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Original scientific paper

Investigation of the physico-chemical and microstructure changes of beef meat during frozen storage at −23°C

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A b s t r a c t: Freezing beef meat is the most effective way to extend its storage life. However, there is little information about whether this practice alters the microstructure of beef and its effects on meat quality. For this reason, the object of our research was to determine the effect of frozen storage (one year at −23°C, with meat examined every two months) on physical, chemical and microstructural properties of beef in cuts of 20 Biceps femoris muscles. Significant physical changes were detected at different frozen storage durations, including increases in pH and yellowing (b), as well as decreases in water activity, lightness (L*), and redness (a*). In terms of chemical characteristics, the protein solubility in the beef reduced, but lipid oxidation (TBARS) values considerably rose with frozen storage duration. The width of ice crystals in frozen beef steadily increased as storage time was extended to 12 months, indicating structural changes in the frozen meat.*

Keywords: *beef, physico-chemical parameters, microstructure, freezing, storage time.*

Introduction

Meat is an important part of a well-balanced and varied diet, since its nutritional components fulfil the demands of the majority of people for essential nutrients, including protein, minerals and vitamins (*Rahman et al.,* 2015). The worldwide beef meat export industry is valued over US$ 13 billion, and demand is growing at a rate of 3.5 % each year (*Leygonie et al.,* 2012a). Freezing is frequently used in the food industry to preserve perishable substances for lengthy periods of time (*Wang et al.,* 2020). Freezing, in particular, is important for keeping meat safe, allowing the meat industry to tailor its production to customer demand, adapt meat output to processing rates, and export meat to all parts of the world. The freezing technique employed in meat preservation is mainly concerned with slowing the growth and proliferation of meat spoilage microorganisms as well as delaying other deleterious changes to colour, odour, texture, moisture and ultrastructure (*Zhang et al.,* 2019).

As a result of freezing, ice crystals cause mechanical damage, structural changes and protein denaturation in meat (*Jeong et al.,* 2011). Ice crystals disrupt the ultrastructure of meat and concentrate solutes, causing metabolic changes and influencing the meat's physical qualities (*Leygonie et al.,* 2012b). One of these changes is a reduction in water-holding capacity that reduces the tenderness and the overall eating quality of the meat, lowering its commercial value (*Kim et al.,* 2015)frozen only, and 3 or 4. weeks ageing at −1.5°C then frozen. Protein oxidation also lowers meat quality by causing fragmentation or aggregation of proteins and diminishing protein solubility *(Sun et al.,* 2002). Phospholipids containing a wide variety of fatty acids including polyunsaturated fatty acids are the lipid component of meat that is most sensitive to oxidation. As a result, radical secondary lipid oxidation can be produced during meat freezing (*Medić et al.,* 2018), resulting in undesired alterations in meat quality (*Deng et al.,* 2021). Therefore, researchers must have a complete understanding of the physicochemical and structural changes caused by frozen storage of meat (*Bertram et al.,* 2007).

Numerous studies have reported on the impact of long-term preservation on physicochemical and biochemical changes in meat of different animal species (*Muela et al.,* 2015; *Medić et al.,* 2018;)although the influence of frozen holding temperatures was negligible. LL carbonyl, and nitrate and nitrite content responses were variable and yet broadly reflected an increased incidence of protein oxidation

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across increasing chilled storage and ensuing frozen storage periods — this aspect meriting future exploration. Total myoglobin content and the estimated myoglobin redox fractions (metmyoglobin, deoxymyoglobin, and oxymyoglobin. Regardless, there are just a few studies in the literature on the impact of ice crystal development on physico-chemical changes during long-term frozen beef storage. It appears important to highlight the consequences of these significant alterations due to freezing, in order to anticipate the quality of frozen beef. The aim of this study was to investigate the effect of prolonged frozen storage on the quality of beef by looking at the physicochemical changes of the meat and its tissue structure.

Materials and methods

Twenty randomly selected beef carcasses from a commercial slaughterhouse in the Algerian city of Batna were sampled. At 24 h postmortem, sections of the femoral biceps were selected. Each sample was divided into portions of equal approximate weight that were individualy vacuum packed and frozen at −23°C in a freezer (CRF-NT64GF40, Condor, Algeria). The temperature during frozen storage was monitored with an infrared thermometer (TIA 101, China). Fresh samples (n = 20) were analysed before freezing then portions from the same muscle were analysed after 2, 4, 6, 8, 10 and 12 months of storage.

Determination of physico-chemical changes on fresh and frozen/thawad beef

Meat pH

Meat pH was measured according to the procedure of *Zhu et al.* (2020). A sample of beef (fresh or frozen/thawed) was minced, and 5 g of the mince was mixed for 1 min in 50 mL distilled water. The pH of the mixture was measured using an INOLAB digital pH meter.

Water activity (a_w)

Water activity ($\mathbf{a_w}$) was measured using a BT-RS1 Rotronic Hygroscope as indicated by (*Lakehal et al.,* 2019). The beef was cut into small pieces and placed in a three-quarter capacity sample cup. The probe was immediately placed in the sample cup. The result was read as soon as the humidity and temperature readings stabilized.

Colour measurement

The surface colour of beef samples was determined by a computer vision system (CVS) as described by *Tomasevic et al.* (2019), using a digital camera (Canon DS126621) mounted in a black box supplied with standard illumination (6500 K) positioned at an angle of 45° from the sample to obtain uniform lighting. The colour was analysed quantitatively using Adobe Photoshop CS3 software to assess lightness (L*), redness (a*) and yellowness (b*).

Protein solubility

Protein solubility was measured according to the method of *Zhang et al.* (2017) with minor modifications. Total protein was extracted from a 1 g sample of the beef using 10 mL of ice-cold solution (1.1 M potassium iodide in 0.1 M phosphate buffer, pH 7.2). The samples of beef meat were minced, homogenised in the ice-cold solution in an ice bath, and then refrigerated for 20 h. Following centrifugation (2600 *g*, 30 min, 4°C), the protein in the supernatant was quantified using the Biuret assay. Protein solubility was calculated in milligrams of protein per kilogram of beef.

Thiobarbituric acid reactive substances (TBARS)

TBARS measurements indicated fat oxidation in the frozen meat according to the protocol described by *Buege and Aust,* (1978). Minced beef (4 g) and 40 ml distilled water were mixed for 1 min. After that, 1 ml of the solution was combined with 2 ml of a solution containing TCA-TBA-HCI reagent (15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N hydrochloric acid). The mixture was boiled for 20 min in a water bath. The resulting solution was cooled for 10 min nnder running water. The absorbance of the resulting top layer was measured at 532 nm. The amount of TBARS was expressed as nmol of malondialdehyde/g of beef using a molar extinction value of 1.56×10^{-5} M^{-1} cm^{-1}.

Microstructure of meat

The microstructure of meat was studied according to the method of *Su et al.* (2014) fresh shrimp and porcine liver were frozen by PSF at 100 MPa (−8.4°C. To begin, meat samples were fixed in Clarke's solution (75% absolute ethanol and 25% glacial acetic acid) at −23°C for 24 h. After that, the samples were equilibrated to room temperature, then dehydrated with ethanol gradients according to the protocols and techniques defined previously (*Luna,* 1968) and then were immersed in xylene and paraffin at 58°C. After that, each sample was paraffin-embedded and trapped

in paraffin blocks. Blocks were sliced into 6 µm thick slices with a microtome (Leica, Jung-histocut 820) and resultant sections were placed on glass slides, dewaxed, and stained with Calleja solution for later microscopic analysis. Finally, a microscope (Zeiss Axioscope) equipped with a digital camera (SLS-Mvision) was used to examine all of the prepared the sectioned tissues. The intracellular location of each ice crystal, as well as the number and average diameter of ice crystals in each cell, was computed after the intracellular ice crystals (white voids) were delimited.

Statistical analysis

Results were statistically evaluated using the SPSS software version 20 (IBM SPSS Statistics v22). Analysis of variance (one-way ANOVA) techniques and Tukey's multiple comparison tests were employed to examine for differences among the data acquired. Results are presented as means with standard deviations.

Results and discussion

Beef meat pH

It was observed that the pH of beef samples increased with the extended frozen storage period, as shown in (Figure 1). According to *Ho et al.* (2020), accumulation of free amino acids, ammonia and organic sulphides derived from the hydrolysis of proteolytic amines might be considered to be the primary cause of the elevated pH. In this study, amino acids were not measured, but in another study, the changes of free amino acids during frozen storage of lamb meat were measured, and authors concluded that the increase in pH was due to the increase in free basic amino acids (*Braggins et al.,* 1999). Commonly, the increase of pH could be mainly associated with the increase of alkaline substances (*Farouk and Swan,* 1998).

Water activity (a_w)

Figure 2 shows the water activity (a_w) in the beef changed during frozen storage. The a_w in fresh samples averaged 0.945, but a_w in all beef samples decreased dramatically as storage duration increased after two months. Although the a_w dropped after freezing, it rose for about ten months before dropping again at the conclusion of storage (12 months). Variations in a_w are linked to fluid movement and ice crystallization (*He et al.,* 2015). Our results are in agreement with those of others (*Medić et al.,* 2018), who detected a change in pork a_w after 18 months' freezing. Nonetheless, one study (*Coombs et al.,* 2017)at 24 h post-mortem, and assigned to five chilled storage periods (0, 2, 4, 6 and 8 weeks found no changes in a_w in frozen lamb meat for two freezing temperatures (−12 and −18°C) during a 52-week period. It has been demonstrated that unfavourable reactions are frequently linked to a food's a_w rather than its water content (*Black and Jaczynski,* 2008) chicken breast meat, and trout fillets was modified to intermediate (aw 0.98–0.99.

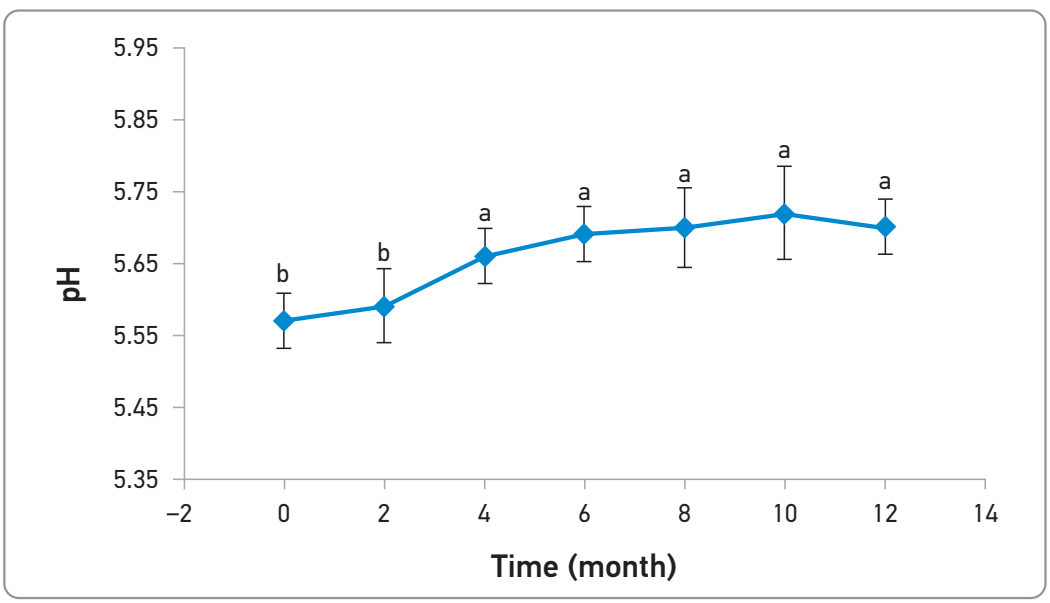

Figure 1. Beef pH during frozen storage.

Legend: a, b different letters show a statistically significant difference ($p<0.05$)

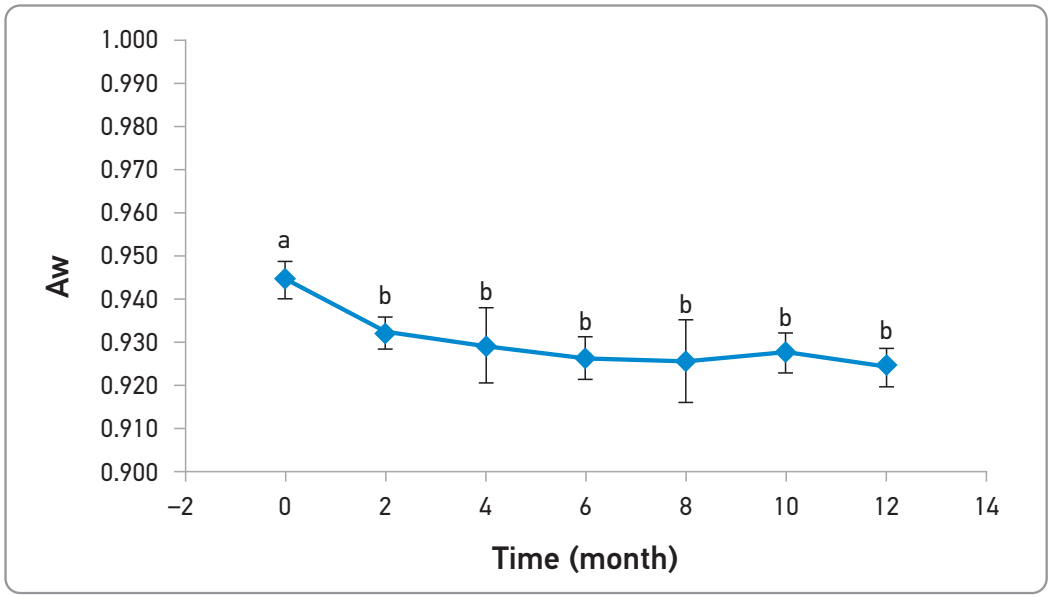

Figure 2. a_w of frozen beef during storage.

Legend: [a, b] different letters show a statistically significant difference ($p<0.05$)

Colour evaluation

Table 1 shows colour value changes (CIE L*, a*, and b*) occurred during frozen storage. Our results showed that from the fourth month of frozen storage, a significant decrease of (L*) occurred. Decrease in meat lightness due to freezing was reported earlier (*Muela et al., 2015*; *Hou et al.,* 2020) and was explained by the structural changes in the meat caused by myofibrillar protein degradation during the storage (*Wang et al.,* 2020). After 11 months of frozen storage, the values of (a*) were significantly lower than in the fresh meat ($p<0.05$). *Hansen et al.* (2004) also found that, after 30 months, (a*) levels decreased with frozen storage. According to *Alonso et al.* (2016), the lowering in (a*) values could be related to myoglobin denaturation during frozen processing. On the other hand, in our study, (b*) values were obviously increased by frozen storage from the 6th month ($p<0.05$). Several previous studies reported an increase in meat yellowness (b*) (*Hansen et al.,* 2004; *Vieira et al.,* 2009; *De Paula Paseto Fernandes et al.,* 2013; *Coombs et al.*, 2017). This change could be explained by the oxidation of fat and protein degradation during frozen storage (*Estévez,* 2011; *Muela et al.,* 2015). Meat colour is a crucial factor for consumers when evaluating meat quality, and it is also the most popular determinant of whether or not to buy frozen meat (*Zhang et al.,* 2019).

Protein solubility

With increasing frozen storage duration, protein solubility in frozen/thawed beef samples showed a significant tendency to decrease ($p<0.05$) (Figure 3). Protein solubility dropped from 111.65 m/g to 77.25 mg/g by the end of storage. *Nahar et al.* (2014) and *Farouk and Swan* (1998) found similar reductions in protein solubility in frozen chicken and beef meat, respectively. In the food sector, protein solubility is an extremely important parameter. Protein solubility has been frequently employed as a good indicator of

Table 1. Changes i CIE L*a*b* of froze beef uscles during frozen storage

	Frozen storage time (Months)						
	Fresh meat	**2**	**4**	**6**	**8**	**10**	**12**
CIE L*	42.83[a]	42.67[a]	40[ab]	38.50[b]	38.33[b]	39.17[ab]	38.16[b]
CIE a*	15[a]	15[a]	15.67[a]	15.67[a]	15.5[a]	12.83[b]	12[b]
CIE b*	5.17[c]	5.5[c]	7[bc]	9.43[a]	9.67[a]	8.93[a]	9.69[a]

Legend: CIE L*a*b* — light reflectance scores; [a,b,c] different letters in the same row show statistically significant difference ($P<0.05$).

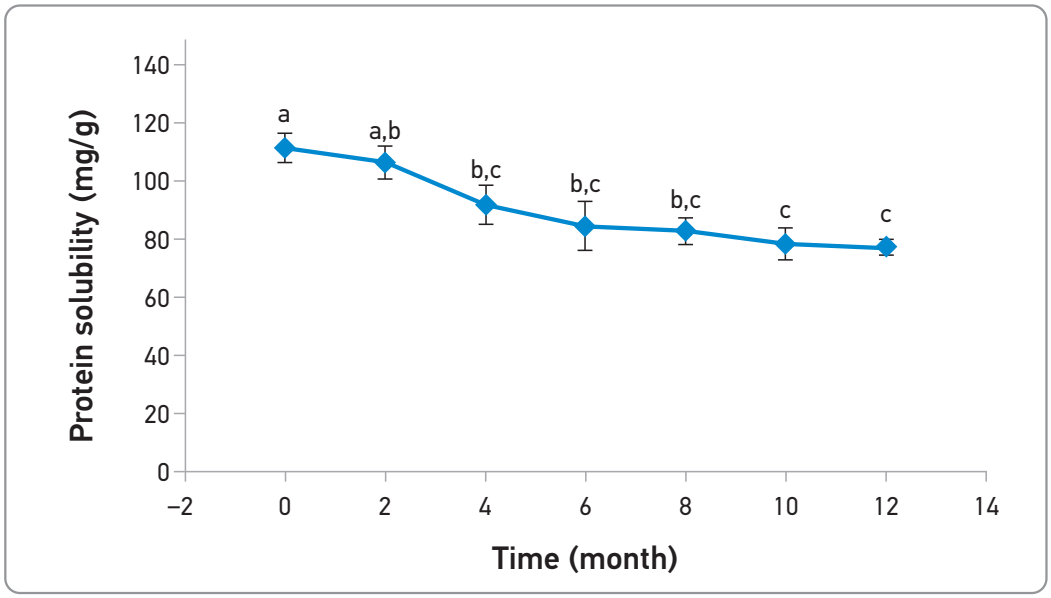

Figure 3. Protein solubility of beef during frozen storage.

Legend: [a, b, c] different letters show a statistically significant difference ($P < 0.05$).

protein denaturation (*Nahar et al*., 2014) because it is easy to test and has a high association with meat texture (*de Koning and Mol,* 1991). According to *Chan et al.* (2011), protein solubility is significantly reduced during freezing storage, and this reduction could be associated with sensitivity of proteins to temperature-induced aggregation. It is also worth noting that the dynamics of ice formation had an impact on the solubility of proteins (*Farouk and Swan,* 1998). In addition, the freezing process effectively contributes to protein damage, as it is the main culprit in the appearance of intracellular ice crystals and, thus, it mechanically weakens meat's protein structures and causes their further fragmentation (*Zhou et al.,* 2018).

TBARS evaluation

Figure 4 shows the mean TBARS value was 0.172 mg/kg on day 0 and it increased significantly ($p<0.05$) during frozen storage up to a maximum value at 12 months (0.870 mg/kg). Despite the fact that no sensory analysis was undertaken in this

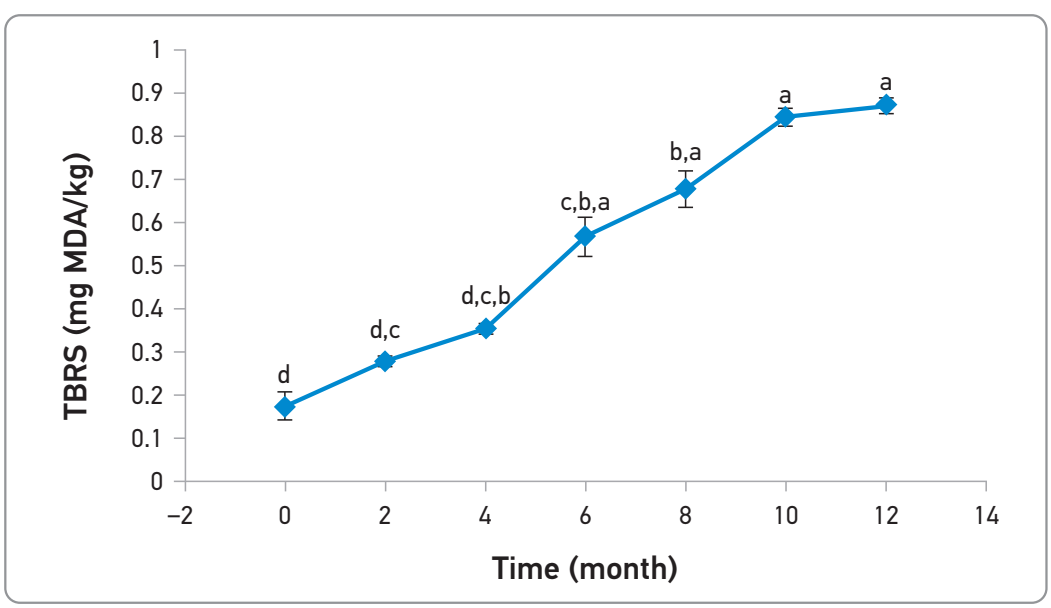

Figure 4. TBRS values in beef during frozen storage.

Legend: [a, b, c, d] different letters show a statistically significant difference ($P < 0.05$)

study, the final TBARS values achieved in this work did not reach the flavour criterion (>1.0 MDA mg/kg) above which an undesirable odour and a rancid taste could result (*Ripoll et al.,* 2011). The TBARS parameter indicates secondary oxidation products, which result from the hydrolysis of polyunsaturated fats, and which relate to unpleasant flavours in meat and meat products (*Sun et al.,* 2019). Also, oxidation of lipids can cause denaturation of proteins, which changes their structure, causes peptide scission (*Saeed and Howell,* 2002) and changes their functional qualities, such as their water holding capacity (*Cao et al.,* 2018; *Zhou et al.,* 2018). The TBARS value is the main indicator of fat oxidation in food (*Turgut et al.,* 2017).

Microscopic observation

Figure 5(A–G) shows microscopic images of the beef meat at different frozen storage durations. Figure 5A shows a typical microscopic image of the beef meat before freezing for the purpose of comparison

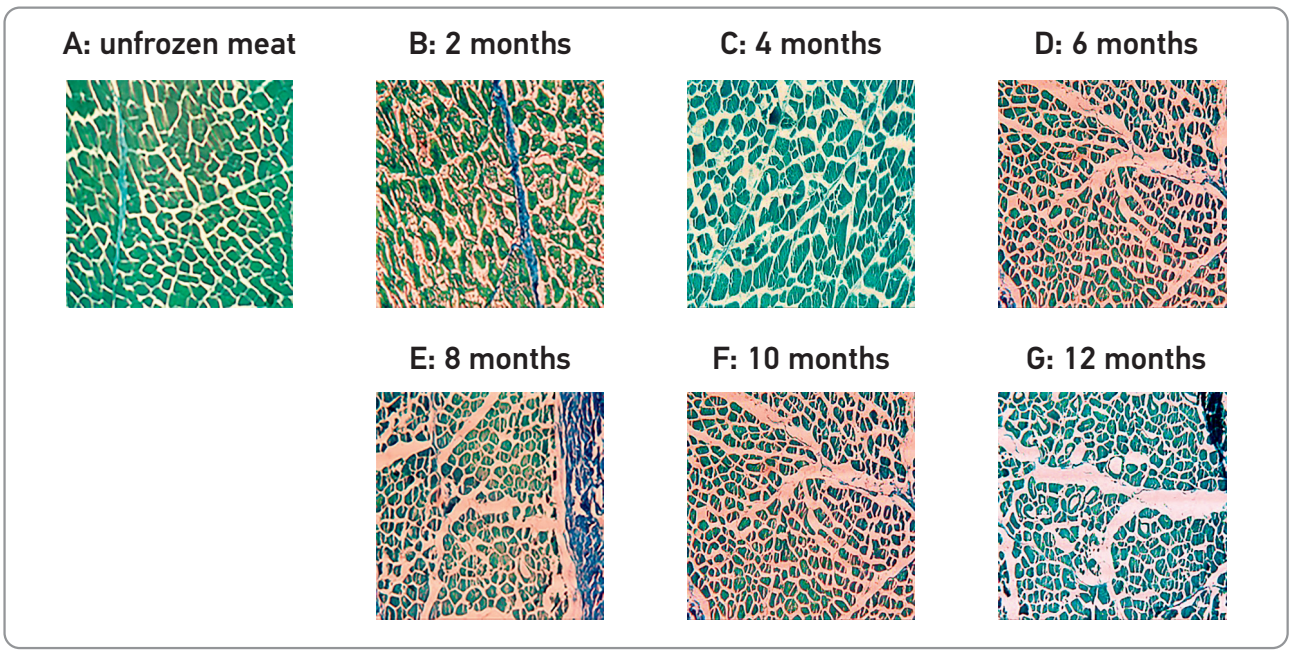

Figure 5. Changes in microstructural cross section of frozen beef meat with time. *Calleja stain*; ×100.

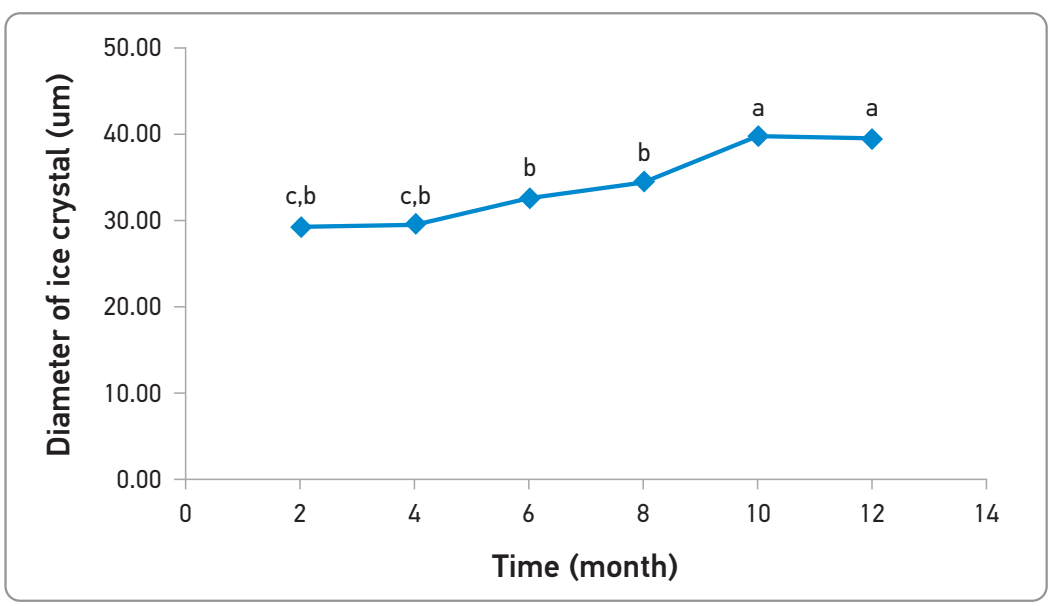

Figure 6. Mean diameter of ice crystals during frozen storage

Legend: [a, b, c,] different letters show a statistically significant difference (p<0.05)

with frozen meat (Figure 5B-G). The fresh, unfrozen beef (Figure 5A) had uniform distribution of regularly formed fibres. At the end of two months of frozen storage, small vacuoles, formed from ice crystals in the meat, were observed (Figure 5B). After 12 months of storage, the intracellular ice crystals appeared larger and the space between fibres was greater (Figure 5G). The main factor affecting the texture of meat is structural changes related to the relationship between proteins and water molecules (*Nakazawa et al.,* 2019). Generally, lowering the storage temperature is a good technique to keep meat fresh for a long time (*Zhang et al.,* 2019). However, the crystallization of water in the tissue might result in an increase in the solute concentration during frozen storage, followed by the separation of the endomysium and the expansion of the perimysium, which results in a decrease in cell size and an expansion of the extracellular space (*Shi et al., 2018*; *Tolstorebrov et al.,* 2016).

By analysing the data obtained from microscopic images (Figure 5), we noted that ice crystal diameters showed an ascendant trend as storage duration prolonged. The diameter of ice crystals increased from 29.09 μm after 2 months to 39.34 μm after 12 months, with significant differences ($p > 0.05$) between them (Figure 6). *Jiang et al.* (2020)distribution of water and freshness properties of grass carp during frozen storage was investigated. The freezing methods contained air-blast freezing (AF demonstrated that recrystallization during frozen storage could cause expansion of voids inside muscle fibres.

Conclusion

In general, our results showed that the physical properties of beef meat are significantly affected by frozen storage, and these effects evolve during the meat's frozen storage at −23°C. Freezing increased the pH and yellowing (b*) of the meat, while the lightness (L*), redness (a*) and a_w decreased. Based on TBAS values, it was deduced that the beef was of a quality that was adequate for processing after 12 months of frozen storage. However, a continuous decrease in protein solubility was observed during the 12 months of storage. The microstructure of the beef visually showed a gradual increase in the diameter of intra-tissue ice crystals with increased frozen storage duration, which means the muscle tissue was undergoing fracture.

Ispitivanje promena fizičko-hemijske i mikrostrukture goveđeg mesa tokom skladištenja na niskoj temperaturi

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A p s t r a k t: Zamrzavanje goveđeg mesa je najefikasniji način da se produži dužina odn. trajanje njegovog skladištenja. Međutim, malo je informacija o tome da li ova praksa menja mikrostrukturu govedjeg mesa, kao i o njenom uticaju na kvalitet. Iz tog razloga, cilj našeg istraživanja je bio da se utvrdi uticaj šest perioda skladištenja u zamrznutom stanju na fizička, hemijska i mikrostrukturna svojstva goveđeg mesa u komadima mišića biceps femoris (20). Značajne fizičke promene su otkrivene u različitim vremenima skladištenja, uključujući povećanje pH i intenziteta žute boje (b), kao i smanjenje aktivnosti vode, svetlost (L*) i crvene boje (a*). Zatim, u pogledu hemijskih karakteristika, rastvorljivost proteina u uzorcima govedjeg mesa je smanjena, ali su vrednosti oksidacije lipida (TBA) znatno porasle. Širina ledenih kristala u smrznutim uzorcima stalno se povećavala kako se vreme skladištenja produžavalo na 12 meseci, što ukazuje na strukturne promene u smrznutim mišićima.*

Ključne reči: *goveđe meso, fizičko-hemijski parametri, mikrostruktura, zamrzavanje, vreme skladištenja.*

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