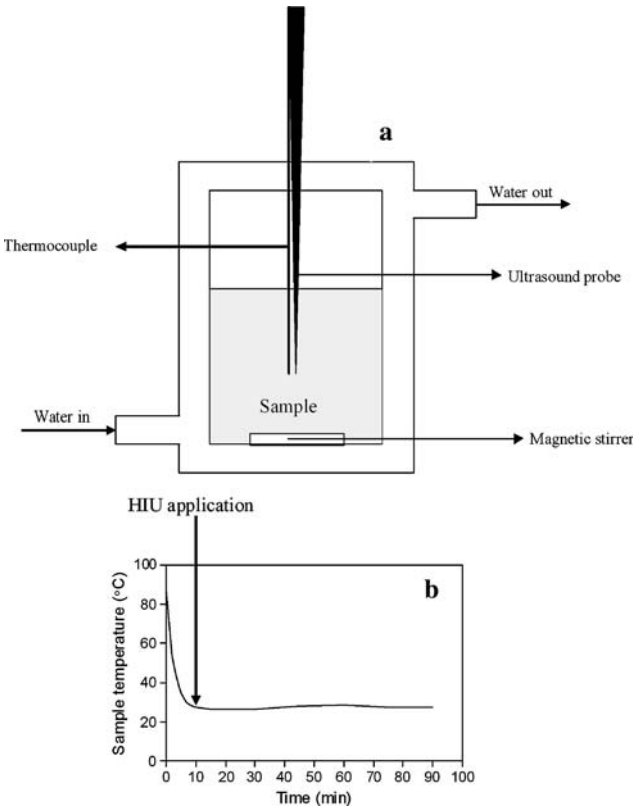

Fig. 1 Crystallization cell used to evaluate the effect of HIU on the crystallization behavior and morphology of AMF (**a**). Thermal history of the sample and HIU application (**b**)

HIU Application

HIU was applied as soon as the sample reached T_c (Fig. 1b). Preliminary experiments performed in this laboratory at 26 and 28 °C showed that 50 W of power applied for 10 s significantly induced the crystallization in AMF with the generation of smaller crystals. Therefore, the application of 50 W of power for 10 s was used as the starting point for the experiments detailed in this manuscript. HIU was applied using a Misonix S-3000 sonicator (Misonix Inc., NY) using an acoustic frequency of 20 kHz. Ten and five second pulses were applied using different acoustic power (50, 30, 20 and 5 W). The progress of AMF crystallization was followed using a PLM.

PLM Measurements

When the first crystals were detected by the naked eye of an experienced observer a drop of the lipid was placed in a

thermostatized slide and cover-slide and observed in a (PLM, Olympus CX 31) provided with a digital camera. Induction times were determined as the time at which the first crystals were observed with the naked eye and confirmed by microscopy. A 20× magnification was used. Samples from the crystallizing material were taken as a function of time every 5 min until no further crystallization was observed, usually 90 min.

Image Analysis

The morphology of AMF crystals was quantified using Image J software (Image J 1.38×, http://www.rsb.info.nih.gov/ij/). The area fraction occupied by the crystals shown in the micrographs was used as the quantification tool.

Viscosity Measurements

After samples were kept at T_c for 90 min, viscosity was measured using a Brookfield Viscometer (Model DV-II+) with spindle H7 at 0.5 rpm for samples crystallized at 22, 24 and 26°C and spindle LV2 at 100 rpm for samples crystallized at 28 and 30 °C. During viscosity measurement, samples were kept in the double walled crystallization cell to avoid temperature fluctuation.

Melting Point Determination

Melting point of AMF was determined as described by AOCS Official Method Cc 1-25. The melting point calculated for AMF was 32.4 ± 0.6 °C.

Statistical Analysis

Significant differences ($P < 0.05$) were evaluated using a two-way ANOVA with Bonferroni's post test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA, http://www.graphpad.com.

Results and Discussion

As described in the Materials and Methods section, samples were crystallized without and with the application of HIU at different crystallization temperatures. The induction times of crystallization, defined as the time interval between the moment the sample is placed in the crystallization cell and the moment the first crystal appears, was determined by the operator and corroborated by PLM (Fig. 2). An increase in the induction time of crystallization was observed as a function T_c when samples were crystallized without the application of HIU. That is, the higher the crystallization temperature the longer the induction time. This is an expected result, since the higher the temperature the lower the driving force (supercooling) for crystallization to occur. When HIU was applied to the samples, a decrease in the induction of crystallization was observed for 24, 26, 28 and 30 °C; being this decrease more significant at 28 °C ($P < 0.001$). On the other hand, an increase in the induction time was observed for AMF crystallized at 22 °C with the application of HIU compared to the same sample crystallized at the same T_c but without the application of HIU. These facts suggest that the effect of HIU on the induction time of crystallization of AMF samples is dependent on the sample supercooling. In particular, HIU did not affect the crystallization behavior of AMF when extreme supercooling were used [high supercooling (22 °C) and low supercooling (30 °C)]. However, HIU affected the onset of crystallization of AMF when crystallized at intermediate supercoolings (24, 26 and 28 °C).

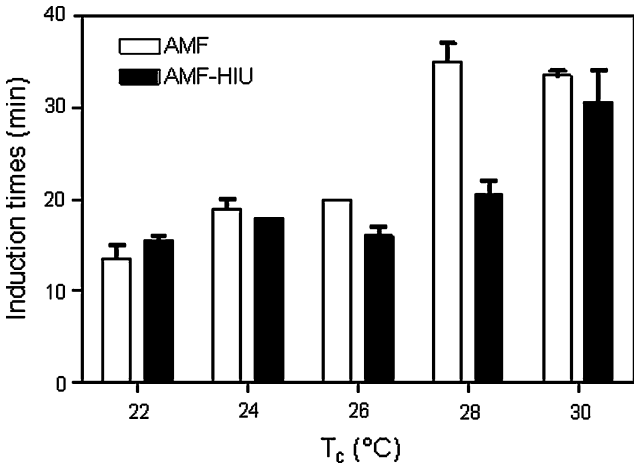

Fig. 2 Induction times of crystallization for AMF samples crystallized without and with the application of HIU

Figure 3 shows the morphology of AMF crystallized at 22, 24, 26, 28 and 30 °C as a function of crystallization time without and with the application of HIU. As expected, the lower the crystallization temperature (higher supercoolings), the smaller the crystals aggregates formed. It can be observed that HIU application significantly affected the crystallization behavior of AMF, especially for T_c of 24, 26 and 28 °C. Not only was an induction on the onset of crystallization observed but also a promotion of crystal growth. The formation of smaller crystals aggregates was also observed as a consequence of HIU application. However, as previously mentioned, for low T_c (22 °C), that is high supercooling, HIU did not induce the crystallization, moreover, a delay on the onset of crystallization and crystal growth was observed.

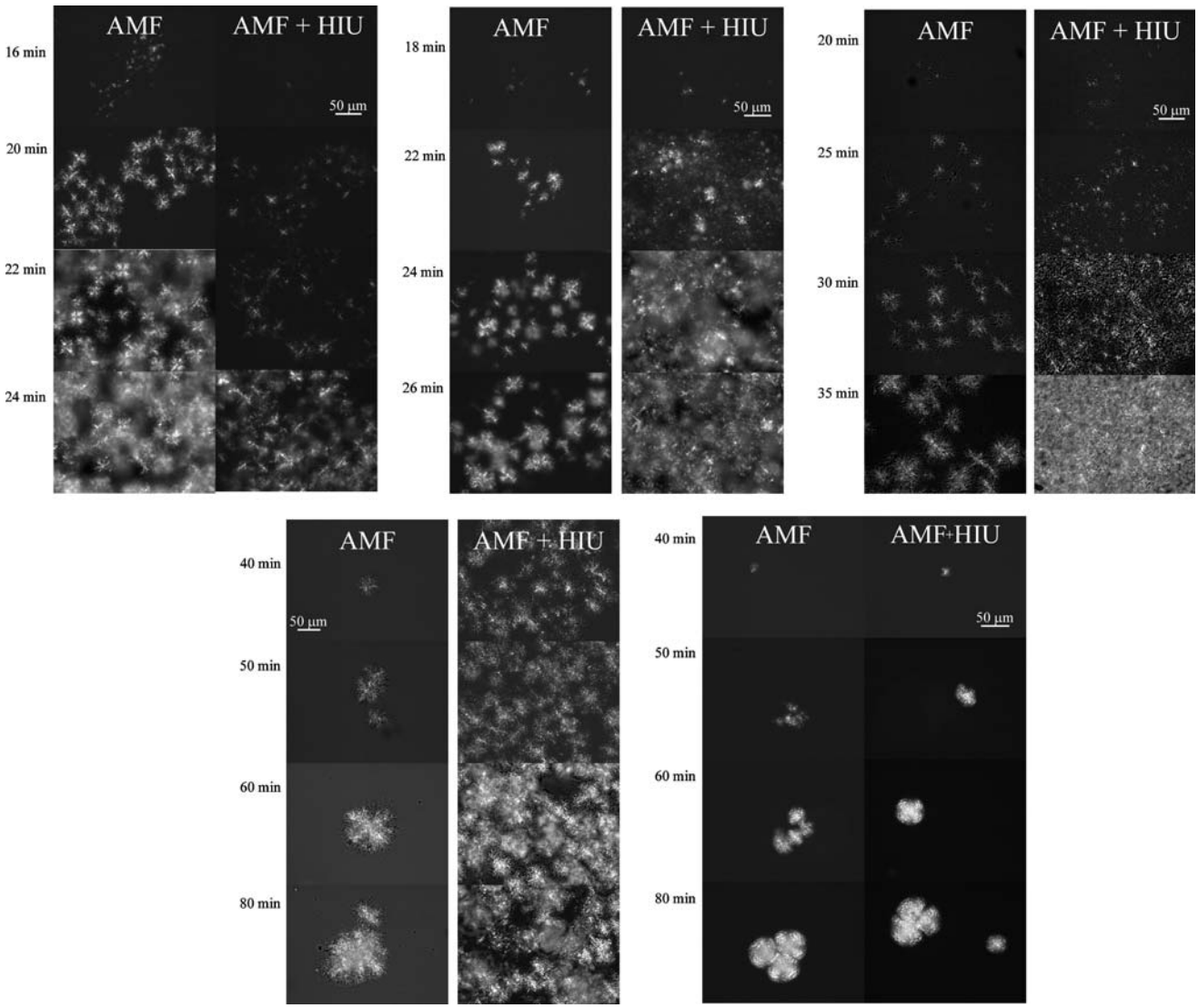

Fig. 3 Microstructure of AMF crystallized at 22, 24, 26 and 28 °C without and with the application of HIU after as a function of crystallization time

The mechanism by which HIU affects the crystallization of lipids is called cavitation. Cavitation refers to the formation and the subsequent dynamic life of bubbles in liquids. Two different types of cavitation phenomena can be generated by acoustic waves: *inertial* and *non-inertial* cavitation. Inertial cavitation involves large scale variations in the bubbles' size (relative to the equilibrium size) over a time scale of a few acoustic cycles, and the rapid growth usually terminates in a collapse of varying degree of violence. Non-inertial cavitation (stable) on the other hand, usually involves small-amplitude oscillations (compared to bubble radius) about the equilibrium radius. Non-inertial cavitation in most instances results in little appreciable bubble growth over a time scale of thousands of acoustic cycles. Non-inertial cavitation can lead to inertial cavitation, and the collapse of an inertial cavity can produce smaller bubbles that undergo stable cavitation [33]. Considering the discussion presented above, the behavior observed in AMF crystallized at 22 °C could be a attributed to two phenomena: (1) a slight temperature increase (0.5–1 °C) during HIU application, (2) higher viscosity of the oil at 22 °C. We propose that when an acoustic field is applied at 22 °C using the conditions described in this work, the bubbles generated are non-inertial in nature and they cannot collapse due to the high viscosity of the medium, having therefore no effect on the induction of crystal formation. In addition, the slight increase in sample temperature results in the melting of crystals and therefore a delay in the sample crystallization. At higher temperatures, the lower viscosity of the samples allows inertial cavitation to occur and therefore, even if the temperature in the system increases (0.5–1 °C) due to HIU

application, the crystallization is induced as a consequence of bubbles' collapse.

The effect of HIU on the crystallization behavior of AMF was quantified using the area fraction calculated using Image J software. The area fraction is defined as the area covered by the lipid crystals in the image expressed as a function of the total area. Considering this definition; the higher the area fraction value, the more lipid molecules crystallized in that specific image. Figure 4 shows the area fraction obtained for AMF crystallized at 22, 24, 26, 28 and 30 °C without and with the application of HIU. It can be seen for AMF crystallized at 22 °C that the crystals formed without the application of HIU resulted in a higher area fraction when compared with the same sample crystallized with the application of HIU (Fig. 4a). This indicates that HIU delayed the crystallization at 22 °C as discussed for Fig. 3. In the same context, when samples were crystallized at 24, 26 and 28 °C without the application of HIU, the area fraction covered by crystals was lower that the one obtained when the samples were crystallized with the application of HIU indicating a promotion of crystallization caused by HIU application. No significant differences were observed in the area fraction of AMF crystallized at 30 °C without and with the application of HIU (Fig. 4e).

Figure 5 shows the viscosity of samples crystallized at the different T_c without and with the application of HIU. As expected, the higher the temperature the lower the viscosity. HIU application to AMF generated a lipid network with significant higher viscosity, especially at lower temperatures (22, 24, and 26 °C). The viscosity of samples crystallized at 28 and 30 °C was significant lower than the ones observed at lower temperatures. In addition, no significant differences were found in these samples as a consequence of HIU application ($P < 0.05$).

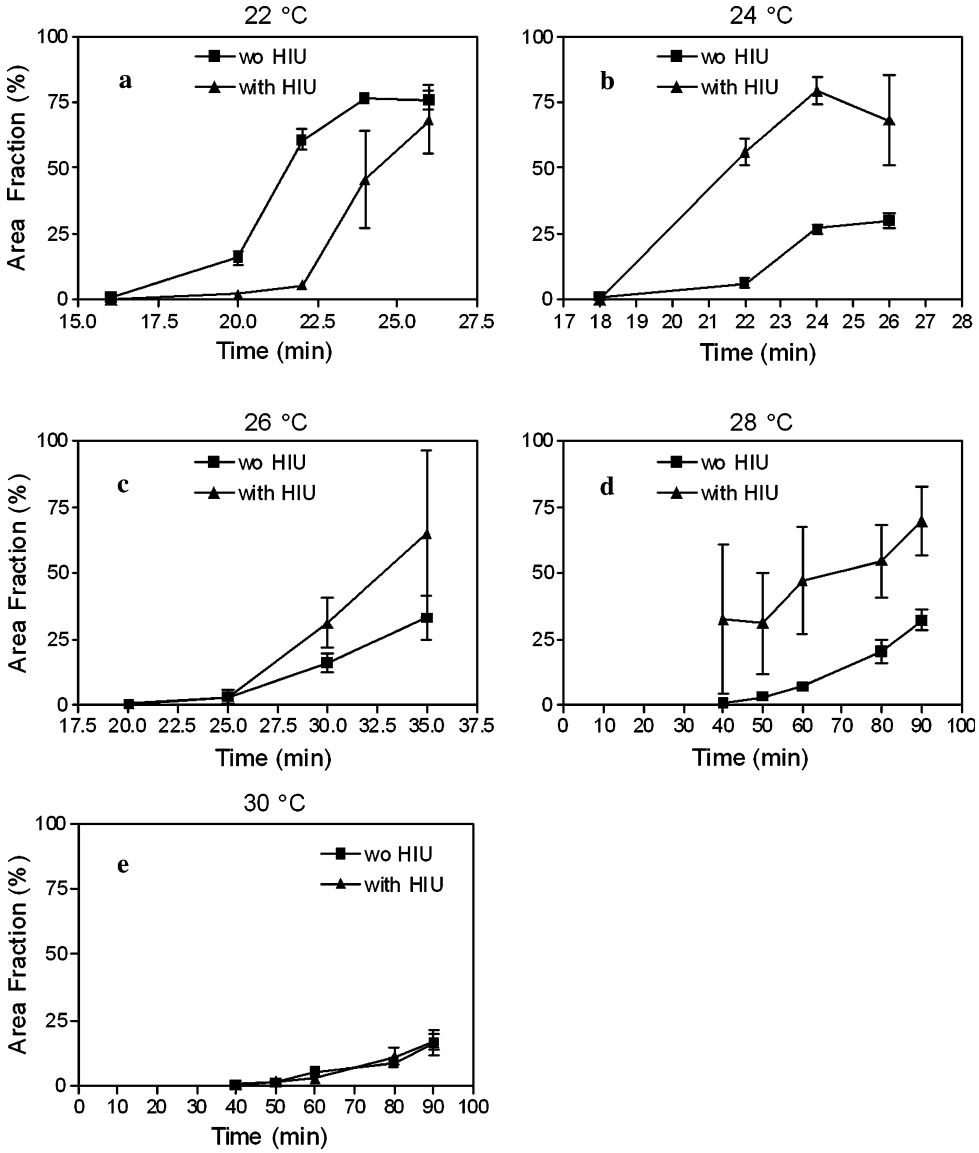

Fig. 4 Area fraction of AMF crystals obtained as a function of T_c and time when crystallized without (*filled squares*) and with (*filled triangles*) HIU application. **a** $T_c = 22$ °C, **b** $T_c = 24$ °C, **c** $T_c = 26$ °C, **d** $T_c = 28$ °C and **e** 30 °C

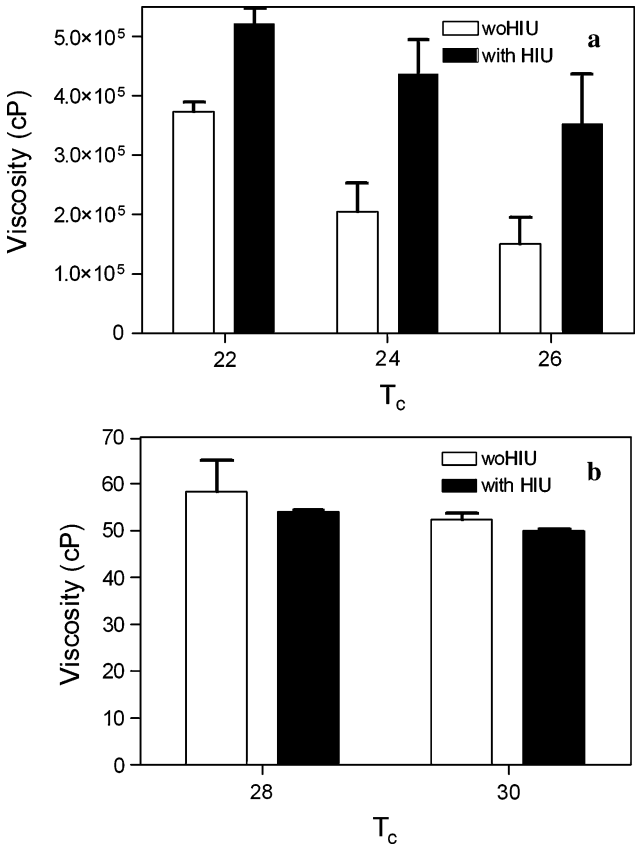

Fig. 5 Viscosity of AMF crystallized at 22, 24, and 26 °C **a**; and 28 and 30 °C **b** without (*white bars*) and with (*black bars*) the application of HIU

When samples were crystallized at 30 °C, HIU did not produce any significant change on either the induction time (Fig. 2) or the crystallization behavior of AMF (Fig. 3). This discrepancy can be explained by the way HIU was applied to the sample. In this case, when the sample reached T_c, HIU was applied, however, because at 30 °C the supercooling is very low, the sample does not crystallize immediately at this temperature showing an induction time of approximately 40 min. During this period of time, the effect of ultrasound (collapsing of bubbles) is dissipated and no effect on the crystallization is observed. That is, even if inertial bubbles are formed, when they collapse, the triacylglycerides molecules are not yet in their right conformation to crystallize (due to the low supercooling) and therefore, no effect on the crystallization behavior is observed.

To further evaluate the effect of HIU in AMF at lower supercoolings, AMF was crystallized at 30 °C with application of HIU, but this time, HIU was applied just after the first crystals were observed and using different time and power settings (Fig. 6). These conditions were chosen to decrease the amount of heating in the sample during HIU application that will melt the growing crystals and still generate inertial bubbles (non-stable) that will collapse when the lipid molecules achieved the right conformation to crystallize. As shown in Fig. 6, smaller crystals aggregates and a promotion of the crystallization was observed when HIU was applied under these conditions. When lower power (20 and 5 W) and shorter application times (5 s)

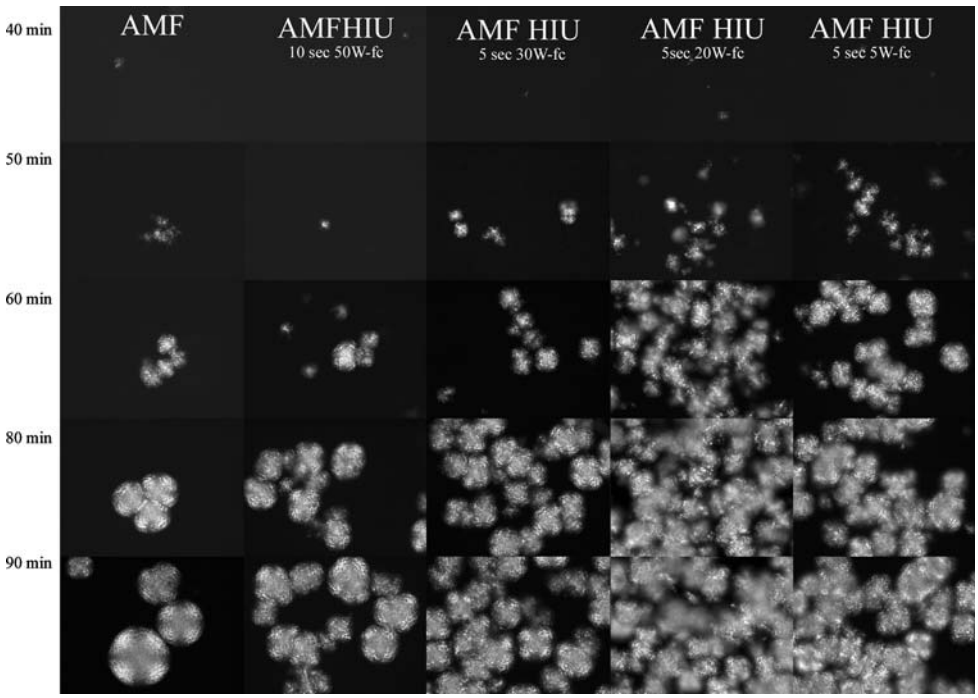

Fig. 6 Microstructure of AMF crystallized at 30 °C without and with the application of HIU after 40, 50, 60, 80 and 90 min at T_c. HIU was applied when the first crystals were observed (fc). Power levels used are 50, 30, 20 and 5 W for 10 and 5 s

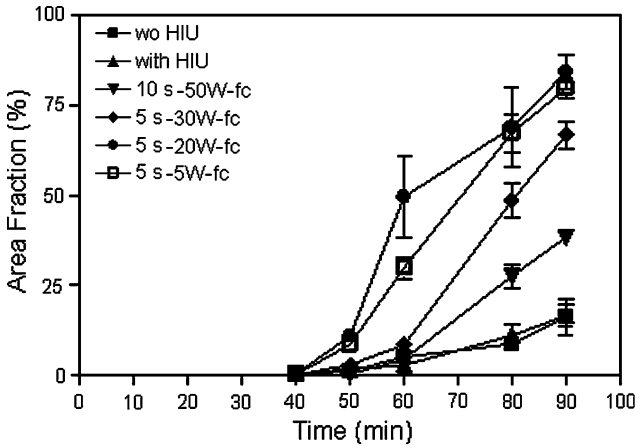

Fig. 7 Area fraction of AMF crystals obtained at 30 °C when crystallized without (*filled squares*) and with the application of HIU just after the first crystals were observed (fc). Power levels used are 50, 30, 20 and 5 W for 10 and 5 s

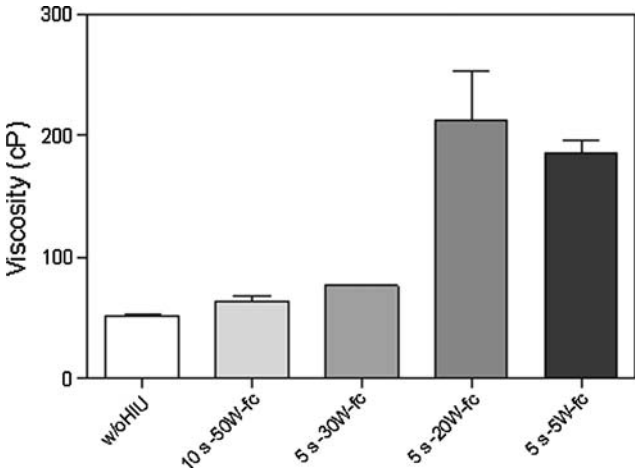

Fig. 8 Viscosity of AMF crystallized at 30 °C using different HIU settings. HIU was applied when the first crystals were observed (fc). Power levels are the same as in Fig. 6

were used, the crystallization was promoted in a greater extent. This data supports our hypothesis that to have an effect on the crystallization of lipids, HIU has to be applied very close to the moment when the first crystals appear. In addition, it can be confirmed that the heating generated through HIU has some effect on the induction of crystallization since lower power and less time of HIU application generated more crystals.

Figure 7 shows the microstructure quantification of the crystals obtained when crystallizing AMF under the condition described in Fig. 6, expressed as area fraction. This figure corroborates that the lower the HIU setting and the shorter the HIU application the greater the effect on crystallization induction. However, significant differences were not found between the two lowest power levels (20 and 5 W).

In addition to changes on the microstructure and crystallization behavior of AMF, HIU affected the viscosity of the samples crystallized at 30 °C. Similar to the microstructural tendencies observed in Fig. 7, the viscosity significantly increased when HIU was applied for shorter periods of time and using lower settings (Fig. 8).

In conclusion, HIU can modify the crystallization behavior of AMF. Depending on the supercooling and on the HIU settings, an induction of the crystallization onset and an increase in the sample's viscosity can be achieved. These results suggest that HIU can be used as an additional processing variable to improve the crystal network of lipids and therefore their physicochemical characteristics of lipids with low contents of saturated and *trans*-fatty acids. In particular, using HIU to obtain a more viscous material has the potential application in lipid-based foods such as mayonnaise, margarine and spreads that need to be formulated with healthier lipids while maintaining their physicochemical and sensory characteristics.

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