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# FIXING CONDITIONS IN THE FREEZE SUBSTITUTION TECHNIQUE FOR LIGHT MICROSCOPY OBSERVATION OF FROZEN BEEF TISSUE

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## Abstract

The freeze substitution histological technique allows the indirect light microscopic observation and the quantitative evaluation of ice crystal size in frozen tissues. The use of chemical fixatives in substituting fluids improves morphological and histochemical preservation of the tissue. Fixation conditions become important since this step can introduce modifications in crystal sizes. Effects of temperature on: a) diffusion rate of fixing solution in tissue, b) recrystallization rate of ice in frozen beef and c) variations of the frozen water fraction were analyzed, establishing that isothermal freeze fixation constitutes an appropriate method for histological observation. Fixing at temperatures lower than that of the sample, in an attempt to reduce recrystallization effects, involves an increase of the frozen water fraction in the tissue leading to modifications in size of ice crystals and to changes in the histological structure.

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## Introduction

Examination of frozen tissue by light microscopy shows differences in the cell structure with regard to the location of ice crystals (intra- or extra - cellular) and fiber disruption. This examination can be performed in two ways: a) direct observation of ice crystals formed in the tissue (microscope equipped with cryo-stage) and b) indirect observation which considers voids to represent the spaces occupied by ice crystals in the specimen (freeze - substitution and freeze - drying techniques). Indirect observation is the more common method of choice.

Freeze substitution is based on the dissolution of ice within the tissue at low temperature in a fluid solvent containing chemical fixing agents which contribute to the morphological and histochemical preservation of the tissue. Fixing agents act mainly by introducing new cross-linkages between and within the molecules of the tissue constituents, thus immobilizing its structure (Feder and Sidman, 1958).

Traditionally, freeze substitution for both light and electron microscopy required that frozen tissues be cooled to very low temperatures, down to -20°C(Fernández-Morán, 1960; Van Harreveld and Crowell, 1964; Van Harreveld et al., 1965; Rebhun, 1972; Asquith and Reid, 1980). Richardson and Scherubel (1908) first reported observations on the use of ethanol as a substituting agent to examine ice crystal formation in frozen tissues. Van Hulle et al. (1965) compared direct observation with the fixing - staining procedure showing that tissue voids represent the size and location of ice crystals and that the freeze substitution technique did not result in any gross misrepresentations of the frozen structure. If fixation temperature differs from that of the sample, melting of ice or additional freezing of water can occur during fixation step, thus changing the ice crystal measurements (Kaess and Weidemann, 1961; MacKenzie, 1972, 1975; MacKenzie et al., 1975; Lampila et al., 1985).

Bevilacqua et al. (1979) and Bevilacqua and Zaritzky (1980) used the isothermal freeze substitution technique to analyze the effect of freezing on the ice morphology of beef muscle.

Freezing is one of the more common methods of food preservation. Storage temperatures lower

than - 18°C are indicated to retard frozen food deterioration during prolonged periods but involve considerable energy costs (Jul, 1984). When only short storage times are required the technique of partial freezing (super-chilling or light freezing) with storage temperatures close to - 5°C can be used to reduce energy consumption (Aleman et al., 1982).

Histological analysis of partially frozen tissues can be performed using isothermal freeze fixation technique. Since the diffusion rate of a fixing solution is not instantaneous, one concurrent phenomenon such as recrystallization could modify crystal sizes and shapes during fixation, thus altering subsequent measurements (MacKenzie et al., 1975).

Recrystallization (grain growth) is determined by the average grain size enlargement without increasing the total crystalline mass. Driving forces are essentially differences in surface energy of the grain boundaries, which allow the growth of the larger crystals at the expense of the smaller ones (Luyet, 1966; Love, 1966, 1968; Fennema, 1973). Recrystallization rate in muscle tissue increases with temperature and becomes important at thermal levels above - 7°C (Sy and Fennema, 1973; Voyle, 1974; Gerrits and Jansonius, 1975; Bevilacqua and Zaritzky, 1982).

The objective of the present study was to establish adequate fixing conditions (time and temperature) for application of the freeze substitution technique in the measurement of ice crystal sizes in frozen beef tissues especially at high subzero temperatures. Analysis was based on the evaluation of the following phenomena occurring in the samples: variation of the frozen water fraction with temperature, diffusion of the fixing solution and ice recrystallization rate in tissue.

## Materials and Methods

### Freezing Procedure

Beef samples (Semitendinosus muscle, 60 hours post-mortem) were frozen in a heat exchanger where methanol from a Lauda UK 50 D-W Cryostat was circulated with a temperature control of + 0.1°C. The freezing rate was regulated by modifying the temperature of the refrigerant (- 20 to - 30°C) and interposing acrylic slabs between the meat and the heat exchanger. Heat flux from both sides was unidirectional and parallel to the muscle fibers. Lateral insulation was provided by the use of expanded polystyrene 5 cm thick. The corresponding thermal histories were monitored by copper-constantan thermocouples which were inserted in the border and center of the meat pieces. Freezing rate was represented by the time necessary for the central point of the sample to change its temperature from - 1 to - 5°C (characteristic freezing time, $t_r$).

### Determination of the Fixative Diffusion Coefficient

The freeze substitution method was applied using Carnoy fluid as the fixative (absolute ethyl alcohol 60%, chloroform 30% and glacial acetic acid 10%, v/v). Ethanol solutions of picric acid (1%) and mercuric chloride (1%) were also assayed. According to the results of Bello et al. (1981) picric acid denatures the proteins of the tissues and coagulates them, but causes extreme shrinkage; mercuric chloride distorts the tissue less than picric acid does. The general appearance of the tissue was improved using Carnoy fluid as in previous works (Bevilacqua et al, 1979; Bevilacqua and Zaritzky, 1980, 1982).

The diffusion coefficient of the Carnoy fluid in frozen beef tissue was measured at different temperatures in meat samples simulating unidirectional mass transfer conditions. Beef samples of 3.5 x 3.5 x 1 $cm^3$ were frozen as described above reaching final uniform temperatures of -4, - 10, - 20 and - 26°C. Carnoy fluid with Methylene blue (0.1% W/V), used as an indicator to facilitate visualization of diffusion through the tissue, was allowed to cool in storage chambers to the same final temperatures. Specimens were transferred to a volume of fixative that was 100 times the tissue volume and freeze fixation was allowed to proceed isothermally. Depth of penetration ($\delta$) of the fixative was measured photographically under an S.R. Zeiss stereomicroscope equipped with a Zeiss MC 63 Camera at 6 hour intervals. Experiments at the assayed temperatures were performed in triplicate.

### Histological Method. Effect of Fixing Temperature

Beef samples 5 cm in diameter and 1 cm thick were frozen as described above reaching a uniform final temperature of -5°C (typical of a partially or lightly frozen tissue). For histological analysis (freeze substitution method) small pieces of 0.5 cm in diameter and 1 cm thick were cut from the initial specimens in a cold chamber at - 5°C to avoid thermal changes. These specimens were fixed at two different temperatures (- 5 and - 15°C) to analyze the effects of this step on ice crystal morphology. Experiments were performed in quadruplicate.

Fixation times at each temperature (t) were obtained from the results of the diffusion coefficient experiments previously described. Once this step was accomplished, samples were brought to room temperature, dehydrated using a series of gradually increasing concentrations of ethyl alcohol, cleared in benzene and embedded in paraffin (56-58°C). The sectioning was done with a rotary microtome (American Optical, model 820) using a steel knife; section thickness ranged from 8-10 µm. Sections were mounted on glass slides and stained with haematoxylin-eosin.

### Ice Crystal Measurements

Micrographs of the frozen specimen sections were obtained with a Leitz Ortholux II microscope equipped with a Leitz Vario-Orthomat Camera . A Zeiss Morphomat 30 semi-automatic image analyzer working on the principle of coordinate measurement by quantifying the propagation time of magnetically induced waves (magnetostriction), was used to obtain, using an adequate internal program, the equivalent diameter of the holes left by the crystals in the tissue and the total area occupied by ice. The equivalent diameter ($D_e$) was defined as the diameter of the circle which has the same surface area as the measured figure.

Mean crystal diameters and standard deviations were obtained from a frequency distribution of more than one hundred crystals per field. Confidence limits for the mean crystal diameter were calculated using the Student "t" test ($P > 0.05$).

## Results and Discussion

### Effects of Fixing Temperature on Histological Structure of Frozen Beef Tissue

Histological transverse sections of bovine semitendinosus muscle (60 hours post-mortem), frozen at - 5°C with a characteristic freezing time $t_c$ = 20 min, and fixed at - 5 and - 15°C, are illustrated in Fig. 1 a) and b) respectively. The analysis of the micrographs in the image analyzer equipment showed that samples fixed at - 15°C have a significantly ($P < 0.05$) higher proportion of ice and larger mean crystal diameter than those at - 5°C. The experimental ratio of areas occupied by ice at - 15°C with respect to - 5°C was 1.20 and the corresponding mean equivalent diameters were $D_e$ = 20.46 ± 1.71 µm at - 15°C and $D_e$ = 12.41 ± 2.84 µm at - 5°C. These results are attributed to the effect of temperature on the frozen water fraction in the tissue. According to Fig. 2, 85% of total water in beef muscle is frozen at - 15°C and only 74% at - 5°C (Riedel, 1957), leading to a theoretical ratio of frozen water fraction at these temperatures of 1.18 close to the experimental value of 1.20 obtained from the micrographs.

It can be observed that fixing at temperatures lower than that of the sample in an attempt to reduce recrystallization effect at high subzero temperatures, involved an increase of the frozen water fraction of the tissue which led to a different histological structure.

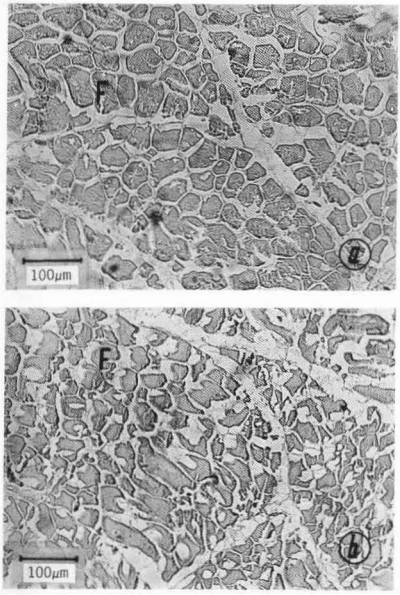


Fig. 1. Transverse section of beef (Semitendinosus muscle) frozen at - 5°C. a) fixed at - 5°C, b) fixed at - 15°C. Dark zones correspond to area occupied by fibers (F).

### Determination of the Diffusion Coefficient of Fixative in Beef Tissue

To compare the use of low fixing temperatures with isothermal freeze fixation at high subzero temperatures, fixative diffusion coefficient and influence of recrystallization during fixation have to be evaluated.

A typical photograph indicating Carnoy fluid penetration in frozen beef tissue is shown in Fig. 3. The system can be mathematically considered as a semi-infinite media since during the

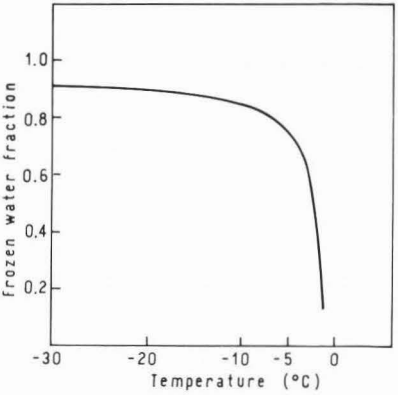


Fig. 2. Equilibrium freezing point curve for beef tissue. Initial water content of the tissue on wet basis: 74%. From Riedel, (1957) and Mascheroni and Calvelo, (1978).

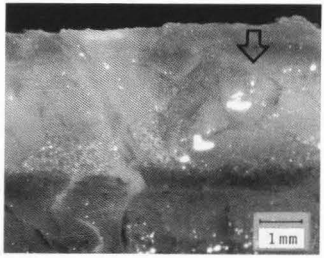


Fig. 3. Diffusion of the fixative in frozen tissue. The light zone shows penetration depth ($\delta$) of Carnoy fluid with methylene blue ($\delta$ = 0.34 after one day of fixation at - 10°C). Arrow in the border of the tissue indicates direction of the diffusion flux.

experiment fixing penetration depths were small in comparison with sample thickness in the direction of mass transfer. Values of fixing solution penetration ($\delta$) as a function of time were used to determine diffusion coefficients (Crank, 1957) according to:

$$\delta = 4\sqrt{\mathcal{D}t} \qquad (1)$$

where: $\delta$ : depth of fixative penetration (m); $\mathcal{D}$ : diffusion coefficient of fixative ($m^2/s$), t : time (s). Values of $\mathcal{D}$ were calculated at different temperatures (Table 1). Activation energy ($E_a$) for the diffusion process of fixing solution was determined according to an Arrhenius type dependence on temperature:

$$\mathcal{D} = \mathcal{D}_0 \, e^{-Ea/RT} \qquad (2)$$

where $\mathcal{D}_0$ is the pre-exponential factor. The activation energy was estimated from the linear regression of ln $\mathcal{D}$ versus 1/T (Fig. 4) obtaining Ea = 57,560 ± 2390 J/mole. Eqs. (1) and (2) allowed the calculation of total fixation times ($t_f$) expressed in hours at different temperatures:

$$t_f = \frac{\delta^2 \; 1.736 \; 10^{-5}}{\mathcal{D}_0 \; e^{-Ea/RT}} \qquad (3)$$

Values of $t_f$ (Table 2) were obtained considering $\delta = 0.25 \times 10^{-2}$ m, which corresponded to the radius of the samples used for the histological treatment.

The determination of diffusion coefficients for the fixing solution in frozen beef allowed estimation of total fixation times at different temperatures, accelerating the operation of the freeze substitution technique.

Table 1. Effects of temperature on the diffusion coefficient of fixing solution ($\mathcal{D}$).

| T (°C) | $\mathcal{D} \times 10^{11}$ ($m^2/s$) |
|---|---|
| - 4 | 1.460 ± 0.007[a] |
| - 10 | 0.834 ± 0.006 |
| - 20 | 0.454 ± 0.124 |
| - 26 | 0.125 ± 0.006 |

a = 95% confidence limits of mean values calculated from three replicates.

Table 2. Effects of recrystallization on mean crystal diameters during fixation.

| T (°C) | $t_f$ (hrs) | Changes of ice crystal diameter due to recrystallization Δ (μm) $D_0$ = 30.0[a] ± 2.6[b] μm | $D_0$ = 50.0 ± 3.2 μm |
|---|---|---|---|
| - 5 | 8 | 0.79 | 0.48 |
| - 10 | 12 | 0.84 | 0.51 |
| - 15 | 21 | 0.97 | 0.59 |
| - 20 | 35 | 1.08 | 0.66 |

T: fixing temperature; $t_f$: total fixation time (correspond to specimen radius = 0.25 cm); $\Delta = D - D_0$ with $D_0$: initial mean crystal diameter and D: mean crystal diameter at total fixation time; a: mean values from four replicates; b: 95% confidence interval for $D_0$ value.

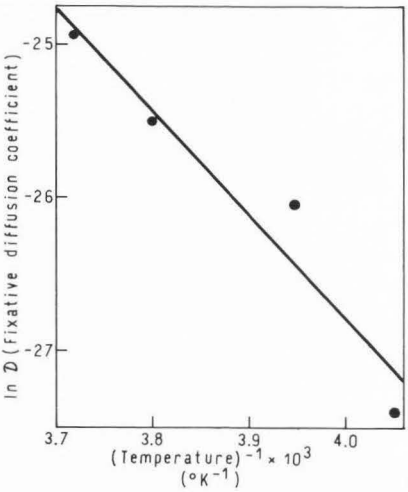


Fig. 4. Determination of the activation energy for the diffusion of the fixing agent in frozen beef tissue. Correlation coefficient r = 0.97. Each point is the mean of three replicates.

Recrystallization Effects during Fixation

Recrystallization could modify the crystalline structure during the diffusion of the fixative particularly at temperatures near -1.1°C (initial freezing point of beef tissue). To estimate this effect, increments in mean ice crystal diameters during fixation time were calculated according to the ice recrystallization kinetics in frozen beef proposed by Bevilacqua and Zaritzky (1982):

$$dD/dt = K/D \qquad (4)$$

that gives the following:

$$D^2 - D_0^2 = 2 \, K \, t \qquad (5)$$

where: D : mean ice crystal diameter at fixation time t; $D_0$ : initial mean ice crystal diameter; K : recrystallization kinetic constant, with:

$$K = K' \, e^{-EaR/RT} \qquad (6)$$

where:

K' (preexponential factor)= $1.67 \times 10^{-5}$ $m^2/s$; $Ea_R$(activation energy of recrystallization phenomenon)= $4.35 \times 10^4$ J/mole.

The difference (Δ) between the average ice crystal diameter at the total fixation time and the initial diameter ($\Delta = D - D_0$) should indicate the enlargement of ice crystals due to recrystallization during fixation.

Effects of initial crystal diameters and fixing temperature on Δ were estimated using Eq. (5) with $t = t_f$ (total fixation time). Two

typical average values of initial extracellular ice crystal with their confidence limits were considered (Table 2). As can be observed large initial crystal diameters led to smaller changes in $\Delta$ because the driving force for recrystallization increased with the average curvature of the surface (reciprocal of the mean crystal diameter, Eq. 4). Changes in the size of ice crystals during fixation at different temperatures were not statistically significant ($P > 0.05$) because increments in size due to recrystallization laid in the 95% confidence interval for the corresponding $D_o$ value (Table 2).

Results confirmed that isothermal freeze fixation was an appropriate method for analyzing the histological structure and ice crystal pattern in frozen tissues. It was demonstrated that changes in crystal size caused by recrystallization were not statistically significant ($P > 0.05$) even when sample temperature was as high as - 5°C since the simultaneous increase of the fixative diffusion rate reduced total fixation times. When the temperature of the sample is lower than - 15°C isothermal freeze fixation is also recommended. In this case, however, effects of recrystallization and percentage conversion of water to ice during the fixing step were notably reduced and an eventual fluctuation of the fixing temperature would not introduce any significant change in ice crystal sizes.

## Conclusions

Determination of temperature effect on fixative diffusion coefficients allowed estimation of total fixing times thus accelerating the operation of the freeze substitution technique. Isothermal freeze fixation was an appropriate method for analyzing ice crystal patterns in frozen beef tissue even at high subzero temperatures (lightly frozen tissues). Fixing at temperatures lower than that of the sample in an attempt to reduce recrystallization effects (grain growth) involved an additional freezing of water in the tissue that changed ice crystal measurements. Experiments showed that recrystallization effects during isothermal freeze fixation produced non-significant ($P > 0.05$) changes in ice crystal diameters even when sample temperature was close to - 5°C because fixation times were notably reduced.

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## References

Aleman M, Kakuda K, Uchiyama H. (1982). Partial freezing as a means of keeping freshness of fish. Bull. Tokai Reg. Fish. Res. Lab. 106: 11-26.

Asquith MH, Reid DS. (1980). The preparation of frozen samples for electron and light microscopy by isothermal freeze-fixation at temperatures down to - 20°C. Cryo-Letters 1: 352-359.

Bello RA, Luft JH, Pigott GM. (1981). Improved histological procedure for microscopic demonstration of related changes in fish muscle tissue structure during holding and freezing. J. Food Sci. 46: 733-737, 740.

Bevilacqua AE, Zaritzky NE. (1980). Ice morphology in frozen beef. J. Food Technol. 15: 589-597.

Bevilacqua AE, Zaritzky NE. (1982). Ice recrystallization in frozen beef. J. Food Sci. 47: 1410-1414.

Bevilacqua AE, Zaritzky NE, Calvelo A. (1979). Histological measurements of ice in frozen beef. J. Food Technol. 14: 237-251.

Crank J. (1957). The Mathematics of Diffusion, Oxford University Press, London, 30-37.

Feder N, Sidman RL. (1958). Methods and principles of fixation by freeze-substitution. J. Biophys. and Biochem. Cytol. 4 (5): 593-609.

Fennema OR. (1973). Nature of the freezing process. In Low Temperature Preservation of Foods and Living Matter, Fennema OR, Powrie WD, Marth EH (eds), Marcel Dekker, Inc., New York, 202-207.

Fernández-Morán H. (1960). Low temperature preparation techniques for electron microscopy of biological specimens based on rapid freezing with liquid Helium II. Ann. N.Y. Acad. Sci. 85: 689-713.

Gerrits AR, Jansonius FAT. (1975). Recrystallization of ice crystals in frozen muscle tissue of broilers. Spelderholt Medeling 233 (16): 1-9.

Jul M. (1984). Energy and Cost. In the Quality of Frozen Foods, Harcourt Brace Jovanovich (ed), Academic Press, London, 256-260.

Kaess G, Weidemann JF. (1961). Freezer burn as a limiting factor in the storage of animal tissue. II. Histological study of freezer burn of liver. Food Technol. 15 (3): 129-133.

Lampila LE, Mohr V, Reid DS. (1985). Scanning electron microscopic study of rockfish preserved at either ambient temperature or by isothermal freeze-fixation. Food Microstructure 4: 11-16.

Love RM. (1966). The freezing of animal tissue. In Cryobiology, Meryman HT (ed), Academic Press, London, 317-405.

Love RM. (1968). Ice formation in frozen muscle. In Low Temperature Biology of Foodstuffs, Hawthorn J, Rolfe EJ (ed), Pergamon Press Ltd., Oxford, U.K., 105-124.

Luyet BJ. (1966). Anatomy of the freezing process in physical systems. In Cryobiology, Meryman HT (ed), Academic Press, London, 115-138.

MacKenzie AP. (1972). Freezing, freeze-drying, and freeze substitution. Scanning Electron Microscopy, 1972: 274-280.

MacKenzie AP. (1975). The physico-chemical environment during the freezing and thawing of biological materials. In Water Relations of Foods, Duckworth RB (ed), Academic Press Inc. Ltd., London, 477-503.

MacKenzie AP, Kuster TA, Luyet BJ. (1975). Freeze-fixation at high subzero temperatures. Cryobiology 12: 427-439.

Mascheroni RH, Calvelo A. (1978). Modelo de descenso crioscópico en tejidos cárneos. La Alimentación Latinoamericana 11 (111): 34-39,42.

Rebhun LI. (1972). Freeze-substitution and freeze-drying. In Principles and Techniques of Electron Microscopy: Biological Applications, Hayat MA (ed), Van Nostrand Reinhold, N.Y., 2: 3-49.

Richardson WD, Scherubel E. (1908). The deterioration and commercial preservation of fresh foods. First paper: General introduction and experiments on frozen beef. J. Am. Chem. Soc. 30: 1515-1564.

Riedel L. (1957). Kalorimetrische untersuchungen uber das gefrieren von fleisch. Kaltetechnik 9: 38-40.

Sy DH, Fennema OR. (1973). Rates of recrystallization in beef liver tissue. In Proceedings of the International Congress of Refrigeration, 13th Washington, 3, AVI Publ. Co. Inc., Westport, CT. 199-204.

Van Harreveld A, Crowell J. (1964). Electron microscopy after rapid freezing on a metal surface and substitution fixation. Anat. Rec. 149: 381-386.

Van Harreveld A, Crowell J, Malhotra SK. (1965). A study of extracellular space in central nervous tissue of freeze-substitution. J. Cell. Biol. 25: 117-137.

Van Hulle G, Fennema O, Powrie WD. (1965). A comparison of methods for the microscopic examination of frozen tissue. J. Food Sci. 30: 601-603.

Voyle CA. (1974). Structural and histological changes associated with the freezing of meat. In Meat Freezing - Why and How?, Proc. M.R.I. Symposioum No 3, Cutting CL (ed), Meat Research Institute, Langford, Bristol, 6.1-6.6.

## Discussion with Reviewers

M. Brown: Why do you consider the evaluation of recrystallization effects important?
Authors: Reported data showed that recrystallization rates in frozen beef tissues are relevant at high subfreezing temperatures. Since fixative diffusion in freeze-fixation proceeds with a finite velocity, recrystallization phenomenon during fixation time can increase ice crystals modifying the initial pattern. Therefore, one of the objectives of this study was to evaluate this change. However, finally we demonstrated that recrystallization effect was negligible because diffusion rate of the fixative increases at high subfreezing temperatures reducing fixing time and the possibility of recrystallization during this stage of the histological analysis.

S. Cohen: How do the authors define lightly frozen tissue?
Authors: Lightly frozen or partially frozen beef tissue is that submitted to temperatures between - 5 to - 1.1°C (high subfreezing temperatures), having therefore a low proportion of water converted to ice. This kind of tissue becomes important since partial freezing improves the quality of the products with respect to the refrigeration process.