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Microscopy and calorimetry as complementary techniques to analyze sugar crystallization from amorphous systems

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Abstract

A comparison of microscopic and macroscopic techniques to evaluate sugar crystallization kinetics is presented using amorphous lactose and lactose–trehalose mixtures. Polarized light video microscopy (PLV) and differential scanning calorimetry (DSC) were applied to measure crystallization kinetics, induction times and time for complete sugar crystallization at different storage temperatures (60–95 °C). DSC was also employed to measure the glass transition temperature (T_{ag}) of the systems. PLV permitted direct observation, in real time, of growth of individual crystals and morphological aspects at a scale not detected by DSC. Taking the average of several microscopic observations, the results for temperature dependence of crystallization rate and time to complete lactose crystallization were similar to those obtained by DSC. Both PLV and DSC techniques showed that the presence of trehalose delayed lactose crystallization, without affecting the T_{ag} value. For the analysis of sugar crystallization in amorphous systems, PLV and DSC proved to be complementary techniques. Validation of results obtained by PLV with results from DSC opens a new area of microstructural analysis of crystallizing systems.

Keywords: Optical microscopy; DSC; Lactose; Trehalose; Crystallization; Microstructures; Freeze-drying.

1. Introduction

Stabilization of labile biomolecules is important in the development of food and pharmaceuticals, and it is related to the properties of their amorphous or crystalline phases. Amorphous sugars have been found to be optimal in protecting enzymes, antibodies, liposomes and microorganisms during drying and later in storage.[1–5] Several authors related the stabilizing effect of sugars to the noncrystalline states of the matrices that are formed.[6,7] Otherwise, in food products, the size and shape of crystals is sometimes important in formation of microstructures and in texture perception. To control the crystalline microstructure in pharmaceutical and food systems, an understanding of the interactions between components and the rates of their reactions during processing and storage is required.[8]

Temperature of storage, magnitude of temperature fluctuations, and relative humidity can all affect the changes in crystalline structures and rate of crystallization or re-crystallization that take place during the storage and distribution of products. Crystallization rates may also depend on the formulation of the product, since many ingredients influence nucleation and growth rates.[8] Some authors indicate that the addition of a second sugar, polymer or salts to an amorphous sugar matrix delays crystallization during storage, thus extending the protective capability of the sugar.[7,9–12]

There are many techniques available to follow or characterize the rate of crystallization and to measure the size, amount, or type of crystals present in a system.[8] Microscopy is probably the most widely used technique for characterizing structure, especially crystalline content, in foods.[13–15] Under the right circumstances, particle sizing through image analysis can also be performed on the images obtained by microscopy. Thus, microscopy has the potential both to visually document the nature of the crystalline structure in a

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food and to give quantitative information regarding the size distribution of crystals.[16] Polarized light microscopy allows one to distinguish crystals from non-crystalline material.[8] Microscopy can also be used to evaluate rates of crystal growth directly. Numerous studies[17–19] have used optical microscopy to study the rates of ice crystal growth and the effects of various components on crystal growth rate. Budiaman and Fennema[18] evaluated the effects of hydrocolloid suspensions on the linear rate of advance of a freezing front. Optical microscopy techniques have also been used to characterize the growth of lipid crystals in the presence of impurities.[8 20]

Calorimetry is another method to evaluate crystallinity in foods. Although several types of calorimeters may be used to study crystallization, differential scanning calorimetry (DSC) is most frequently used in food systems. The primary advantages of studying crystallization by DSC are the small sample size required and the high sensitivity. DSC is most commonly used to verify the type and amount of crystalline material present in a food. The onset and shape of enthalpic transitions depend on the crystallization kinetics of the sample and the rate of scanning, the latter being general chosen to allow adequate detail for the purpose of the study.[8]

Knowledge and control of sugar crystallization is relevant in biological, pharmaceutical and food sciences. Particularly, the study of lactose crystallization is important in a wide variety of products, and there is a need for suitable methods to determine the degree of crystallization that would occur under specific processing and storage conditions.[21]

The objective of the present work was to compare results from direct microscopic analysis and DSC to evaluate the crystallization kinetics of lactose and lactose–trehalose mixtures initially amorphous and heated to the supercooled state.

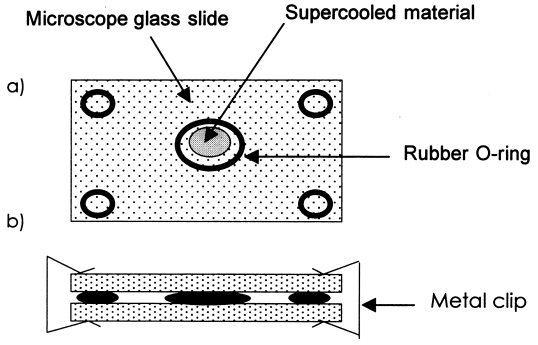

Fig. 1. Device employed for the PLV analysis. (a) Samples were placed inside the central rubber O-ring; (b) after rehumidification the samples were hermetically sealed with metal clips between two microscope glass slides.

2. Experimental

2.1. Preparation of model systems

Amorphous systems were obtained by freeze-drying solutions containing 20% (w/v) of lactose (L) (Mallinckrodt, St. Louis, USA) or mixtures lactose–trehalose (Pfanstiehl, Pfanstiehl Laboratories, Inc. Waukegan, IL, USA) (LT) in the ratio 80:20 w/w. Aliquots of 1 mL of each model solution were placed in 3-mL vials, frozen at -26 °C and immersed in liquid nitrogen (temperature $= -190$ °C) before freeze-drying. An Heto–Holten A/S, cooling trap model CT 110 freeze-dryer (Heto Lab Equipment, Denmark) was operated at -110 °C and at a chamber pressure of 4×10^{-4} mbar. After freeze-drying, samples were transferred into a vacuum desiccator and exposed for 1 week over a saturated salt solution of $MgCl_2$ at 33% relative humidity (RH).

2.2. Polarized light videomicroscopy (PLV)

Polarized light video microscopy was used to determine the isothermal crystallization kinetics of lactose in both model systems. Images were acquired using an Olympus BX50 light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) with polarizer filters and a JVC video camera model TK-128E (JVC Europe Ltd., London, United Kingdom) connected to the video entry port of a computer. In heating experiments a Linkham hot-stage model THMS 600 (Linkham TMS 92, Linkham Scientific, Surrey, UK) coupled to the microscope was used, and the temperature and rate of heating of the sample were controlled with a Linkham HFS 91 temperature programmer (Linkham Scientific, Surrey, UK).

Between 10 and 15 mg of the freeze-dried samples were transferred into a vacuum desiccator and exposed over a saturated salt solution of $MgCl_2$ during 48 h at 25 °C (Fig. 1(a)). After rehumidification the samples were hermetically sealed between two microscopic slides separated by rubber O-rings (Fig. 1(b)) to maintain constant water content during the isothermal treatment in the hot-stage. Initially amorphous samples were rapidly heated (50 °C/min) from room temperature to the selected constant temperature (60–110 °C $\pm$ 2 °C) and crystallization in supercooled conditions was observed as a function of time.

Lactose crystallization kinetics were evaluated by determining the area of the growing crystals as a function of heating time using the software Scion Image (Release Beta 4.0.2). Images were taken with a 10-X objective that allowed following the growth of single crystals. The change in area of single crystals with time was evaluated to the time when it was no longer possible to distinguish individual crystals. Each condi-

tion was analyzed in triplicate, and the growth of about 1–5 crystals was followed per sample.

The curves of crystal area versus time of isothermal storage were fitted by linear regression, and the slope was calculated for each case. Average values from the slopes of several runs were pooled to describe the growth rate at a given temperature. The induction time of crystallization was taken as the intercept of the regression line with the time axis. The time required for complete sugar crystallization was taken as that at which the observed field (4–6 mm diameter) was fully covered by crystals.

2.3. Differential scanning calorimetry (DSC)

A DSC analysis system Mettler TA 4000 with a TC11 TA processor and Graph Ware TA72 thermal analysis software (Mettler Instrument AG, Greifensee, Switzerland) was used for all the measurements. Analysis involved samples (between 10 and 15 mg) placed in 40-μL hermetically sealed aluminum pans. An empty pan was used as a reference, and the instrument was calibrated using indium. Two replicates of each sample were analyzed, and the average value was reported.

The dynamic method was used to determine glass transition temperatures (T_g) of the sugar systems. Each sample was heated at a rate of 10 °C/min in a temperature range from 40 °C below the expected T_g value, up to 150 °C. Glass transitions were recorded as the onset temperature of the discontinuities in the curves of heat-flow versus temperature.

Isothermal DSC measurements were performed to determine crystallization kinetics, and temperature dependence of crystallization time. The samples were placed in the DSC sample holder once the desired temperature was reached and the exothermal heat flow corresponding to lactose crystallization was recorded versus time during isothermal storage. The crystalline fraction (α) of the sugar was calculated from the ratio of the exothermic peak of crystallization at different times and the total heat of crystallization of the sugar at the selected storage temperature.[12] The induction time of crystallization was the time after which the exothermal peak of crystallization started. The time for complete sugar crystallization was taken as the time at which the exothermal peak finished.

2.4. Crystallization kinetics. The Avrami equation

From the isothermal DSC runs sugar crystallization kinetics were calculated by integrating the exothermal peaks with respect to time to determine the heat of crystallization. The crystalline fraction was plotted against time and analyzed using the Avrami equation:[22]

$$1-\alpha=\exp(-Kt^n) \tag{1}$$

where α represents the fraction of the sugar crystallized at time t ($\alpha=1$ corresponds to the total peak area); K is the rate constant of isothermal crystallization [(time)$^{-n}$] which depends primarily on the crystallization temperature, and n is known as the Avrami index, a parameter characteristic of nucleation and growth mechanisms of the crystals. The Avrami equation is particularly valuable because it provides important information about the crystallization mechanisms when crystals form from a relatively pure melt.[23] In the present work, the crystals had a different composition than the melt, and the Avrami model was used as a tool for the kinetic analysis. Time t was taken as the time of the experiment minus the induction time. The induction time normally corresponds to the time until a stable crystal nucleus starts to grow. Taking the end of the induction period as time 0, both Avrami parameters can be obtained from the linear portion of the experimental data. The numerical value of K is directly related to the overall rate of crystallization, and reciprocal crystallization half-time values ($\tau_{1/2}$) were calculated using the following equation:

$$K=\frac{0.693}{(\tau_{1/2})^n} \tag{2}$$

On the other hand, graphical determination of the values for K and n can be made from a double logarithmic plot of the Avrami equation:

$$\ln[-\ln(1-\alpha)]=\ln K+n\ln t \tag{3}$$

3. Results

Fig. 2 shows a sequence of images obtained by PLV for lactose crystallization from the supercooled liquidus state at 70 °C. The use of polarized light allowed one to enhance the visualization of the sugar crystals and to distinguish them from the supercooled material. The crystals appeared as a bright region surrounded by the dark background. At any time crystals varied greatly in size, even in the same sample, and grew until they became in contact with one another. Small crystals that appeared later were observed as bright points on the background. Crystallization rate of lactose and 8:2 lactose–trehalose mixtures in freeze-dried systems exposed at 33% RH was followed by PLV at several storage temperatures (60, 63, 65, 75 and 80 °C for pure lactose systems and 70, 80, 90 and 95 °C for the mixture LT). As previously observed,[12] the T_g values determined for the lactose and lactose/trehalose samples were not different from the T_g of pure lactose humidified at 33% RH, which was close to 40 °C. Isothermal heating temperatures were selected between T_g and T_m. At temperatures close to T_g the crystallization time was too long for practical purposes, and at temperatures

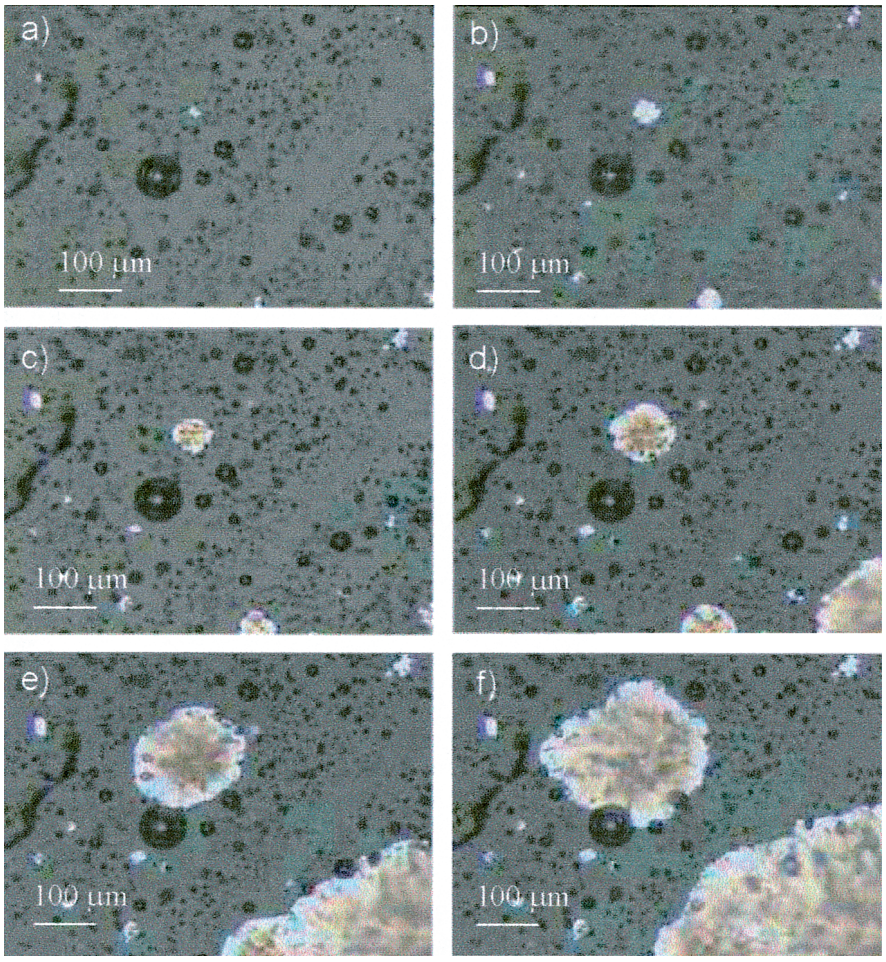

Fig. 2. Sequence of images obtained by PLV for lactose crystallization at 70 °C from a freeze-dried system exposed at 33% RH. Time of isothermal heating: (a) 360; (b) 720; (c) 960; (d) 1080; (e) 1140; (f) 1170 s.

near ($I_g + 60$ °C) almost instantaneous crystallization occurred. Crystallization was not observed in pure trehalose systems exposed at 33% RH in the analyzed temperature range. These results were in agreement with previous DSC measurements.[12] Thus, crystallization in the lactose–trehalose systems at those conditions was assigned to lactose crystallization. Fig. 3(a–d) shows the area of several single crystals as a function of time during isothermal storage for pure lactose at 65 and 75 °C, (Fig. 3(a and b), respectively), and for the lactose–trehalose mixture at 90 and 95 °C, (Fig. 3(c and d), respectively). The data, both for crystal growth and induction time, showed that lactose crystallization from the supercooled occurred at different rates in different zones of the samples (Fig. 3) (note that the y-axis scales in Fig. 3(a–d) are different). These observations are probably related to microdomains in the supercooled phase caused by an uneven distribution of water, different degree of local supersaturation or localized packing of sugar molecules in different regions of the sample. Considering that nucleation is also a non-equilibrium, spontaneous and random event, a distribution of induction times and rates is to be expected. This results in individual crystals growing at different rates in the same environments based on internal imperfection or lattice stresses. Thus, the analysis of a limited number of crystals gives a rough average of the kinetic parameters due to a growth rate dispersion which is well know in sugar systems. The curves of crystal size versus time of isothermal storage (Fig. 3(a and b)) were adjusted by linear regression ($r^2 = 0.800–0.998$), the corresponding slopes were calculated and an average value was used to describe the crystallization rate for each temperature. A one-dimensional growth rate, which could also take place was also analyzed, but the correlations did not improve when square or logarithm transformation of the data were plotted. Fig. 4 shows the rate of crystal growth versus the storage isothermal temperature for the lactose and 8:2 lactose–trehalose systems exposed at 33% RH (bars indicate the standard deviation of the values). The crystallization process presented less variability at lower temperatures, in terms of spatial distribution of crystals and their growing rate, and this was reflected by the smaller standard

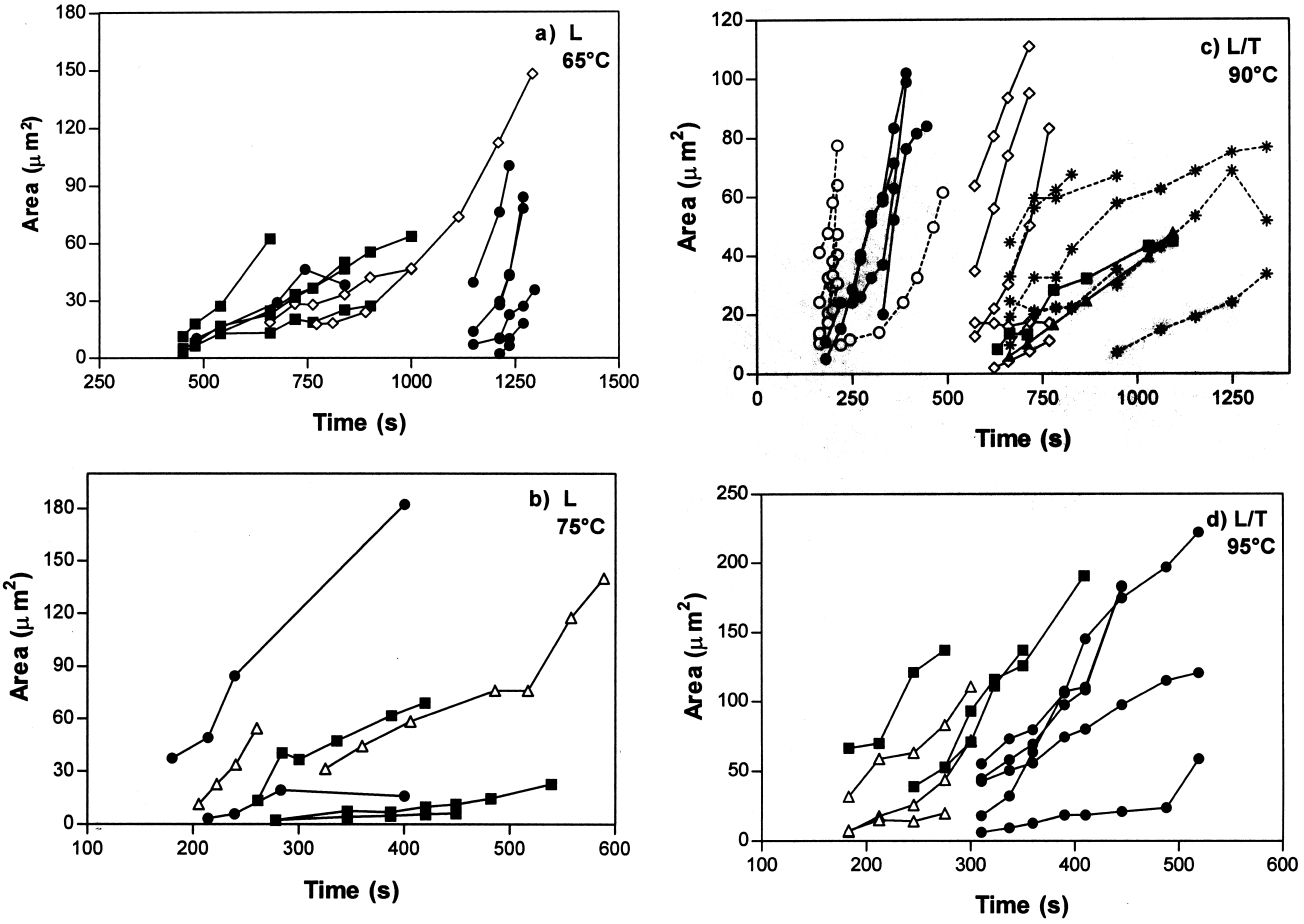

Fig. 3. Area of several single crystals of lactose from freeze-dried pure lactose systems (a, b) or 8:2 lactose–trehalose mixtures (c, d) exposed at 33% RH as a function of time of storage at the indicated temperatures. Replicate samples are indicated with different symbols, the curves with the same symbols correspond to the growth of different crystals in a given sample.

deviation determined for the lower temperatures. Considering the wide distribution of crystal growth rates at intermediate–high temperature range, discussed in relation to Fig. 3, the data in Fig. 4 are a gross approximation of the growth of lactose crystals in the systems studied, which makes it impossible to take absolute values. However, there was a clear trend of increasing crystal rate when increasing heating temperature. There is a release of the mobility constraint and a decrease in the supersaturation driving force as temperature is increased above T_g. Also, as previously observed by DSC measurements,[12] the presence of trehalose clearly delayed lactose crystallization without affecting the T_g value of the system. Arvanitoyannis and Blanshard[23] also found a delay of the half crystallization times in amorphous lactose–sucrose mixtures, and they attributed this effect to the higher T_g value (and hence lower molecules mobility) of the mixture with respect to the pure sucrose. The delay of lactose crystallization was not related with an increase of the T_g value of the mixture and was attributed to thermodynamic, geometric or kinetics factors, associated to the modification of the molecular environment in the combined systems that probably affect nucleation and/or crystal growth. Shamblin et al.[24] showed that at certain levels, additives (particularly polymeric materials) may significantly infl-

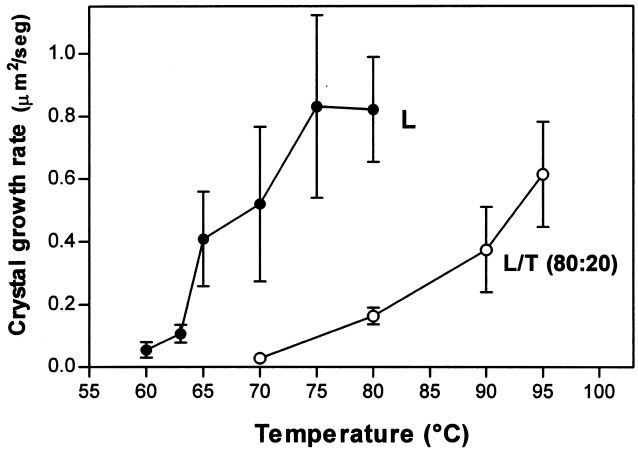

Fig. 4. Crystal growth rate versus the isothermal storage temperature for lactose and 8:2 lactose–trehalose systems exposed at 33% RH.

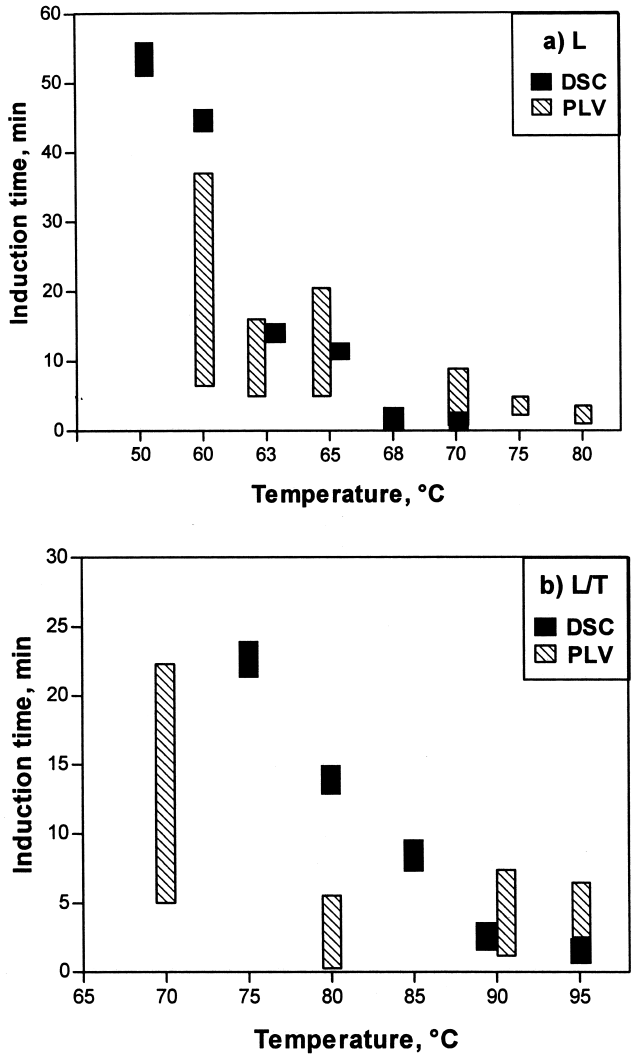

Fig. 5. Range of induction times of crystallization determined by PLV and DSC at different storage temperatures for pure lactose systems (a) or 8:2 lactose–trehalose mixtures (b) exposed at 33% RH.

uence nucleation without having any effect on T_g. Jouppila and Roos[25] showed that the various components in milk (protein, salts, etc.) had no effect on T_g of the amorphous phase, yet caused significant inhibition of lactose crystallization (compared with a pure lactose system).

Fig. 5 compares the range of crystallization induction times determined by PLV and DSC at different storage temperatures for pure lactose systems (Fig. 5(a)) or 8:2 lactose–trehalose mixtures (Fig. 5(b)) exposed at 33% RH. The size of the boxes shows that there was a large range for induction time, mainly for PLV measurements. Nevertheless, through direct examination of the sample, PLV allowed to topographically identify zones with different tendency to crystallize. While big crystals were observed in a given part of the sample, there were regions where lactose crystallization had not been started. Both in the pure system and in the mixture at low temperatures, the induction times determined by DSC were higher than those determined by PLV. At these temperatures, a given sample had crystals large enough to be detected by PLV, but did not have enough crystallized mass to be detected by DSC. There may be a few nuclei and crystals formed initially, without giving off sufficient energy for DSC to sense a change. DSC can only sense the massive onset of crystallization, but it is known that there may be formation of a few nuclei and crystals before this point. These crystals are those seen by microscope. Although PLV and DSC are techniques based on the measurement of different properties, the values of induction times for crystallization, presented similar temperature dependence. As also observed in Fig. 5 the microscopic detection of the beginning of lactose crystallization appeared to be less sensitive to heating temperature than the detection by DSC.

Fig. 6 shows the crystallization rate determined by PLV and DSC at different storage temperatures for pure lactose (Fig. 6(a)) or 8:2 lactose–trehalose mix-

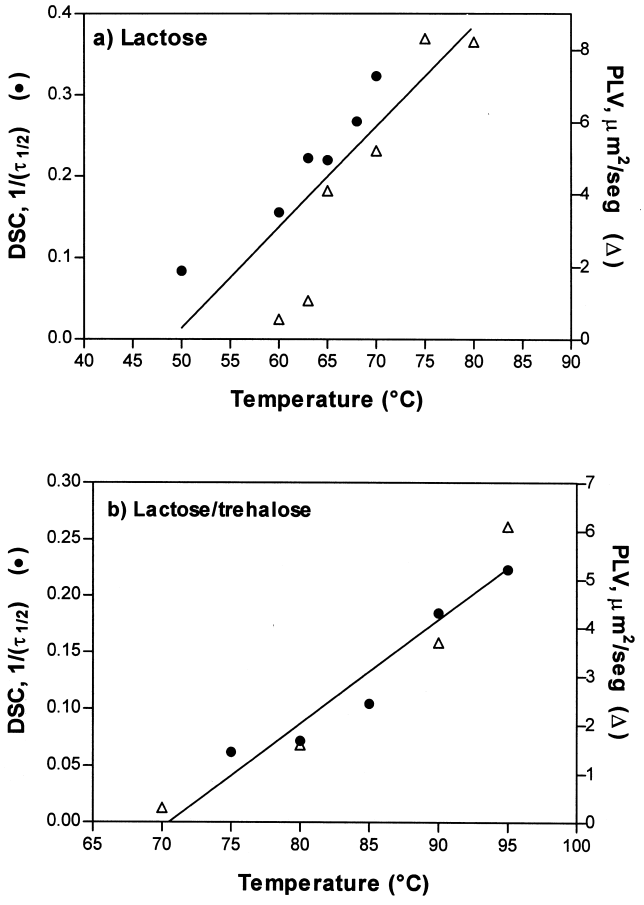

Fig. 6. Crystallization rate of lactose determined by PLV and DSC at different storage temperatures for pure lactose systems (a) or 8:2 lactose–trehalose mixtures (b) exposed at 33% RH.

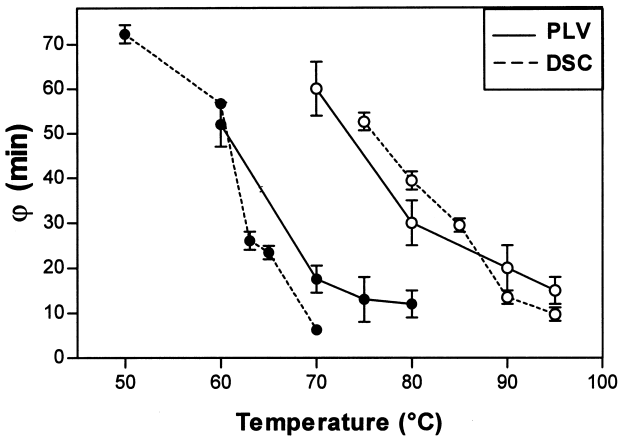

Fig. 7. Temperature dependence of the time required for complete lactose crystallization (φ) in supercooled systems determined both by PLV and DSC for pure lactose (●) and 8:2 lactose–trehalose mixtures (○) exposed at 33% RH.

tures (Fig. 6(b)) exposed at 33% RH. Even when differences were observed for the crystallization rates measured by PLV or DSC, the average values calculated for PLV observations were close to the results obtained by DSC. The analysis of a large number of samples by PLV described the global process and showed similar temperature dependence behavior to the DSC data.

The time required for complete lactose crystallization (including induction time and crystal growth) in the supercooled systems presented also a similar pattern when determined by PLV or DSC (Fig. 7). The time for complete crystallization diminished as the heating temperature increased, and crystallization at a certain temperature was delayed in presence of trehalose.

Taking the average of several microscopic observations, the results for temperature dependence of crystallization rate (Fig. 6) and time for complete lactose crystallization (Fig. 7) were similar to those obtained by DSC. Microscopic observations were particularly useful to get within the microstructure of the material and to obtain information about crystal size and crystallization rate distribution in the sample that could not be detected by DSC (Fig. 3).

4. Discussion

DSC analysis allowed one to obtain precise data of crystallization enthalpies, induction times, glass transition temperatures and to study sugar crystallization kinetics in model systems. This technique provides information of an overall average property at the supramolecular or suprastructural level. Its use is sometimes limited in foods or complex systems since other transitions (i.e., gelatinization, water evaporation, chemical reactions, etc.) can also occur during heating, which result in complex thermograms that are often difficult to interpret.[8] A particularly valuable use of microscopic observations and image analysis in the supercooled sugar systems studied was the possibility to observe, in real time, changes in the microstructure of the sample. PLV analysis showed that lactose crystallization process takes place at different rates in different zones of the sample (Fig. 3). There are several examples in which microscopy has been instrumental for the understanding of many phenomena that depend on the microstructure rather than on bulk conditions.[15,16] In fact, the development of the Avrami equation for the kinetics of crystal growth is based on microscopic considerations.[22] Although optical techniques are relatively easy and simple to implement, proper sampling, adequate preparation of samples (for quantitative image analysis) and careful interpretation of results are required to obtain meaningful information.[8]

There is always a compromise between the versatility of optical techniques and the convenience of thermal methods, so that for the analysis of sugar crystallization from amorphous systems PLV and DSC can best be regarded as complementary techniques. The fact that minimal quantities of a sample are required both by DSC and PLV constitute an advantage for these techniques when studying crystallization from the solid state.

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