

THE EFFECT OF REPEATED FREEZE-THAW CYCLES ON THE MEAT QUALITY OF RABBIT

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Abstract: We investigated the effect of repeated freeze-thaw cycles on the quality of rabbit meat. Twenty-five Hyla rabbits were slaughtered using standard commercial procedures. A freeze-thaw procedure—i.e., seven days frozen at -18°C followed by thawing at 4°C for 12h— was repeated 5 times, and 9 *Longissimus thoracis et lumborum* muscles were randomly selected at pre-set cycles (0, 1, 2, 3, and 5). The *Longissimus lumborum* muscles were used to determine meat quality parameters, while the *Longissimus thoracis* muscles were used for chemical analysis. During the repeated freeze-thaw process, muscle pH, redness, hardness, and water holding capacity gradually decreased, whereas meat lightness and yellowness gradually increased. The amount of total volatile basic nitrogen significantly increased ($P<0.05$) and exceeded the threshold value for frozen meat after 5 repeated freeze-thaw cycles. The metmyoglobin proportion, thiobarbituric acid-reactive substances (TBARS) and protein carbonyl content in rabbit meat samples increased with a higher number of freeze-thaw cycles ($P<0.05$), and the proportions of these compounds were positively correlated. During the repeated freeze-thaw process, extractable haeme iron levels significantly decreased ($P<0.05$), and non-haeme iron levels markedly increased ($P<0.05$). An sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis indicated that the degradation of both water- and salt-soluble proteins was more prevalent in samples subjected to higher numbers of freeze-thaw cycles. Additionally, a principal component analysis identified good correlations between physicochemical properties (TBARS, protein carbonyl levels and metmyoglobin content) and quality parameters (thawing loss, redness, lightness and hardness). Taken together, we conclude that the repeated freeze-thaw process can strongly affect rabbit meat quality as well as its physicochemical properties.

Key Words: rabbit, freeze-thaw cycles, meat quality, myoglobin oxidation, lipid oxidation, protein oxidation.

INTRODUCTION

At present, consumer demand for food products is often determined by customer nutritional preferences, due to the association between diet and health. Rabbit meat is rich in essential amino acids, vitamin B family nutrients and polyunsaturated fatty acids. Moreover, rabbit meat also contains low amounts of cholesterol and monounsaturated fatty acids (Dalle Zotte and Szendrő, 2011), and is a suitable choice for health-conscious consumers. China is a major producer of rabbit meat; according to FAOSTAT (2018), in 2014 China produced 762,627 tons of rabbit meat, thus accounting for 48.89% of world rabbit meat production. However, the consumption of rabbit meat in China is lower (Xie *et al.*, 2016), and a large quantity of rabbit meat produced in China was exported to other countries.

Frozen preservation is a widely used storage method for meat and meat products. Hansen *et al.* (2004) suggested that the temperature of -55°C is an ideal preservation condition for frozen meat to prevent storage conditions from affecting meat quality. At this temperature, microbial activity, enzymic reactions and oxidative deterioration are minimised, and there is almost no deterioration in meat quality during storage (Zhou *et al.*, 2010). However, a temperature of -18°C is a commonly used commercial storage condition. At this temperature, some chemical and biochemical processes

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in the meat may still occur. Such reactions —mainly involving lipid oxidation and discoloration— are responsible for the deterioration of meat quality during frozen storage (Turhan *et al.*, 2017). Moreover, it has been reported that frozen storage has an effect on protein carbonylation, which in turn affects specific meat attributes such as colour, texture and water holding capacity (WHC) (Utrera *et al.*, 2014). The freezing process can affect meat properties due to mechanical damage from ice crystal formation in extracellular locations, as well as volume alternations in cellular structures (Dalvi-Isfahan *et al.*, 2016). Bianchi *et al.* (2006) reported that some changes in the sensory attributes of rabbit meat, such as off-flavour and rancidity, present during frozen storage. Dalle Zotte *et al.* (2016) revealed that the pH, a^* and b^* colour values of frozen rabbit meat tend to be lower than in unfrozen meat. Lan *et al.* (2016) found that the shear force, lightness, muscle microstructure integrity and WHC decreased as frozen storage time increased.

Due to poor cold-chain conditions, the problem of repeated cycles of freezing and thawing caused by temperature fluctuations during transport, storage and retail is a serious issue for frozen meat, especially in developing countries. Numerous studies have reported that repeated freeze-thaw cycles affect many qualities of meat and related products, including colour, texture and WHC (Benjakul and Bauer, 2001; Xia *et al.*, 2009; Jeong *et al.*, 2011; Qi *et al.*, 2012; Ali *et al.*, 2015; Rahman *et al.*, 2015; Wang *et al.*, 2015). In a study of chicken breast meat, Ali *et al.* (2015) revealed that freeze-thaw cycles cause significant lipid and protein oxidation. Benjakul *et al.* (2001) suggested that the freeze-thaw cycle results in damage to the cell, including to haeme proteins, which release pro-oxidants upon denaturation. Zhang *et al.* (2017) found that the freeze-thaw process can cause changes in the protein structure and water distribution within meat. Xia *et al.* (2010) reported that the freeze-thaw process had a detrimental effect on the functionality of porcine myofibrillar proteins, which are closely related to protein denaturation and aggregation. Each of these alternations may contribute to changes in meat quality. However, to the best of our knowledge, there is no published work examining the effect of the freeze-thaw cycle on the quality of rabbit meat. Therefore, the objective of the present work was to determine the effect of repeated freeze-thaw cycles on rabbit meat quality and physicochemical makeup.

MATERIALS AND METHODS

Materials

Twenty-five Hyla rabbits (with masses ranging from 2600 to 2900 g per live rabbit) were slaughtered using standard commercial procedures at A Xing Ji Food Co. Ltd. (Chongqing, China). The carcasses were immediately transported to the laboratory using a chilled box (approximately 6°C) within 4h and were cold-stored for another 20 h to dissipate rigor mortis.

Sample preparation

After aging, a total of 50 *Longissimus thoracis et lumborum* (LTL) samples were obtained by dissection. Nine LTL samples were randomly selected as control (e.g. fresh meat). All other samples were packaged in polyethylene plastic bags and stored at -18°C for 7 d. The samples were then thawed at 4°C for 12 h, and another 9 LTL samples were selected randomly as the first time point to be measured (i.e. 1 freeze-thaw cycle). The rest of the samples were again stored at -18°C for another 7 d. This freeze-thaw procedure —i.e., 7 d frozen at -18°C followed by thawing at 4°C for 12 h— was repeated for 5 cycles, and 9 LTL samples were randomly selected at pre-set cycle numbers (1, 2, 3, and 5 freeze-thaw cycles) to be analysed later. For each experiment, 9 LTL were randomly selected. Nine *Longissimus lumborum* (LL) muscles were used to determine quality parameters (colour, texture and WHC) and were considered as experimental units (i.e. $n=9$). Nine *Longissimus thoracis* (LT) were minced and used as a composite sample for physicochemical analysis. The composite sample was divided into 4 replicates, each of which were considered separate experimental units (i.e. $n=4$).

pH

pH was evaluated according to the method described by Lan *et al.* (2016), with minor modifications. One gram of minced rabbit meat sample was homogenised in 10 mL potassium chloride (0.1 M, pH 7.0) using a high-speed disperser (XHF-D, Ningbo Xingzhi Biotechnology Co., Ltd., Zhejiang, China). A digital pH meter (UB-7, Sartorius AG, Goettingen, Germany) was used to measure pH.

Colour

The surface colour was measured according to the method of Lan *et al.* (2016). Briefly, a colorimeter (UltraScan PRO, Hunter Associates Laboratory, Inc., Virginia, USA) with a D65 light source, a 10° angle observer and a measuring area with a diameter of 10 mm at room temperature was calibrated using black and white plates before measuring lightness (L^* value), redness (a^* value) and yellowness (b^* value). The data were evaluated at 3 different points for all samples.

Thawing loss and cooking loss

The method from Barbin *et al.* (2013) was used to evaluate thawing loss, (W_f is the weight of fresh meat before freezing, and W_t is the weight of repeated freeze-thaw cycles samples after thawing).

$$\text{Thawing loss (\%)} = \frac{W_f - W_t}{W_f} \times 100$$

Cooking loss was measured according to the method of Peiretti *et al.* (2013) with some modifications. A meat cube (5 g) was placed in a plastic bag, and was then heated at 90°C for 30 min. Cooking loss was calculated by the following equation, where W_b is the weight of meat before cooking, and W_a is the weight of meat after cooking.

$$\text{Cooking loss (\%)} = \frac{W_b - W_a}{W_b} \times 100$$

Texture analysis

Texture analysis was carried out using a TA-XT2i texture analyser (Stable Micro Systems, Surrey, England) equipped with a cylindrical probe (10 mm diameter) using the method of Isleroglu *et al.* (2015) with minor modifications. Briefly, 2 cycles of compression (15×15×15 mm) were carried out on each sample in turn. The instrument parameters were set as follows: pre-test speed: 5 mm/s; test speed and post-test speed: 1 mm/s; trigger force: 10 g; and final distance: 4 mm.

Total volatile basic nitrogen (TVB-N)

TVB-N of meat samples was evaluated by the method of the National Standard of China (GB/T 5009.228 -2016).

Myoglobin content

The extraction of myoglobin from rabbit meat was carried out according to the method of Dai *et al.* (2013) with minor modifications. Five grams of minced rabbit meat were mixed with 25 mL phosphate buffer (0.04 M, pH 6.8), followed by homogenising at 3000 rpm for 30 s. The resulting sample was then centrifuged at 3000 g for 30 min at 4°C (Avanti J-30L, Kurt Backman, USA), and the supernatant was filtered. The filtrate absorbance was read at 503, 525, 582, and 557 nm by a spectrophotometer (Shimadzu, UV-16001, Tokyo, Japan). The proportions of deoxymyoglobin (DeoMb), oxymyoglobin (OxyMb) and metmyoglobin (MetMb) were calculated using the method of Tang *et al.* (2004), where $R_1 = A_{582} / A_{525}$, $R_2 = A_{557} / A_{525}$, $R_3 = A_{503} / A_{525}$.

$$[\text{DeoMb}] = C_{\text{DeoMb}} / C_{\text{Mb}} = -0.534R_1 + 1.594R_2 - 0.522R_3 - 1.329$$

$$[\text{OxyMb}] = C_{\text{OxyMb}} / C_{\text{Mb}} = 0.722R_1 - 1.432R_2 - 1.659R_3 + 2.599$$

$$[\text{MetMb}] = C_{\text{MetMb}} / C_{\text{Mb}} = -0.159R_1 - 0.085R_2 + 1.262R_3 - 0.520$$

Absorption spectra of myoglobin in the Soret band

Absorption spectra at 380 to 450 nm were measured using the UV-visible spectrophotometer (Shimadzu, UV-16001, Tokyo, Japan). The scanning speed was set as 1000 nm/min, and the phosphate buffer was used as a blank.

Haeme iron content

Haeme iron content was determined using the method described by Ramos *et al.* (2012) with minor modifications. Four grams of rabbit meat sample were weighed and placed in a glass tube, and 20 mL of acidified acetone (an acetone:water:HCl ratio of 45:4:1) was added. After vortexing (15 s), the tube was sealed and incubated at room temperature in the dark for 2 h. Next, the mixture was filtered, and the filtrate's absorbance was read at 640 nm.

Non-haeme iron content

The non-haeme iron of rabbit meat was extracted using the method of Dai *et al.* (2013) with minor modifications. One gram of minced rabbit meat was mixed with 40 μ L of 0.39% (w/v) sodium nitrite, and a mixture (10 mL) of 20% trichloroacetic acid (TCA) and 3 M HCl (ratio of 1:1 [v/v], prepared freshly) was added and the tube cap was tightened. After incubation at 60°C for 24 h, the tube was cooled down to room temperature. Non-haeme iron content was measured using the method of the National Standard of China (GB/T 9695.3 -2009). Briefly, 5 mL of extracted liquid containing non-haeme iron was transferred to a capacity bottle and 2.5 mL of fresh hydroxylamine hydrochloride solution (5%) was added. After incubating for 10 min, 1 mL of tartaric acid solution (10%), 2.5 mL of sodium acetate solution (50%) and 5 mL of 1, 10-phenanthroline solution (0.25%) were added in succession. The mixture solution was fixed to 25 mL with distilled water, and the absorbance was read at 510 nm.

Thiobarbituric acid-reactive substances (TBARS)

The method from Lan *et al.* (2016) with minor modifications was used to measure TBARS. Ten grams of minced sample were firstly homogenised (10000 rpm, 60 s) in 20 mL of 20% TCA. The sample was then centrifuged at 5500 rpm for 15 min at 4°C, and the supernatant was filtered using qualitative filter paper. A 5 mL volume of filtrate was mixed with 5 mL of 20 mM 2-thiobarbituric acid. A solution prepared by mixing 5 mL of TCA and 5 mL of 2-thiobarbituric acid solution was used as a blank. Next, both sample and blank were heated for 20 min in a boiling water bath, and they were then cooled to room temperature. Their absorbances were measured at 532 nm.

Carbonyl content

Carbonyl content was evaluated according to the modified 2,4-dinitrophenylhydrazine (DNPH) carbonyl assay described by Soglia *et al.* (2016). One gram of rabbit meat was mixed with 10 mL of ice-cold potassium chloride solution (0.15 M) and homogenised at 3000 rpm for 60 s in a water-ice bath. Two aliquots (100 μ L/each) were taken from the homogenate and mixed with 1 mL of 20% TCA. After centrifugation (5000 g, 5 min), the supernatant was removed and 400 μ L of 5% SDS was added. The resulting samples were ultrasonicated for 60 min at room temperature. Next, one sample was treated with 800 μ L of 3 M HCl containing 0.3% (w/v) DNPH, while the other sample was treated with 800 μ L of 3 M HCl (as a blank). After incubation for 30 min, proteins were precipitated by adding 400 μ L of 40% TCA, followed by centrifugation (5000 g, 5 min). Next, the pellets were washed with 1 mL of ethanol-ethyl acetate (ratio of 1:1 [v/v]) solution and then centrifuged (10000 g, 5 min). This washing process was repeated 3 times. Next, the pellets were dissolved in 1.5 mL of 20 mM NaH₂PO₄ (pH 6.5) containing 6 M guanidine hydrochloride. These solutions were incubated overnight, and their absorbances were read at 280 and 370 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The method from Duun and Rustad (2008) was used to extract water-soluble and salt-soluble proteins. The Biuret method (Robinson *et al.*, 1940) was used to evaluate protein concentration. The protein concentration was adjusted to 2 mg/mL. One millilitre of sample solution was mixed with 4 mL of sample buffer. Next, samples were heated for 3 min in a boiling water bath and were subsequently centrifuged (1800 g, 10 min). Ten microlitres of resulting supernatant were loaded to the well in 4% stacking gel, and samples were separated using a 10% running gel. Molecular weights were determined by a protein marker ranged from 9 to 245 kDa.

Statistical analysis

Statistical analysis was performed using SPSS 19.0. A One-way Analysis of Variance was used to analyse the differences between different freeze-thaw cycles. The significance threshold for all statistical analyses was set at $P < 0.05$. A principal component analysis (PCA) was also carried out using SPSS 19.0.

RESULTS AND DISCUSSION

Changes in pH value

As illustrated in Table 1, pH gradually declined with an increased number of freeze-thaw cycles. A similar result was reported by Ali *et al.* (2015), who examined chicken meat. The pH is used to evaluate the concentration of free hydrogen ions (H^+) in a solution. A decrease in pH therefore indicated that the ionic balance of rabbit meat samples changed as the number of repeated freeze-thaw cycles increased. Leygonie *et al.* (2012) suggested that water loss from meat muscle tissue may lead to an increase in the concentration of solutes, which consequently caused a decline in pH. Moreover, the release of H^+ due to the denaturation of proteins (Ali *et al.*, 2015) and the acidification of rabbit meat due to the formation of free fatty acids during storage (Lan *et al.*, 2016) also contributed to a decrease in pH.

Changes in WHC

The effect of repeated freeze-thaw cycles on thawing loss and cooking loss of rabbit meat was also shown in Table 1. From the results, thawing loss significantly increased ($P < 0.05$) as the number of repeated freeze-thaw cycles increased, indicating that repeated freeze-thaw cycles may lead to reduced WHC in rabbit meat. This result agreed with the findings of Ali *et al.* (2015) regarding chicken breast meat. WHC is defined as the ability of meat to retain natural moisture, and factors such as net charge effect, steric effect, and ion exchange can influence meat WHC. During freezing process, protein oxidation (as measured by carbonyl) and the alternation of tissue as well as proteins iso-electrical point (Muela *et al.* 2015) may occur, and these processes may be responsible for thawing loss. As for cooking loss, it is believed that moisture loss is related to the condition of muscle tissue (Leygonie *et al.*, 2012). Vieira *et al.* (2009) reported that fat melting and protein denaturation during cooking together contributed to the liberation of chemically bound water. Repeated thawing and regeneration of ice in the present study may have resulted in serious mechanical disruption of muscle cells, which is clearly detrimental to muscle tissues (Wang *et al.*, 2015). Here, increased cooking moisture loss was observed as the number of freeze-thaw cycles increased from 0 to 3 ($P < 0.05$). However, after 3 repeated freeze-thaw cycles, the cooking moisture loss gradually decreased, which was similar to the finding of Wang *et al.* (2015) in common carp. This may be explained by the fact that too much water was exuded during repeated freeze and thaw processes and, in turn, the water content in the rabbit meat samples was significantly reduced.

Changes in hardness

Meat texture qualities, including hardness, cohesiveness, springiness and chewiness are critical characteristics for consumers. Among these, hardness is the most important, as it imparts the commercial value of a meat (Dalvi-

Table 1: Changes in the physical properties of rabbit meat subjected to repeated freeze-thaw cycles.

Item	Freeze-thaw cycles				
	0	1	2	3	5
pH ¹	5.87±0.01 ^c	5.85±0.02 ^c	5.84±0.02 ^{bc}	5.81±0.015 ^b	5.75±0.01 ^a
Thawing loss (%) ¹	-	4.71±0.34 ^a	9.32±0.43 ^b	15.35±1.19 ^c	23.83±2.02 ^d
Cooking loss (%) ²	32.64±0.63 ^a	33.58±0.92 ^b	36.33±0.42 ^c	39.27±0.58 ^d	35.93±0.91 ^c
Hardness (g) ²	1294.83±51.71 ^e	1039.11±40.19 ^d	832.26±73.19 ^c	691.79±31.33 ^b	462.98±30.20 ^a
TVB-N (mg/100 g) ¹	9.24±0.24 ^a	10.54±0.35 ^b	11.81±0.29 ^c	13.39±0.30 ^d	18.67±0.58 ^e

Values are presented as means±standard deviation. ^{a-e}Different letters in the same row indicate significant differences between different numbers of repeated freeze-thaw cycles ($P < 0.05$). (n¹=4, n²=9). TVB-N, total volatile basic nitrogen.

Isfahan *et al.*, 2016). The effect of repeated freeze-thaw cycles on the hardness of rabbit meat is presented in Table 1. The hardness of rabbit meat significantly decreased ($P < 0.05$) as the number of repeated freeze-thaw cycles increased, implying that the texture of the rabbit meat was significantly affected by freeze-thaw cycles. This result agreed with the findings of Chen *et al.* (2017), who found that the hardness of chicken muscle declined with increased freeze-thaw cycles. Food texture is strongly associated with its structure. It has been reported that the tenderness of meat increases when it undergoes freezing and thawing, and the authors of one study argued that this is caused by the breakdown of muscle fibres by enzymatic action, as well as the loss of structural integrity that results from ice crystal generation (Dalvi-Isfahan *et al.*, 2016). Food texture also depends on physicochemical properties (Huang *et al.*, 2015). Xia *et al.* (2010) reported that repeated cycles of freezing and thawing showed a harmful influence on the functionality of myofibrillar proteins, which are responsible for many of the physicochemical properties of muscle-based meats.

Changes in TVB-N

The TVB-N value can reflect the freshness of meat (Chen *et al.*, 2013). The results of our analysis of the relationship between freeze-thaw cycles and TVB-N are shown in Table 1. The TVB-N value significantly increased ($P < 0.05$) with an increased number of freeze-thaw cycles. The increase in the TVB-N value indicated the generation of ammonia, trimethylamines and amines in meat as a result of spoilage by both enzymic and bacterial degradation (Cai *et al.*, 2011). According to Chinese National Standards for fresh or frozen meat (GB 2707-2016), the TVB-N value should be below a threshold value of 15 mg/100 g. In the present study, the TVB-N value of rabbit meat from samples subjected to 5 repeated freeze-thaw cycles exceeded this value.

Colour changes

Similar to meat texture, meat colour also is a critical characteristic for consumers (Karpińska-Tymoszczyk *et al.*, 2014). Figure 1 showed the influence of repeated freeze-thaw cycles on the colour (L^* , a^* , and b^*) of rabbit meat. With increased freeze-thaw cycles, the a^* value gradually decreased ($P < 0.05$), whereas the L^* and b^* values gradually increased ($P < 0.05$). Similar results were observed by Xia *et al.* (2009) in pork. The decrease in a^* value (redness) indicated that the meat darkened. In general, myoglobin is the principal pigment responsible for the red colour of meat (Suman and Joseph, 2013). When ferrous myoglobin was oxidised to form ferric MetMb (Figure 2), the meat colour changed from cherry-red to brown, and hence the a^* value decreased. It has been reported that lipid oxidation in pork meat exposed to repeated freeze-thaw cycles was closely related to an increase in yellowness (b^*) (Thanonkaew *et al.*, 2006). In the present study, the increase in the b^* value was coincidental with an increase in TBARS (Figure 2). This may be explained by the fact that the yellow pigment in meat can be formed by non-enzymatic browning reactions occurring in amine and lipid oxidation products (Xia *et al.*, 2009). The change in the lightness of rabbit meat may be associated with moisture migration and/or distribution caused by structural changes in muscle (Kim *et al.*, 2017). Given the increased thawing loss during repeated freeze-thaw cycles (Table 1), the migration of moisture from intramuscular space outside may lead to changes in light reflectance of the surface of the rabbit meat, and therefore increased lightness (Farouk *et al.*, 2004).

Changes in MetMb content, TBARS, and carbonyl content

Changes in the MetMb content of rabbit meat muscle subjected to repeated freeze-thaw cycles are shown in Figure 2. The proportion of MetMb in rabbit meat samples increased with the number of freeze-thaw cycles ($P < 0.05$). In meat, the ferrous forms of myoglobin (DeoMb and OxyMb) can be oxidised to ferric states (MetMb). The observed increase in MetMb content indicated that myoglobin underwent oxidation changes during repeated freeze-thaw cycles. Normally, MetMb can be reduced back to ferrous myoglobin in fresh muscle due to the activity of metmyoglobin-reducing enzymes (MREs) (Alonso, *et al.*, 2016). However, the activity of MREs decreases when meat is frozen (Alonso, *et al.*, 2016). Thus, a rapid accumulation in MetMb was observed during the initial 3 repeated freeze-thaw cycles. Thereafter, a slow accumulation in MetMb was observed. This may be explained by the fact that the oxidation of MetMb to other forms occurs due to the action of endogenous oxidants (Wongwichian *et al.*, 2015). Faustman *et al.* (2010) suggested that superoxide, an intermediate generated in the process of OxyMb autoxidation, can rapidly

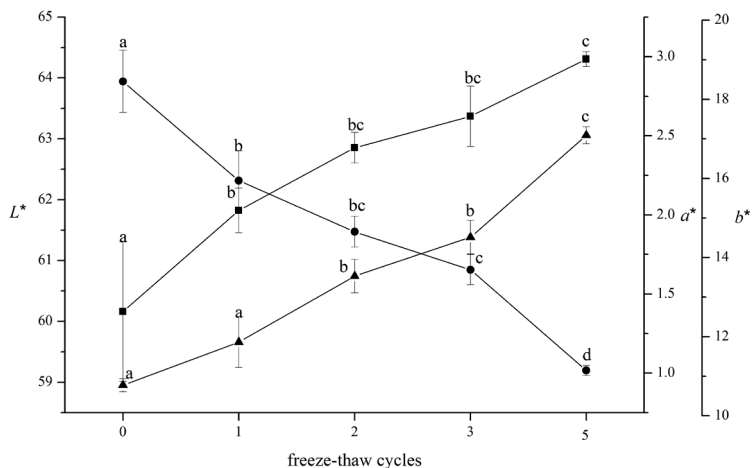


Figure 1: Changes in the colour of rabbit meat subjected to repeated freeze-thaw cycles. ^{a-d}Different letters in the same line indicate significant differences between different numbers of repeated freeze-thaw cycles ($P < 0.05$), ($n=9$). L^* : (—■—); a^* (—●—); b^* (—▲—).

dismutate into hydrogen peroxide, which can further react with MetMb to generate hypervalent products such as ferrylmyoglobin (MbFe(IV)=O).

TBARS assays can be used to measure lipid oxidation. As shown in Figure 2, TBARS gradually increased with an increased number of repeated freeze-thaw cycles ($P < 0.05$). A similar phenomenon was observed in chicken meat by Ali *et al.* (2016). The increase in TBARS indicated an accumulation of secondary lipid oxidation products—including aldehydes and ketones—that contribute to meat off-flavours. Ali *et al.* (2016) suggested that the loss of muscle

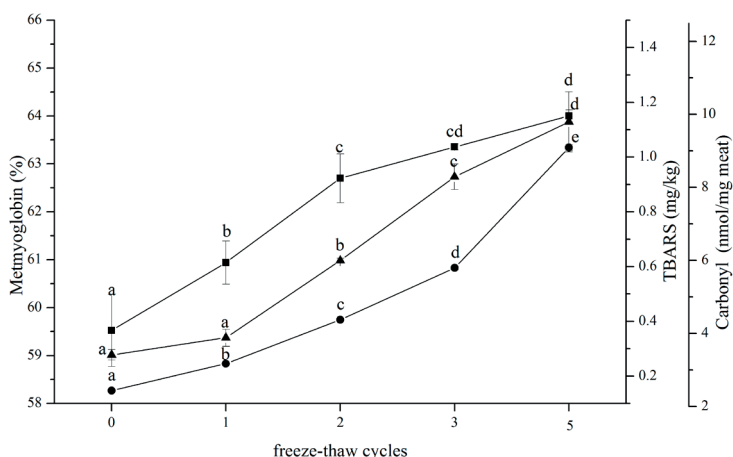


Figure 2: Changes in metmyoglobin (—■—) content, Thiobarbituric acid-reactive substances (TBARS) (—●—), and carbonyl (—▲—) content of rabbit meat subjected to repeated freeze-thaw cycles. ^{a-d}Different letters in the same line indicate significant differences between different numbers of repeated freeze-thaw cycles ($P < 0.05$), ($n=4$).

system integrity caused by repeated freeze-thaw cycles can accelerate oxidation. Moreover, the formation of ice crystals can damage the cell and can result in the release of pro-oxidants that promote lipid oxidation (Wang *et al.*, 2015). According to Rahman *et al.* (2015), the TBARS threshold for the development of rancidity is 1.0 mg/kg. In the present study, the TBARS values of rabbit meat samples subjected to 5 cycles of repeated freeze-thaw had reached this threshold.

Protein carbonyls are the major products of protein oxidation. Figure 2 also presented the effect of repeated freeze-thaw cycles on the total carbonyl content of rabbit meat. Our results showed that protein carbonyl content increased as the number of freeze-thaw cycles increased ($P < 0.05$). Protein carbonyls can be formed via four mechanisms, including the direct oxidation of the side chains of certain amino acids, non-enzymatic glycation, the oxidative cleavage of the peptide backbone and the binding of non-protein carbonyl compounds (Estévez, 2011). The increase in total protein carbonyls suggested that the proteins in the rabbit meat sample underwent oxidation during the repeated freeze-thaw process. It is worth noting that a higher rate of increase in protein carbonyl levels was observed after the first freeze-thaw cycle, as indicated by a sharp curve. This could be explained by the fact that the ultrastructure of the muscle cells was damaged after the first freeze-thaw cycle, which consequently caused the release of mitochondrial and lysosomal enzymes, haeme iron and other pro-oxidants, which then further increased protein oxidation (Leygonie *et al.*, 2012).

Changes in absorption spectra of myoglobin in the Soret band

As shown in Figure 3, an absorption maximum at 413 nm was obtained in fresh rabbit meat. With an increased number of freeze-thaw cycles, the absorption maximum gradually declined. Changes in absorption spectra in the Soret band can reflect the unfolding of haeme proteins (Benjakul *et al.*, 2001). The decrease in the Soret absorption peak suggested that the haeme protein was destroyed, or that the porphyrin was detached from the globins in response to freezing and thawing (Wongwichian, *et al.*, 2015). Moreover, a blue shift from 413 to 409 nm was observed in rabbit meat samples during repeated freeze-thaw process. Benjakul *et al.* (2001) also reported that a blue shift was observed in catfish myoglobin from samples treated by different numbers of freeze-thaw cycles, and concluded that the haeme proteins had been converted to their oxidised states. Therefore, it may be that the oxidation and degradation of haeme proteins in rabbit meat samples occurred simultaneously as the number of repeated freeze-thaw cycles increased.

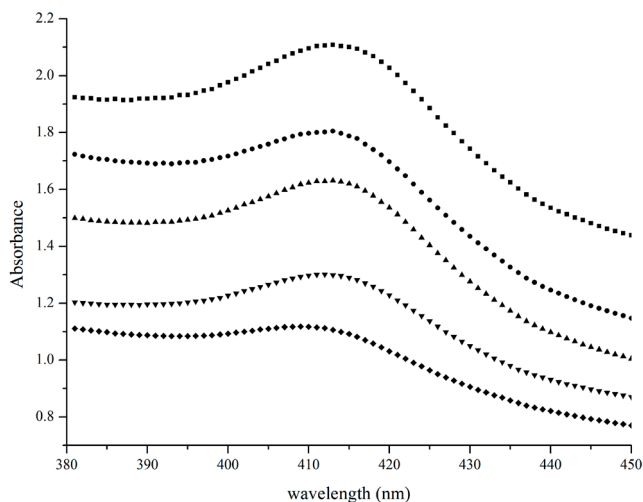


Figure 3: Changes in absorption spectra of myoglobin from rabbit meat subjected to repeated freeze-thaw cycles in the Soret band. (n=4). ■: 0; ●: 1; ▲: 2; ▼: 3; ◆: 5.

Changes in haeme iron and non-haeme iron content

The effect of repeated freeze-thaw cycles on the haeme iron and non-haeme iron contents of rabbit meat were presented in Figure 4. Our results showed that haeme iron content gradually decreased with an increased number of freeze-thaw cycles ($P < 0.05$). This finding agreed with those of Turhan *et al.* (2006) in bluefish. Generally, haeme iron is ligated in the porphyrin ring of haemoglobin or myoglobin. The decline in haeme iron content indicated that in some cases the porphyrin ring may have been disrupted (Dai *et al.*, 2014). Non-haeme iron content showed an opposite trend to haeme iron content. Moreover, changes in both haeme iron and non-haeme iron content were similar to those found by Benjakul *et al.* (2001). Wongwichian *et al.* (2015) suggested that non-haeme iron is itself mainly liberated from haeme proteins. Therefore, in this study we expected that the loss of haeme iron should be proportional to the accumulation of non-haeme iron. Moreover, besides haemoglobin and myoglobin, iron is also present in the insoluble fraction, in ferritin, and in the low molecular weight fraction of muscle meat (Hazell, 1982). Thus, when meat is subjected to repeated freeze-thaw cycles, these components may denature and release iron (Benjakul *et al.*, 2001).

SDS-PAGE

Our SDS-PAGE results showed that salt-soluble protein (Figure 5A), myosin heavy chain (MHC), tropomyosin and actin significantly changed in abundance during repeated freeze-thaw cycles. These findings indicated that these proteins underwent proteolytic degradation during the repeated freeze-thaw cycles. Generally, protein degradation is mainly caused by the activity of endogenous enzymes and microorganisms (Lan *et al.*, 2016). Also, frozen preservation can lead to the degradation of myofibrillar proteins (Ali *et al.*, 2015), which can further damage the structural integrity of muscle cells and subsequently result in the release of numerous internal enzymes, thus promoting protein degradation in turn (Yang *et al.*, 2017).

In the present study, we used SDS-PAGE to investigate water-soluble protein (including sarcoplasmic protein) levels during repeated freeze-thaw cycles (Figure 5B). Generally, sarcoplasmic proteins include many enzymes associating with the glycolytic pathway (Ali *et al.*, 2015). Our SDS-PAGE results showed that the band intensity of some sarcoplasmic proteins changed significantly in samples subjected to repeated freeze-thaw cycles. On the one hand, with increased freeze-thaw cycles, the intensity of some sarcoplasmic proteins such as glyceraldehyde phosphate dehydrogenase, creatine kinase and aldolase were declined. This may be caused by the loss of solubility

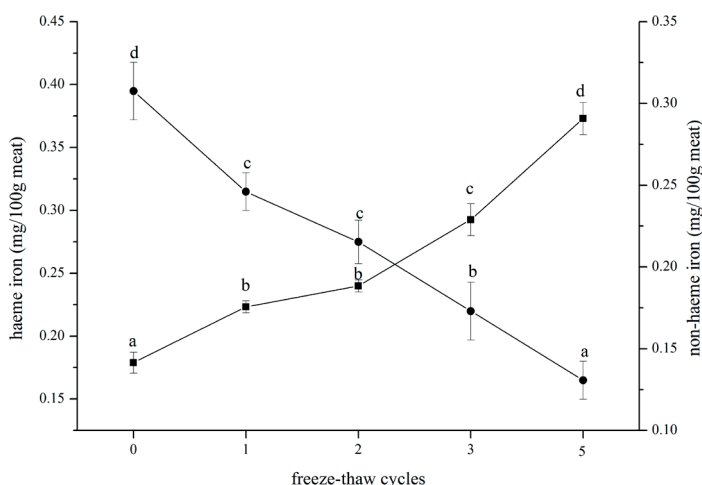


Figure 4: Changes in haeme iron and non-haeme iron content of rabbit meat subjected to repeated freeze-thaw cycles. ^{a-d}Different letters in the same line indicate significant differences between different numbers of repeated freeze-thaw cycles ($P < 0.05$). (n=4). Haeme iron: (●); Non-haeme iron: (■).

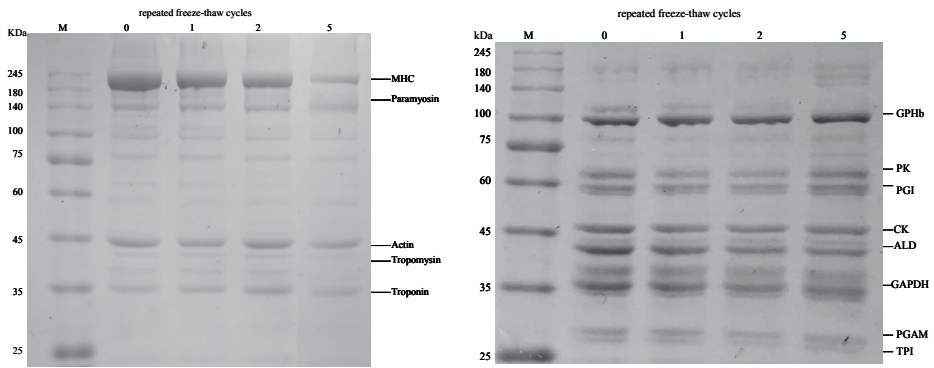


Figure 5: An SDS-PAGE of salt-soluble protein (A) and water-soluble protein (B) from rabbit meat subjected to repeated freeze-thaw cycles. M: Standard protein; GPHb: Glycogen phosphorylase b; PK: Pyruvate kinase; PGI: Phosphoglucose isomerase; CK: Creatine kinase; ALD: Aldolase; GAPDH: Glyceraldehyde phosphate dehydrogenase; PGAM: Phosphoglycerate mutase; TPI: Triosephosphate isomerase.

of sarcoplasmic proteins after denaturation (Marino *et al.*, 2014). On the other hand, our SDS-PAGE results showed that other protein bands—including phosphoglycerate mutase and triosephosphate isomerase—increased as the number of repeated freeze-thaw cycles increased. Similar results were observed by Marino *et al.* (2014), who reported that among young bulls undergoing natural aging, the abundance of some sarcoplasmic proteins declined, while the abundance of other proteins increased.

The relationship among variables of interest

Principal component analysis (PCA) is a technique used to evaluate the relationship among variables. In PCA, the projection of the variables on a space (direction and distance) defined by the 2 first components can provide relevant information about the potential connections among all variables. We performed a PCA on the data we collected during repeated freeze-thaw cycles. Figure 6 illustrated the similarity map of different parameters defined by the 2 first principal components (PC#1 and PC#2), which accounted for 95.45% of the total variance. The first principal

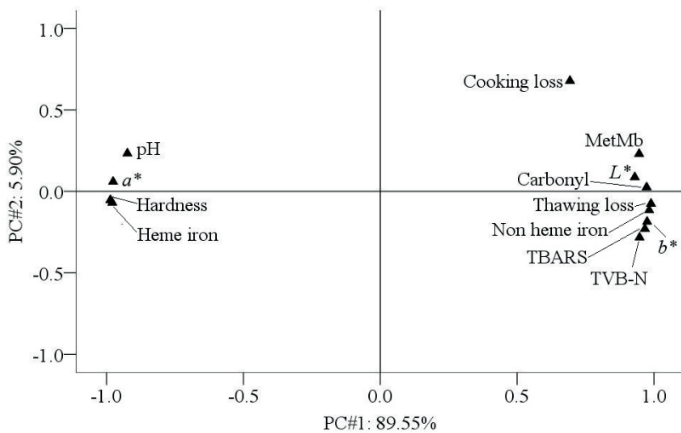


Figure 6: Projection of different indices onto a space defined by principal components PC1 and PC2 (with percentage of total variance explained).

component, which accounted for 89.55% of the total variance, had positive loadings for MetMb, TBARS, carbonyl, thawing loss, L^* , a^* , TVB-N, and non-haeme iron content, and was negatively defined by pH, b^* , hardness, and haeme iron. As shown in Figure 6, myoglobin oxidation (MetMb proportion), lipid oxidation (TBARS) and protein oxidation (carbonyl content) were clustered in the right side, which was expected, as all forms of oxidation are to some degree related to one another (Leygonie *et al.*, 2012). Non-haeme iron was positively correlated with TBARS and carbonyl levels, which was also expected, since non-haeme iron acts as a principal catalyst for lipid and protein oxidation (Oueslati *et al.*, 2016). Additionally, a strongly positive relationship between carbonyl and TVB-N levels indicated a potential association between protein degradation and protein oxidation. Maqsood and Benjakul (2010b) argued that protein oxidation resulted in fragmentation and degradation of structural protein. Berardo *et al.* (2015) also found that protein oxidation could affect proteolysis in a meat model system. Finally, our PCA results showed good correlations between physicochemical properties (TBARS, carbonyl level, and MetMb) and quality indices such as thawing loss, a^* , L^* and hardness, which, taken together, supported the argument that meat quality is closely associated with its physicochemical properties.

CONCLUSIONS

The repeated freeze-thaw process strongly affected rabbit meat quality, as well as its physicochemical properties. Repeated freeze-thaw cycles could reduce pH value, a^* value, hardness, WHC and haeme iron content, and could increase TVB-N, non-haeme iron content, L^* value and b^* value. Moreover, repeated freeze-thaw cycles could lead to the degradation of water- and salt-soluble proteins. During repeated freeze-thaw cycles, increases in MetMb proportion, TBARS and protein carbonyl content were accompanied by meat quality changes. Thus, changes in meat quality due to repeated freeze-thaw cycles were closely associated with physicochemical properties.

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