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LIGHT MICROSCOPY MEASUREMENTS OF ICE RECRYSTALLIZATION IN FROZEN CORN STARCH PASTES USING ISOTHERMAL FREEZE FIXATION

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Abstract

Isothermal freeze fixation was used to analyze ice recrystallization by light microscopy in a 10 % (W/W) frozen corn starch paste during storage at temperatures in the range of -5 to -20 °C.

Different formulations were tested in order to obtain a suitable fixative for this method of indirect observation of the ice crystals. A solution of formaldehyde, ethanol and water (10:45:45 V:V) was selected because it minimized substitution-induced distortion and contraction of the matrix. The diffusion coefficients of the selected fixative in the frozen system were measured at different temperatures in conditions of unidirectional mass transfer in a semi-infinite medium. The activation energy for diffusion was determined ($Ea = 95.11 \pm 1.15$ KJ/mol).

Fixation times for the frozen starch paste at different temperatures were predicted from a mathematical model for unidirectional mass transfer with a discontinuous diffusion coefficient. Matrix contraction during the different stages of the freeze fixation method was evaluated.

Recrystallization of ice in frozen corn starch pastes during storage was analyzed by the measurement of the changes in ice crystal equivalent diameters on the micrographs. A kinetic equation for recrystallization was fitted to the experimental data to obtain the corresponding parameters. Contraction of the matrix affects the kinetic constants but has no effect on activation energy. The effect of recrystallization during fixation on ice crystal measurements was not significant.

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Introduction

Starch granule behavior depends on chemical composition (amylose/amylopectin ratio) and physical arrangement of the components within the granule structure. Gelatinized starch plays an important role in the functional properties of many food products and has been the subject of extensive investigation (Sterling, 1978; Dengate, 1984; Luallen, 1985; Gallant and Bouchet, 1986). In frozen foods, starch is used in sauces, gravies, pie filling, etc., but information about its behavior from a microscopic point of view is limited (Meryman, 1966; Berghofer and Klaushofer, 1976; Holmes and Soeldner, 1981; Przybyl *et al.*, 1983).

During freezing and frozen storage, starch pastes exhibit structure deterioration, showing a spongy matrix and liquid phase separation upon thawing. Several authors (Hanson *et al.*, 1951; Osman and Cummisford, 1959; Brunnek and Koptelova, 1970; Chan and Toledo, 1976) have attributed syneresis problems in starch pastes to retrogradation; other reports have related quality alteration to ice crystal formation during the freezing process (Przybyl *et al.*, 1983) and to modification of the ice crystal pattern during frozen storage (recrystallization).

Ice recrystallization (grain growth) involves the growth of larger ice crystals at the expense of smaller ones, leading to a reduction in the total number of crystals and to an increase in the mean crystal diameter (Sy and Fennema, 1973; Bevilacqua and Zaritzky, 1982; Harper and Shoemaker, 1983; Martino and Zaritzky, 1987, 1988, 1989). In this type of study, where ice crystal measurements are involved, it is difficult to preserve the structure and maintain the location and size of the ice crystals. Two techniques can be applied: **a**) direct microscopic observation of the frozen sample with the aid of a cryomicroscope stage; and **b**) indirect observation of the holes left by the ice crystals in the system (freeze drying or freeze substitution).

Different authors (Varriano-Marston, 1977; Chabot *et al.*, 1978, 1979; Chabot, 1979; Christianson *et al.*, 1982) recommended freeze-etching or freeze drying to prepare samples for microscopic observation.

However, these techniques suffer from serious disadvantages at high subzero temperatures.

Freeze drying requires generally the specimens to be cooled at -45 to -60 °C; in freeze substitution, similar or even lower temperatures are used. Freeze fracture/etching techniques require temperatures of -100 °C or lower (Hunt, 1984). Cooling from high to low subzero temperatures results in formation of additional ice, thus the information obtained by these methods is limited because the amount and distribution of ice crystals have been modified.

Isothermal freeze fixation, an equilibrium technique introduced by MacKenzie *et al.* (1975), preserves structures and retains the conformational relationship of the ice phase in thermodynamic equilibrium with the frozen specimen. This condition is achieved with a fixative solution with a melting point at the temperature of fixation (that is the temperature to which the specimen has been pre-cooled) and the maintenance of these conditions during the fixation period. These formulations interact with the specimen components conferring rigidity to the matrix and ensuring that the ice pattern is preserved (MacKenzie *et al.*, 1975; Hunt, 1984; Lampila *et al.*, 1985; Martino and Zaritzky, 1986). The most discussed aspect of these techniques is the contraction of the matrix during the chemical dehydration. The selection of a fixative that adequately minimizes this problem is important (Hood *et al.*, 1974; Lee, 1984).

The main objective of the present study was to apply the isothermal freeze-fixation to the analysis of the ice recrystallization process in a frozen 10 % (W/W) corn starch paste during storage at different temperatures (-5 to -20 °C) and to determine the changes in ice crystal size distribution. This involved the following specific objectives:

1) To select a fixative system that minimizes matrix distortion during successive stages and maintains the quality of the frozen pattern.

2) To determine diffusion time for the fixation at subzero temperatures to ensure the penetration of the solution into the sample.

3) To determine the influence of the isothermal freeze fixation technique (especially the contraction of the matrix) on the measured recrystallization parameters (kinetic constants and activation energy).

4) To establish the effect of ice recrystallization during the period of fixation on crystal measurements at different temperatures.

Materials and Methods

Preparation of the samples

Commercial corn starch (Refinerías de Maiz, Argentina) with the following composition (% W/W wet basis): water content 11.3, proteins 0.3, lipids 0.6, ash 0.3, amylose/amylopectin = 25/75, was used in the experiments; 10 % (W/W) suspensions were prepared by mixing the starch in cold distilled water. Gelatinization was performed by heating 400 ml batches (in a 7-cm diameter, 10-cm high vessel) in a thermostatic bath. The suspensions were stirred to maintain uniform temperatures with an helical mixer at 680 rpm. This kind of mixer was selected because it minimizes mechanical disruption of the system. Heating rates were 17 °C/min at the lower temperature range (30 to 50 °C) and 8 °C/min at the gelatinization range (62 to 72 °C). The mix reached 80 °C in approximately 6 minutes and remained at an average temperature of 83 °C during 4 minutes. The gelatinization process was followed with a polarizing microscope to verify that all the starch granules had lost their Maltese crosses but kept their granular shape. The pastes obtained were divided into small samples and placed in cylindrical aluminum foil molds 0.8 cm in diameter and 0.5 cm high. The samples were frozen in cold chambers to a final temperature of -20 °C. Heat flux from both sides was unidirectional and parallel to the axis of the cylindrical samples. Lateral insulation was provided by an acrylic sample holder. Thermal histories during heating and freezing were recorded with copper-constantan thermocouples.

Freezing rates were determined according to the IIR (International Institute of Refrigeration, 1972) as the minimum distance from the surface to the thermal center divided by the time elapsed between the moment the surface reaches 0 °C and the moment the thermal center reaches a temperature 10 °C colder than the temperature of initial ice formation in the system. The measured initial freezing point of the starch paste was -0.6 °C.

The freezing rate of the paste samples was 2 cm/h. In commercial practice, 0.5 to 3 cm/h correspond to quick freezing in air blast or plate freezers (IIR, 1972).

Frozen samples were stored at -5, -10, -15 and -20 °C for microscopic analysis at a temperature stability of $\pm$ 0.5 °C.

Microscopic observation: isothermal freeze fixation at subzero temperatures

There are no set rules as to which combination of fixatives and procedures are best for gelatinized starch systems (Kalab, 1983). Generally, it is necessary to test a variety of fixatives and select the one that gives the best overall fixation quality (Bechtel, 1990). A wide range of fixing agents, concentrations, and buffering systems are reported in literature (Varriano-Marston, 1977; Angold, 1979; Chabot, 1979; Falk, 1980; Bechtel, 1990).

However, most of the work on starch pastes, doughs, baked goods, and cereals, reports the use of aldehydes (glutaraldehyde or formaldehyde) as fixatives. Bechtel (1990) stressed that for cytochemical carbohydrate localization, formaldehyde fixation seems to be the best choice, but lack of quality fixation usually forces the use of glutaraldehyde.

In order to select an effective fixative, different formulations were tested (Table 1). Ethanol was used as a co-solute to decrease the freezing point of the solution.

Table 1. Formulation of the fixative solutions tested.

Components	Concentration (V:V)
Carnoy Fluid (chloroform: glacial acetic acid: absolute ethanol)	30:10:60[a]
Glacial acetic acid: formaldehyde (40 %): absolute ethanol: water	5:10:70:15[b,c] 5:10:38:47[d]
Absolute ethanol: formaldehyde (40 %)	90:10[e]
Absolute ethanol: formaldehyde (40 %): water	45:10:45[d]
Absolute ethanol: formaldehyde (40 %): water NaH_2PO_4 Na_2HPO_4	45:10:45[e] 0.35* 0.65*
Glutaraldehyde (25 %): absolute ethanol	8:92[d]

*Concentrations expressed as percent W/V

[a]Feder and Sidman (1958); [b]Woodroof (1939); [c]Van Hulle *et al.* (1965); [d]Present work; [e]Lynch *et al.* (1965)

Ethanol is a preferred dehydrating agent because of its effectiveness and low toxicity. Inclusion of water avoided drastic contraction of the samples during fixation, but limited the variety of formulations that could be used because of miscibility problems.

Glutaraldehyde was tested because it is one of the most commonly used fixatives (MacKenzie *et al.*, 1975; Hunt, 1984; Lee, 1984; Lampila *et al.*, 1985). Phosphate salts were added to one formulation to match the pH of the starch paste (pH = 5). NaCl was used to decrease the freezing point of the solution to -20 °C; however it led to unsatisfactory results because the necessary high concentration (23.3 % W/V) produced a great difference of ionic strength between the solution and the low polarity starch paste.

Frozen samples, stored at -5, -10, -15 and -20 °C, were dipped in the fixative solutions (pre-cooled to these temperatures) to carry out isothermal freeze fixation.

Once the samples were fixed, remaining steps were conducted at room temperature; dehydration was performed with a series of gradually increasing ethanol concentrations (from 70 % V/V to absolute alcohol). Starch samples were cleared in benzene and embedded in paraffin (Paraplast Plus, Monoject Scientific, USA, melting point = 56-58 °C).

The dimensions of the cylindrical samples were measured after dehydration, clearing and embedding processes to determine the changes introduced by the technique. Contraction factors (cf) were evaluated as the ratio between the sample diameter after each stage of the freeze fixation process and the initial value.

Sectioning was done with a rotary microtome (American Optical, Model 820), transversely to the direction in which ice grew through the paste. Sections of 10 μm thickness were mounted, stained with iodine solutions and photographed in a Leitz Ortholux II microscope with a photographic camera Leitz Vario Orthomat. The experiments were performed in triplicate. Fixative selection was based on sectioning capability, rigidity of the sample, cohesion of the material after sectioning, resistance of the material to staining procedures and maintenance of relative sample dimensions.

Diffusion of the fixative

Once the selection of the best fixative was made on the basis of the results previously obtained, fixation times with that fixative at each temperature (-5, -10, -15, -20 °C) were determined.

Experiments to establish fixative diffusion rates were performed by the simulation of unidirectional mass transfer in a semi-infinite medium. Starch pastes were placed in cylindrical molds, 3.5 cm in diameter and 8 cm high, filling 50 % of the total available volume, and were frozen to a final temperature of -20 °C. Frozen starch pastes were transferred to cold chambers at -5, -10, -15 and -20 °C. The fixative solution was precooled at these temperatures and then placed in the cylindrical molds, filling the free space in contact with the frozen starch paste. At different times the advancing front of the fixative was measured on a graduated scale. The diffusion coefficient, in the zone where the fixative replaced the ice, was calculated from equations valid for semi-infinite media at each temperature.

The fixative concentration in the advancing front was determined from the freezing point curve of the solution.

Fixation times for small samples used for microscopic analysis were also calculated. The analysis involved the application of mathematical models based on one-dimensional mass transfer and the diffusion coefficients previously determined.

Recrystallization measurements

Recrystallization experiments were performed with cylindrical starch paste samples 0.8 cm in diameter and 0.5 cm high, located in sample holders of aluminum foil. The samples were wrapped with plastic film to avoid dehydration during frozen storage. Samples were frozen to a temperature of -20 °C with a freezing rate of 2 cm/h and then stored during 60 days at -5, -10, -15 and -20 °C. At different storage times (0-43 days), samples in triplicate were selected at random and processed according to the freeze-fixation method. Ice crystals sizes were obtained from the micrographs by the measurement of the holes left in the system. At least 100 crystals were analyzed with an Image Analyzer

(Zeiss Morphomat 30, Zeiss, Germany) to determine equivalent diameter distributions. The equivalent diameter was defined as the diameter of a circle that has the same surface area as the measured figure. Histograms of the relative frequencies of crystal diameters as a function of equivalent diameter were obtained during storage time at each temperature in order to determine the rate of ice recrystallization, kinetic parameters, and activation energy.

Results and Discussion

Selection of the fixative

Marked contraction of the samples was observed in solutions with high absolute ethanol concentration like Carnoy fluid. This is attributed to the osmotic pressure difference between the sample and the fixative solution. Similar results were reported by Cohen (1979) and Lee (1984).

The fixatives were formulated with the minimum concentration of ethanol compatible with the desired freezing point depression. These formulations have great versatility that permits the modification of the fixative freezing point to minimize the disruption of the frozen system.

Micrographs of the frozen starch pastes fixed with different solutions showed that samples treated with glutaraldehyde disintegrated and distorted; the characteristic network was disrupted and processing was difficult.

The fixing solution that contained phosphate salts produced in certain cases opalescence when temperature decreased and were discarded. Similar problems were reported by Hunt (1984) with respect to phosphate precipitates.

Relative contraction of the samples after each stage of the isothermal freeze fixation method are shown in Fig. 1 for the two solutions with the highest performances. The solution of absolute ethanol: formaldehyde (40 %): water (45:10:45 V:V) was the fixative selected because it showed the lowest matrix contraction. The mean total contraction factor for this fixative at the different tested temperatures was 0.81 ± 0.06 ($p = 0.05$).

Fixative diffusion

The diffusion rate of the fixative in the system at subzero temperatures was determined based on uni-directional mass transfer in a medium that was effectively semi-infinite, since penetration of the advancing front X(t) was very small with respect to the starch sample size in the mass transfer direction (x). In the selected system, the concentration of fixative at the surface $x = 0$ was constant ($c = Ce$). The diffusion coefficient changes discontinuously from a constant finite value D in the zone where ice was dissolved by the fixative solution [$0 < x < X(t)$] to a value $D = 0$ in the frozen zone [$x > X(t)$] where the fixative does not penetrate. According to Fick's law, the diffusion equation in the region $0 < x < X(t)$ is:

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \qquad (1)$$

where c is the fixative concentration at time t and position x with the following initial and boundary conditions:

$t = 0$	$c = 0$	$0 \leq x \leq \infty$
$t > 0$	$c = Ce$	$x = 0$
	$c = Cx$	$x = X(t)$

where Cx is the fixative concentration at the advancing front and Ce is the constant fixative concentration at the surface. The position of the advancing front X(t) and the solution of eq. (1) are given by Crank (1957).

$$X = b\, t^{1/2} \qquad (2)$$

where b is an empirical coefficient and t is the diffusion time.

$$g = \frac{Ce}{Cx} - 1 = \Pi^{1/2} \frac{b}{2D^{1/2}} \exp\left(\frac{b^2}{4D}\right) erf\left(\frac{b}{2D^{1/2}}\right) \qquad (3)$$

Crank (1957) provides a graphical solution of the function g in terms of $b/2D^{1/2}$.

The fixative front was assumed to be at the equilibrium freezing point. Concentration Cx was determined from the experimental freezing point curve for the fixative solution (Fig. 2) plotted as temperature versus C' (concentration of the fixative solution). A constant partition coefficient (s) was assumed in the starch paste ($C = sC'$):

$$\frac{Ce}{Cx} = \frac{C'e}{C'x} \qquad (4)$$

where C'x is the solution concentration when its freezing point coincides with the cold chamber temperature, C'e is the fixative concentration of the solution in contact with the starch paste.

Positions of the advancing front X(t) were measured as functions of time at different temperatures. Plots of X versus $t^{1/2}$ at different temperatures yielded straight lines (Fig. 3); b values were obtained from the slopes of data regression.

Experimental values of b and Ce/Cx were fitted in the graphical solution of eq. (3) to obtain the diffusion coefficients at different temperatures (Table 2). The activation energy for diffusion was estimated according to Arrhenius Law, obtaining $E_a = 95.44 \pm 1.15$ KJ/mol.

Fixation time of the samples

Data of advancing front rate, calculated diffusion coefficients, and fixative concentration distribution were used to predict fixation times of small samples based on simplified mathematical models.

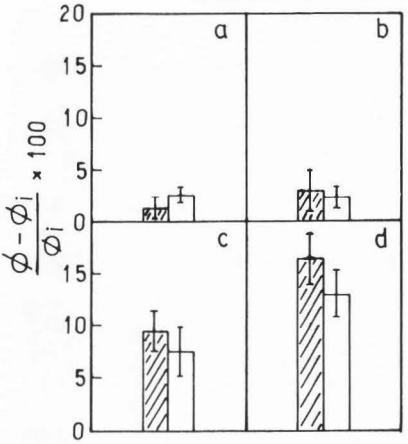

Figure 1. Relative contraction of the samples after each stage of the isothermal freeze-fixation method at -5 °C. ϕ = measured sample diameter after each stage. ϕi = sample diameter prior to fixation. Glacial acetic acid: formaldehyde (40 %):absolute ethanol:water (5:10:70:15 V:V); Absolute ethanol:formaldehyde (40 %):water (45:10:45 V:V). Bars indicate standard errors.

a) 4 days of fixation;
b) 7 days of fixation;
c) after dehydration and clearing;
d) after embedding in paraffin.

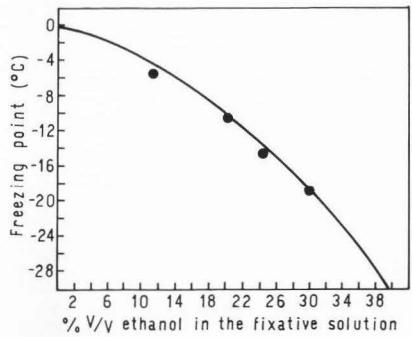

Figure 2. Freezing points of the fixative solution absolute ethanol : formaldehyde (40 %):water (45:10:45 V:V).

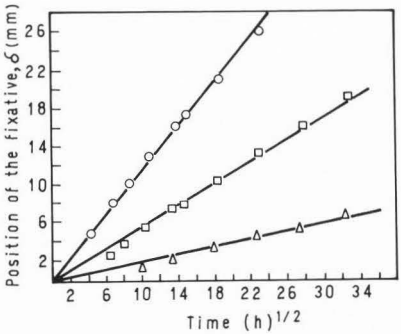

Figure 3. Effect of temperature on the position of the fixative front as a function of (time)$^{1/2}$ in frozen corn starch paste (10 % W/W) considered as a semi-infinite medium; circles: -5 °C; squares: -10 °C; triangles: -20 °C.

Table 2. Effect of temperature on the diffusion coefficient of the selected fixative in frozen corn starch pastes.

T(°C)	D(m^2/sec)
-5	$(1.21 \pm 0.2)\ 10^{-10}$
-10	$(0.60 \pm 0.06)\ 10^{-10}$
-15	$(0.21 \pm 0.05)\ 10^{-10}$
-20	$(0.11 \pm 0.06)\ 10^{-10}$

Fixation times at each temperature were estimated in two steps:

1) The time (t_1) necessary for the front to reach the center of the sample; the concentration profile at this moment ranges from Ce at the border to Cx in the center.

2) The time (t_2) necessary to increase the concentration in the center of the sample to a value c = 0.8 Ce. Previous experiments showed that this concentration was sufficient for fixing the samples.

In order to use mathematical models with analytical solutions unidirectional mass transfer was assumed for both steps. This assumption lead to overestimated values of time because actual systems have bi- or tridirectional mass transfer contributions; however this predicted time ensures fixative penetration.

For the first stage, the time (t_1) was obtained from eq. (2) using experimental data of b; a sample 4 mm thick was considered for calculation.

For the second stage it was considered that once the advancing front reaches the center of the finite sheet the diffusion coefficient is constant over the whole concentration range (Crank, 1957). In this case, the well known mathematical solution for diffusion into a plane sheet with a constant diffusion coefficient but with a

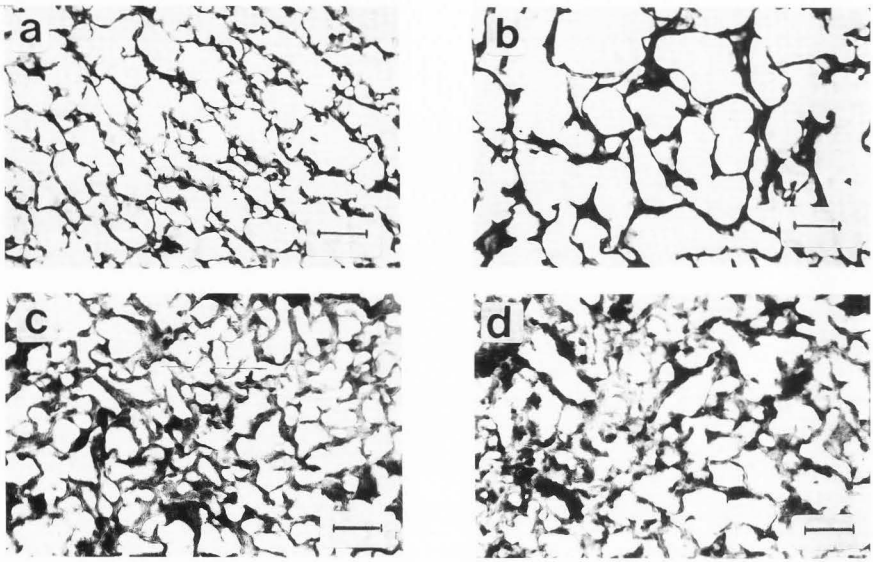

Figure 4. Micrographs of frozen corn starch pastes showing recrystallization phenomena during storage at: **(a)** -5 °C, 6 days; **(b)** -5 °C, 60 days; **(c)** -20 °C, 6 days; **(d)** -20 °C, 60 days. Bar = 100 μm.

Table 3. Effect of temperature on fixation time in a small sample 4 mm thick.

T (°C)	Cx/Ce	Fixation time (hours)		
		t_1	t_2	total
-5	0.29	3.2	5.1	8.3
-10	0.44	7.8	7.9	15.7
-15	0.61	47.2	18.8	66.0
-20	0.70	100.0	22.0	122.0

Cx = fixative concentration in the advancing front;
Ce = fixative concentration at the border of the sample.

given initial concentration distribution, can be used. In order to simplify the mathematical model it was considered that the initial concentration was uniform and equal to Cx, the minimum concentration value. This assumption led again to an over-estimation of the fixing time, but allowed the use of analytical solutions. Values of Cx/Ce at each temperature are shown in Table 3. Carslaw and Jaeger (1978) solution and Heisler charts for plane sheet (Holman, 1976) were used to estimate the time (t_2) necessary to increase fixative concentration in the center of the sample from c = Cx to c = 0.8 Ce. Diffusion coefficients, previously determined at each temperature, were used in this stage to calculate the time from its dimensionless value $t^* = D\ t/l^2$ with l = half thickness of the sample (2 mm).

Obtained values of total fixation times (Table 3) showed the important effect of fixing temperature.

Recrystallization Measurements

Micrographs of frozen starch pastes stored at different temperatures show an enlargement of the ice crystal diameters that is representative of the recrystallization process (Fig. 4). At higher temperatures and longer storage times mean equivalent diameter of ice crystals increased.

Histograms of percent relative frequency (fi) as a function of equivalent diameter derived from the micrographs, confirmed an increase of the larger crystals at the expense of the smaller ones (Fig. 5).

The mean equivalent diameter at each time was calculated as $D = \Sigma\ fi\ Di$. Curves of equivalent ice crystal diameter versus storage time (Fig. 6) showed that at different storage temperatures the tendency is to reach different limit equivalent diameters (Dl).

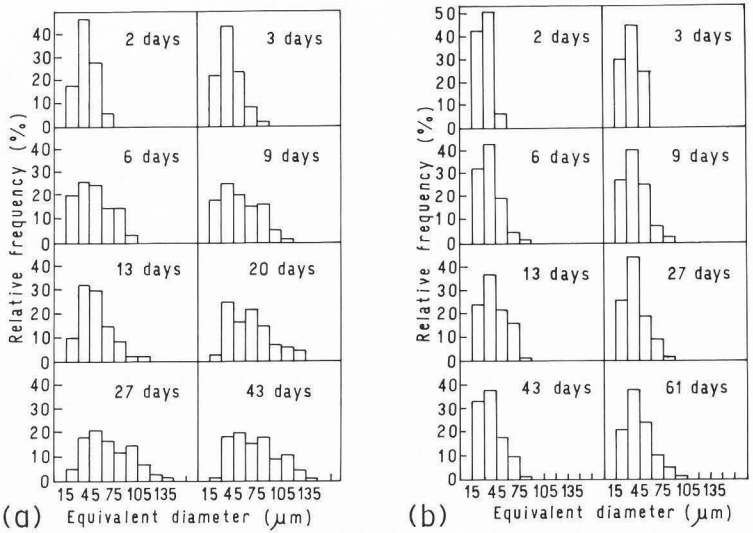

Figure 5. Histograms of percent relative frequencies of ice crystals as a function of equivalent diameters during storage at: (**a**) -5 °C, and (**b**) -20 °C.

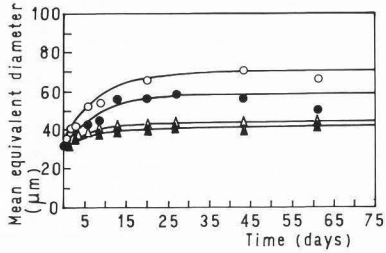

Figure 6. Recrystallization of ice in frozen corn starch pastes. Effect of time and storage temperature on ice crystal diameter. Storage temperatures: hollow circles: -5 °C; solid circles: -10 °C; hollow triangles: -15 °C; and solid triangles: -20 °C. Solid lines represent theoretical model (eq. 6).

A mathematical model that interprets recrystallization of ice in systems where grain growth has an upper limit was fitted to the experimental data; the driving force for the phenomena is the difference between the instantaneous mean curvatures of the system and the limit one (Martino and Zaritzky, 1989), i.e.:

$$\frac{dD}{dt} = k\left(\frac{1}{D} - \frac{1}{Dl}\right) \qquad (5)$$

where D is the mean equivalent diameter at time t, Dl the limit equivalent diameter and k the kinetic constant. Integration of eq. (5) led to the following expression:

$$\ln\left(\frac{Dl-Do}{Dl-D}\right) + \frac{(Do-D)}{Dl} = \frac{kt}{Dl^2} \qquad (6)$$

with Do = initial equivalent diameter.

During grain growth, as crystal size increases, the driving force declines and stops when a stable crystalline size is attained.

Kinetic constants for this model at each tested temperature were obtained by non linear regression analysis of the experimental D versus time data with the Systat program in a IBM PC X-T computer (Table 4). Experimental values of Do and limit diameter (Dl) were fitted in each case.

An Arrhenius Law activation energy (Ea = 39.32 ± 0.45 KJ/mole) was determined by linear regression of ln k = ln ko - Ea/RT.

Table 4. Kinetic constants of the recrystallization phenomena.

T (°C)	k x 10^{15} (m^2/sec) Calculated with measured diameter values	k' x 10^{15} (m^2/sec) Calculated with contraction factors
-5	4.80 (1.20)*	7.30 (1.90)
-10	3.47 (0.98)	5.30 (1.50)
-15	2.38 (0.23)	3.63 (0.35)
-20	1.69 (0.57)	2.57 (0.87)

*Standard errors of the constants between parentheses.

Table 5. Effect of ice recrystallization during fixation on ice crystal size (D) calculated with eq. (6) for an initial diameter Do = 40 μm.

T (°C)	Total fixation time (days)*	D (μm)	Relative error % [(D-Do)/Do]x100
-5	0.3	41.5	3.75
-10	0.6	41.3	2.50
-15	2.7	40.9	2.25
-20	5.0	40.8	2.00

*Obtained from Table 3.

Influence of ice recrystallization during fixation on the measured ice crystal diameters

Several authors (Hunt, 1984; Martino and Zaritzky, 1986) indicated that ice recrystallization during fixation can affect ice crystal measurements. To analyze this effect, the change in ice crystal equivalent diameters produced by recrystallization during fixation, was evaluated with eq. (6). Assuming a typical value of Do = 40 μm, values of D corresponding to the fixation time at different temperatures were estimated.

Table 5 shows that the recrystallization of ice during the fixation process has little influence on final ice crystal sizes, the relative error of the measurements being not higher than 3.75 %.

Effect of sample contraction factors on recrystallization parameters

The influence of sample contraction on the measured recrystallization kinetic constants and activation energy was analyzed on the assumption that, by geometric similarity, the contraction factors (cf) should directly affect hole diameters, that is, ice crystal sizes.

Consider that cf = D/D' = Dl/Dl' = Do/Do' with cf < 1, where the unprimed values are crystal diameters measured in contracted sample and D', Dl', Do' the hypothetical values in the uncontracted sample. This effect on the kinetic values was analyzed using the primed values in eq. (6) to yield the following equation:

$$\ln\left(\frac{Dl'^2-Do'^2}{Dl'^2-D'^2}\right)+\frac{(Do'^2-D'^2)}{Dl'^2}=\frac{kt}{c^4Dl'^2} \qquad (7)$$

As can be observed, the contraction factor affects the kinetic constant (the actual value being k' = k/cf^2) but it has no influence on activation energy of the recrystallization process; k' values (calculated with contraction factors) are also shown in Table 4.

Conclusions

An isothermal freeze fixation method was applied to analyze crystal arrangement in a 10% (W/W) frozen starch paste by light microscopy. The best fixative was the solution absolute ethanol: formaldehyde (40 %): water (45:10:45 V:V), that minimized distortion of the matrix.

The diffusion coefficients of this fixative at different subzero temperatures were determined: values ranged from $0.11x10^{-10}$ to $1.21x10^{-10}$ m^2/sec for temperatures between -20 and -5 °C respectively.

Fixing times were established with the aid of mathematical models of diffusion in one-dimensional systems. Fixation times of 8.3 hours and 122 hours at -5 and -20 °C respectively, ensured the penetration of fixative.

Recrystallization of ice during storage of the frozen starch paste was determined by the measurement of ice crystal diameters. A kinetic equation that interprets the phenomena was postulated on the basis that the driving force is the difference between the instantaneous mean curvature and the limit one. Kinetic constants and activation energy were determined.

Ice recrystallization during the fixation period has no significant influence on crystal sizes and introduces a relative error, not higher than 3.75 %, into the measurements.

The effect of matrix contraction during fixation and its influence on the kinetic constants of ice recrystallization rate was evaluated. No influence on activation energy value was observed.

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Discussion with Reviewers

Z.A. Holmes: What was the gelatinization criteria used with the polarizing microscope? If 100% loss of birefringence was the criteria, was every granule still intact as indicated in the paper? If fragmentation occurred what was the extent?
D.J. Gallant: You said that gelatinization process was analyzed using a polarizing microscope. Please indicate if the starch granules had only lost their Maltese cross, being only more or less swollen and keeping their granular shape, or if the starch granules were totally gelatinized.
Authors: We observed the gelatinized mixtures by polarized light microscopy to check the disappearance of Maltese crosses that indicate loss of birefringence due to gelatinization. Starch granules were swollen but kept their granular shape. This method allowed us to standardize the different processed batches.

D.J. Gallant: Did you verify, using a solution of Congo Red (1 % in water), the presence of swollen starch granules or membrane ghosts? This technique [Schulz AP, Steinhoff G (1932) Beitrage zur mikroskopischen Untersuchung der starke mittels farbungen. Z. Spiritusind. **55**, 162] is elegant, simple, specific for all swollen starch granules or starch granules showing swelling in progress.
Authors: We did not use Congo Red technique to verify gelatinization. Evers (1979) stated that gelatinization temperature is recognized either by loss of birefringence or by uptake of dyes, we used the first criterium. When 98 % of the granules no longer exhibit birefringence, it is commonly taken as the birefringence end-point.

Z.A. Holmes: What was the final gelatinization temperature variation? With the technique used, this is critical. A range of 62 to 72 °C is indicated; however the "mix reached 80 °C in approximately 6 minutes and remained at an average temperature of 83 °C" seems variable.
Authors: Final gelatinization temperature was 72.7 $\pm$ 0.6 °C and was measured with differential scanning calorimetry (DSC).

D.J. Gallant: You wrote that heating rates were 17 °C/min from 30 to 50 °C and 8 °C/min from 62 to 72 °C; the first step corresponds to an heating rate of 0.28 °C/sec, and the second step to 0.13 °C/sec. That is quite fast. In this case, I understand that you needed vigorous stirring (680 rpm) so that the temperature was uniform in the heating batches of 400 ml with 10 % commercial corn starch. In such a case, did you not fear that starch gel network would be disrupted by shearing forces? What are the consequences of this step on the distribution of the two main components of the starch paste, amylose and amylopectin, and what are the consequences on the "fixative" conditions?
Authors: Yes, we used a helical mixer at 680 rpm so that the temperature was uniform. This kind of mixer does not produce significant damage to starch granules. Microscopic observation, with polarized light, of the gelatinized pastes showed that the granules were swollen, but kept their identity. All our batches were obtained with the same conditions. In any case, our opinion is that the order of magnitude of the obtained parameters (diffusion coefficients and activation energy) will not change significantly with the preparation procedure.

Z.A. Holmes: How were the thermocouples placed accurately in the center and on the surface of a sample 0.8 cm in diameter and 0.5 cm height in a foil dish. How was the heating rate calculated?
Authors: We used a control sample holder with an orifice to introduce and locate the thermocouples.

The freezing rate of 2 cm/h was calculated from the experimental data: 6 minutes were necessary to change the temperature from 0 °C at the surface to -10.6 °C at the thermal center of the sample. The distance between these points in the direction of the heat flux was 0.2 cm; hence the 2 cm/h freezing rate.

Z.A. Holmes: What was the role of fixative? How was it selected? What criteria were used?
Authors: Isothermal freeze-fixation was used to maintain the starch paste structure which ensured ice crystal pattern observation.

The different fixatives assayed (Table 1) were selected in order to minimize volume contraction, improve sectioning capability, rigidity and cohesion of the material.

Once the fixative was selected we measured ice crystal sizes, taking into account contraction of the matrix and how it modifies the value of the kinetic constants.

D.J. Gallant: Fixatives you used have antagonistic effects. For example, using pure ethanol on gelatin, volume of gelatin is reduced by half; using pure glacial acetic acid, volume increases three and a half times. For these reasons, Lillie [Bull. Intern. Assoc. Med. Museum, **29**(1), 1949, composition cited in Ganter and Jolles, 1970] determined the formulation of a fixative acting very slightly or without any changes on substrate size. This fixative, spelled "F.A.A.", is very well known and is composed of commercial formaldehyde (40 %), glacial acetic acid, absolute ethanol and water: 10:5:75:10. Why did you not use Lillie's fixative as a reference?
Authors: The inclusion of ethanol in the formulations was necessary to drop the freezing point of the fixative

solution. The formulation 2, used in our work, is actually: acetic acid, formaldehyde 40 %, absolute ethanol and water (5:10:70:15), similar to that used by Lillie (F.A.A.).

D.J. Gallant: In your study you compared actions of several "fixative solutions" including formaldehyde, ethanol and water and, most of time, with the addition of acetic acid. Such components are generally known to react with proteins (as it can be found in several cytochemical handbooks and papers). Please give references and explain the chemical reactions that occur between these chemical components and starch pastes.

Authors: There are no set rules as to which combination of fixatives and procedures are best. Generally, it is necessary to test a variety of fixatives and select the one that gives the best overall fixation quality (Bechtel, 1990). A wide range of fixing agents, concentration and buffering systems are reported in literature (see text references: Varriano-Marston, 1977; Angold, 1979; Chabot, 1979; Falk, 1980; Bechtel, 1990).

Kalab (1983) pointed out that there are no suitable techniques for fixation of starch. However, most of the works on starch pastes, doughs, baked goods and cereals reported the use of aldehydes like glutaraldehyde or formaldehyde as fixatives. Bechtel (1990) stressed that for cytochemical carbohydrate localization, formaldehyde fixation seems to be the best choice, but lack of quality fixation usually forces the use of glutaraldehyde.

Aldehydes, such as formaldehyde, acetaldehyde and acrolein, react bifunctionally to cross-link starch, forming acetals (Rutenberg and Solarek, 1984). Also the formation of methylene bridges ($-CH_2-$) are mentioned by Pearse (1960). The reaction between formaldehyde and reactive hydrogen atoms of the component to be fixed (starch in our case) can be described as follows:

$$RH + CH_2O \rightleftarrows R\,CH_2(OH)$$

$$R\,CH_2(OH) + HR' \rightleftarrows RCH_2R' + H_2O$$

All the mentioned formaldehyde reactions are susceptible to rupture by hydrolysis. However, we worked with frozen starch gelatinized samples, thus, water fraction available for hydrolysis reactions is reduced. Also the presence of ethanol in the fixative formulation decreases water activity. Both factors contribute to the stability of the formaldehyde combination products.

D.J. Gallant: The best fixative is when no recrystallization occurs. Why did you also not try testing cryoprotectants in your formulations and why did you not use, for useful comparison, the very fast freezing rates as commonly used in the freeze-etching techniques? An interesting formulation by Haggis (cited in Nei, 1974) is dioxane, water and glycerol (49.5:49.5:1).

Authors: The aim of our work was to analyze recrystallization of ice in starch pastes during storage at different temperatures that simulate commercial storage of frozen food. Freeze-etching requires very low temperatures that increase the amount of frozen water in the system.

We used the isothermal freeze-fixation because it minimizes ice pattern modifications in the system. However, during diffusion of the fixative (if it is slow) recrystallization of ice can occur, modifying ice crystal sizes (MacKenzie *et al.*, 1975). One of our objectives was to estimate the amount of crystal diameter changes due to recrystallization during fixation.

With regard to the use of cryoprotectants, these substances are commonly applied to reduce or eliminate the development of ice crystals when samples are examined by electron microscopic methods like freeze-fracture. In this technique the most critical step is rapid freezing of the sample. But this is not so in our case because we already have a frozen sample, and we want to alter the ice crystal pattern as little as possible. Moreover, Kalab (1983), pointed out that cryoprotectants are known to introduce artifacts in unfixed samples and for this reason it is recommended to fix samples in advance.

D.J. Gallant: Knowledge of what happens in frozen paste samples using isothermal freeze fixation is indeed of certain interest. But you have chosen the indirect way only. Are you certain that between the step you called "fixation", conducted at low temperature, and other steps, conducted at room temperature, no recrystallization occurred? Why did you not compare your results with direct observations obtained using cryostages either with light microscope or with scanning electron microscope? Or more simply, if such equipment was not available, why did you not compare indirect results with results obtained with very small samples: first frozen with very fast freezing rate, then lyophilized, and finally, embedded in plastic or paraplast?

Authors: When the fixative reaches the center of the sample, ice disappears and further recrystallization does not occur because there is no ice present in the sample. Once fixation is completed, the other steps of the technique can be conducted at room temperature because the frozen pattern was retained.

We could not compare our results with direct observations using cryostages with light microscopy because the obtained images were not clear enough to compare crystal diameter sizes. In previous works we used direct observation of ice crystals in solutions obtaining satisfactory results (Martino and Zaritzky, 1987, 1989).

Several experiments were performed with lyophilized samples but the results obtained were not satisfactory.

H.G. Schwartzberg: Frozen starch solutions have very high collapse temperatures and can be freeze dried using temperatures that are far higher than -45 to -80 °C. I have done so many times. -20 °C or even -10 °C, can be used as a condenser temperature.

Authors: The freeze drying method gave us unsatisfactory results, because the samples did not maintain their

integrity to stand embedding and sectioning procedures. Temperatures of -45 to -80 °C are recommended in general to prepare freeze dried samples for microscopic observation, we agree that a particular product like starch can be processed at higher temperatures.

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