Effect of freezing treatment in tea preparation using *Camellia sinensis* leaves

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Abstract This study was conducted to examine the effect of freezing in the tea preparation using *Camellia sinensis* leaves in order to develop new technology for improving tea quality. Effects of single and combined treatments of freezing (F: $-20\text{ }^{\circ}\text{C}$, 15 days), steaming (S: $100\text{ }^{\circ}\text{C}$, 3 min), and hot-air drying (HD: $50\text{ }^{\circ}\text{C}$, 12 h) on the physicochemical characteristics of tea (powder and hot-water leachate) prepared from treated *Camellia sinensis* leaves were investigated. The L^* , b^* , and C^* values of the powder were the highest in SHD-treated leaves, followed by HD-, SFHD-, and FHD-treated leaves. Redness ($+a^*$ value) was only seen in FHD-treated leaves and greenness ($-a^*$ value) was the highest in SHD-treated leaves. Browning index of the leachate was significantly lower in SHD- and SFHD-treated leaves than in FHD-treated leaves ($p < 0.05$). Soluble solids, acidity, and phenolic compounds of FHD-treated leaves were significantly lower than in SHD- and SFHD-treated leaves ($p < 0.05$). Sucrose was highest in SHD-treated leaves and glucose was highest in FHD-treated leaves. Sensory preference was better evaluated in FHD-treated leaves than in SHD- and SFHD-treated leaves. These results suggest that freezing treatment in tea processing promotes browning and component

decomposition and enhances sensual preference. Therefore, freezing has a potential application in the tea manufacturing.

Keywords *Camellia sinensis* · Tea · Freezing · Steaming · Leachate

Introduction

Camellia sinensis is an evergreen shrub that can be grown in tropical and subtropical climates. Young leaves of *C. sinensis* are picked and processed into various tea forms, which contain catechin, caffeine, protein, amino acids, organic acid, sugar, vitamin A and C, and inorganic salts that produce a unique flavor in harmony with astringent, bitter, umami, and sweet tastes (Kang 2011; Kim et al. 2014b). Recently, public interest has been increasing in the health functionalities of this tea, such as its antioxidant, anticancer, antibacterial, and antitoxin properties (Yang and Liu 2013; Friedman 2007). Therefore, it is necessary to diversify tea processing methods and improve tea quality, aiming at increasing tea consumption.

Tea is generally prepared by treating *C. sinensis* leaves through heating (roasting, steaming), wilting, rolling, fermentation, and drying (Kim et al. 2009). The raw material remains the same but it acquires different flavors and health functionalities according to the kind of treatment and degree of application of the processing method (Yang and Liu 2013; Ruxton 2013; Jo et al. 2006). Among these different types of tea, green tea is unfermented tea prepared by intensive application of heat treatment to prevent oxidation, by deactivating enzymes in tea leaves and preserving the green color. On the other hand, fermented tea is called black tea, which is wilted, rolled and fermented

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without heating, and gains a reddish brown color and a distinctive flavor through oxidation and polymerization of catechins by oxidase (Ruxton 2013). Wilting, rolling and fermentation, which are key processes in the process of tea making, are complex processes requiring experience and skills to enable tissue destruction, enzyme activity, and oxidation (Mohammed et al. 1986). This necessitates research on the effects of parallel treatments with existing and new treatment methods for improving the processing and quality of tea products and developing new products. Such studies should include freezing treatment, which has an alternative effect to that of the existing mechanical rolling and fermentation process because oxidation is promoted through the destruction of plant tissue during freezing (Chassagne-Berces et al. 2009; Delgado and Rubiolo 2005). Freezing suppresses chemical and microbial degradation by converting the moisture in food tissues from liquid to ice, and is mainly used for preservation; however, it causes tissue destruction due to the accompanying ice particle formation and growth (Chassagne-Berces et al. 2009; Delgado and Rubiolo 2005). Tissue destruction caused by freezing needs to be suppressed as much as possible during food preservation, but there are some processing methods in which tissue destruction is induced. For example, tissue destruction caused by freezing has increased the juice yield and drying speed of *Prunus mume*, as well as improved the osmotic extraction process (Chung et al. 2010; Chung et al. 2013), and it has increased the yield of apple juice (Nadulski et al. 2016). However, it has been reported that the product has brown coloration caused by oxidation. Although the oxidation effect of freezing treatment is a problem in the general food manufacturing process, it is a necessary phenomenon in the tea making process. Freezing treatment is expected to be effective in processing tea leaves; however, no information available on this aspect.

Therefore, to obtain basic information necessary for the manufacture and development of new products using *C. sinensis* leaves, this study investigated the effect of freezing on the physicochemical and sensory characteristics of tea and compared these with the effects of steaming and hot-air drying, which are existing manufacturing methods.

Materials and methods

Materials

C. sinensis leaves were harvested on April 22, 2018, from a commercial farm in the Hadong region of Korea and immediately transported to the laboratory. The leaves were used for the experiments described below. Folin–Ciocalteu reagent was purchased from Junsei Chemical Co. (Tokyo,

Japan), and the catechin, sucrose, glucose, and fructose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All the chemicals used for analyses were of high-purity grade.

Treatments and tea preparation

C. sinensis leaves were treated to change their physicochemical properties by using a single method or combination of three different methods (steaming, freezing and drying). Sets of 200 g *C. sinensis* leaves were submitted to four different treatments: a) hot-air drying for 12 h at 50 °C (HD), b) steam blanching for 3 min at 100 °C followed by HD (SHD), c) freezing for 15 days at − 20 °C, followed by HD (FHD), and d) steam blanching for 3 min at 100 °C and freezing for 15 days at − 20 °C, followed by HD (SFHD). Samples for steaming or drying treatment were spread to single layer and sample for freezing was packed in nylon/polyethylene bag of 0.08 mm thickness. The treatment conditions were obtained from preliminary tests to achieve approximately effective conditions. After each treatment, the dried *C. sinensis* leaves were ground by a mill (J-NCM, Jisico Co., Seoul, Korea), sieved to a size of 0.351 mm and the powder was used for analysis of color properties. The powdered samples (1.5 g) were leached with 100 mL of distilled water for 10 min at 80 °C under atmospheric pressure. The leachate was filtered through filter paper (No. 2, Advantec, Tokyo, Japan) and kept at 4 °C until use for analyzing browning, soluble solid content, titratable acidity, phenolic compound content, and free sugar content. Samples for sensory test were maintained at 60 °C until use. Photographs of the powder and hot-water leachate prepared from *C. sinensis* leaves treated with different methods are presented in Fig. 1.

Analysis of color properties

The color properties of the powdered *C. sinensis* leaves were determined using a colorimeter (CR-400, Konika Minolta Inc., Tokyo, Japan) fitted with a CIE illuminant C and featuring a measuring aperture 8 mm in diameter, which was calibrated using a standard white plate ($L^* = 97.79$, $a^* = -0.38$, and $b^* = 2.05$) (Chung et al. 2014). Three readings for L^* , a^* , and b^* were recorded for each sample. Here, L^* represented the lightness, while $+a^*$, $-a^*$, $+b^*$ and $-b^*$ represented the redness, greenness, yellowness and blueness of the sample, respectively. The L^* , a^* , and b^* values for each sample were converted into C^* and h° values according to the following equations. Furthermore, C^* was a measure of the purity or saturation of the color and was calculated as $C^* = (a^{*2} + b^{*2})^{1/2}$. Lastly, h° was a measure of the color tone, which was defined as red–purple: 0°, yellow: 90°, bluish-green: 180°,

Fig. 1 Photographs of dried powder (upper) and hot-water leachate (lower) prepared from *Camellia sinensis* leaf treated with different methods. HD, treated with hot-air drying for 12 h at 50 °C; SHD,

steam blanching for 3 min at 100 °C followed by HD; FHD, freezing for 15 days at – 20 °C followed by HD; SFHD, steam blanching for 3 min at 100 °C and freezing for 15 days at – 20 °C followed by HD

and blue: 270° , and was calculated as $h^\circ = \tan^{-1} (b^*/a^*)$, when $a^* > 0$ and $b^* > 0$, or as $h^\circ = 180^\circ + \tan^{-1} (b^*/a^*)$ when $a^* < 0$ and $b^* > 0$.

Analysis of browning

The browning index of the leachate was measured as a function of light absorbance at 420 nm using a spectrophotometer (UV1800, Shimadzu, Kyoto, Japan) (Kim et al. 2014a).

Analysis of soluble solids

For the determination of soluble solids, a portion (5 mL) of the leachate was transferred into a weighing dish and dried at 105 °C by a convection oven (VS-1202D3N, Vision Scientific Co., Bucheon, Korea) to constant weight. The content of soluble solids was expressed as a ratio of weight of extracted solids against leachate sample volume (Jang et al. 2006).

Analysis of titratable acidity

The titratable acidity was measured by titrating the diluted leachate with 0.1 mol/L NaOH to pH 8.2 and it was expressed as a percentage of malic acid (Chung et al. 2013; Choi and Choi 2003).

Analysis of free sugars

The free sugar content was analyzed using the following procedure (Choi and Choi 2003). First, the leachate was diluted with deionized water, passed through a Sep-Pak C₁₈ cartridge (Waters) and a 0.45-μm membrane filter (Millipore Co., Bedford, MA, USA), and injected into an HPLC (600E, Waters Co., Milford, MA, USA). Next, a Sugar-Pak I column (Waters) was used at a temperature of 90 °C. Then, for the mobile phase, 0.005 g/L Ca-EDTA (0.5 mL/min), a refractive index detector (RI 410, Waters) was used. Finally, the peaks were quantified by the external standard method.

Analysis of phenolic compounds

The content of phenolic compounds was measured according to the Folin-Ciocalteu procedure (Singleton and Rossi 1965). First, 2 mL samples of the leachate were transferred into test tubes. Then, 2 mL Folin-Ciocalteu reagent was added to each test tube, the content of which was thoroughly mixed. After 3 min, 2 mL of 10% Na₂CO₃ solution was added to each test tube and the mixtures were incubated for 1 h. The absorbances of the resulting solutions were measured using a spectrophotometer (UV1800, Shimadzu) at 760 nm. The content of phenolic compounds was measured by comparing the absorbances of the samples to those of different concentrations of catechin used as standard.

Analysis of sensory preferences

A sensory test was conducted using 30 university students as study subjects. The test was carried out in a room illuminated with a fluorescent lamp. The leachates were served to the subjects in a random order on white cups, each labelled with a 3-digit code. The subjects were instructed to cleanse their mouths between samples using distilled water. The subjects evaluated the sensory preferences of the leachate, such as color, aroma, taste, and overall acceptability, using a 9-point scale (1 = extremely dislike and 9 = extremely like).

Statistical analysis

All experiments were performed in triplicate, and the data are presented as the mean $\pm$ standard deviation (SD). The effect of the study treatments on the quality characteristics of the tea from *C. sinensis* leaves was assessed using analysis of variance (ANOVA), and the mean values were compared using Duncan's multiple range test ($p < 0.05$). All statistical analyses were performed using IBM SPSS statistics software (23, IBM Corp., Armonk, NY, USA).

Results and discussion

Comparison of color and browning index

Visual color is a primary factor in general quality judgment of foods and may be assessed in terms of quantitative parameters such as L^* , a^* , b^* , h° , and C^* values. Color parameters of the tea powder prepared from *C. sinensis* leaves treated with single or combined treatments of steaming (S), freezing (F), and hot-air drying (HD) are listed in Table 1. Based on the ANOVA results, the L^* , a^* , b^* , C^* , and h° values of the tea powder were significantly affected ($p < 0.05$) by the treatment method. The lightness

(L^* value) was the highest in the SHD-treated sample, followed by the HD-, SFHD-, and FHD-treated samples. The redness ($+a^*$ value) was only seen in the FHD-treated sample and greenness ($-a^*$ value) was the highest in the SHD-treated sample. Considering that the L^* value and the $+a^*$ value are generally used as an indicator of browning in food processing (Chung et al. 2014; Castaner et al. 1999), these results suggest that the steaming treatment inhibited browning of the powder from *C. sinensis* leaves, but that the freezing treatment promoted the loss of greenness and browning of the powder. Steaming before freezing can suppress the discoloration effect of the freezing treatment to some extent. The L^* value is decreased and a^* value increased with increasing fermentation time in tea processing (Jo et al. 2006), which suggests that the freezing treatment also induces browning like the fermentation treatment in black tea processing. The b^* value and C^* value were the highest in the SHD-treated sample, followed by the HD-, SFHD-, and FHD-treated samples. The color tone (h° value) was the highest in the SHD-treated leaves and the lowest in the FHD-treated leaves. The reason for the changes in color properties in tea processing is known to be because chlorophyll, flavonoids and tannins, which are the main pigments of *C. sinensis* leaves, are browned by oxidation and heat treatment (Choi et al. 2003; Jo et al. 2006). The results suggested that combined freezing and steaming could be a useful method to control the color changes in *C. sinensis* leaves.

The browning index is an indirect measure of the contents of pigment compounds produced from browning reactions. The browning of tea products is mainly caused by enzymatic reactions. The degree of browning is closely related to sensory preference score and is higher in black tea than in green tea (Yeo et al. 1995). The browning index of the leachate from the powdered *C. sinensis* leaves that were treated with single or combined treatments of steaming (S), freezing (F), and hot-air drying (HD) are shown in Fig. 2. The browning index of the leachate from

Table 1 Color properties of powder prepared from *Camellia sinensis* leaf treated with different methods

Property	Treatment method ^a			
	HD	SHD	FHD	SFHD
L^*	39.83 $\pm$ 0.09 ^b	43.69 $\pm$ 0.32 ^a	32.29 $\pm$ 0.56 ^d	39.01 $\pm$ 0.03 ^c
a^*	- 1.16 $\pm$ 0.06 ^b	- 4.96 $\pm$ 0.09 ^d	2.24 $\pm$ 0.07 ^a	- 2.67 $\pm$ 0.02 ^c
b^*	19.78 $\pm$ 0.02 ^b	20.79 $\pm$ 0.44 ^a	13.23 $\pm$ 0.20 ^d	16.62 $\pm$ 0.14 ^c
h°	93.36 $\pm$ 0.16 ^c	103.41 $\pm$ 0.08 ^a	80.38 $\pm$ 0.32 ^d	99.12 $\pm$ 0.12 ^b
C^*	19.81 $\pm$ 0.02 ^b	21.37 $\pm$ 0.44 ^a	13.42 $\pm$ 0.20 ^d	16.83 $\pm$ 0.14 ^c

Mean $\pm$ SD ($n = 3$) in a row followed by the same letter are not significantly different by Duncan's multiple range test ($p < 0.05$)

^aHD, treated with hot-air drying for 12 h at 50 °C; SHD, steam blanching for 3 min at 100 °C followed by HD; FHD, freezing for 15 days at - 20 °C followed by HD; SFHD, steam blanching for 3 min at 100 °C and freezing for 15 days at - 20 °C followed by HD

Fig. 2 Browning index of hot-water extracts prepared from *Camellia sinensis* leaf treated with different methods. Mean $\pm$ SD ($n = 3$) with a different letter above the bar are significantly different by Duncan's multiple range test ($p < 0.05$). HD, treated with hot-air drying for 12 h at 50 °C; SHD, steam blanching for 3 min at 100 °C followed by HD; FHD, freezing for 15 days at -20 °C followed by HD; SFHD, steam blanching for 3 min at 100 °C and freezing for 15 days at -20 °C followed by HD

the SHD- and SFHD-treated leaves was significantly lower than that from only HD-treated and that of the FHD-treated leaves was significantly higher than that of HD-treated leaves. There was no significant difference in the browning index between the SHD- and SFHD-treated leaves. This effect of steaming in restraining browning could be explained by the inactivity of polyphenol oxidase and peroxidase induced by the high temperature of steam (Severini et al. 2004). The elevated browning effect of freezing is attributed to the increased contact between the substrate and the enzyme because of cell tissue destruction following freezing (Chung et al. 2010; Zocca and Ryugo 1975). It is well known that the higher brownness of fermented compared to non-fermented tea raises the sensory color quality (Choi et al. 2003). Thus, in this study the high brownness in FHD-treated leaf samples may be related to their overall sensory quality, which could be improved by freezing treatment.

Comparison of soluble solids and titratable acidity

Soluble solid content is an approximate measure of the sugar content in food. Titratable acidity is a quantitative measure of organic acids present in food. The soluble solid content and titratable acidity greatly affect the sensory quality of beverages. General extraction yield was estimated as the content of soluble solids in the water extract. Soluble solid content and titratable acidity of hot-water leachate prepared from *C. sinensis* leaf treated with different methods are presented in Table 2. The soluble solid

content and titratable acidity were significantly affected ($p < 0.05$) by the treatment method. The soluble solid content of the HD-treated sample was significantly lower than those of the SHD- and SFHD-treated samples and higher than that of the FHD-treated sample. There was no significant difference in the soluble solid content between the SHD- and SFHD-treated samples. This effect of increasing soluble solids after steaming may be due to the increased leaching rate because of the thermal degradation of tissue and decreased reaction rate because of enzyme inactivation (Ramana et al. 1992; Rejano et al. 2007). In contrast, the decrease in soluble solids after freezing may be related to a browning reaction, which can convert soluble low molecule compounds to insoluble high molecule compounds. These results suggest that steaming increases the hot-water leaching yield of soluble solids from *C. sinensis* leaves, whereas freezing decreases it. This effect of freezing was controlled by using a treatment of combined steaming and freezing. Titratable acidity was the highest in the SHD- and SFHD- treated samples, followed by the FHD- and HD-treated samples. There was no significant difference in titratable acidity between the SHD- and SFHD-treated samples. These results suggest that the titratable acidity of hot-water leachate is increased by steaming or freezing.

Comparison of free sugars

Free sugar content of foods is considered an index in processing and quality control. The main free sugars in fresh *C. sinensis* leaves are fructose, glucose and sucrose, and their content is changed during tea preparation (Kim et al. 1983). The free sugar content of hot-water leachate prepared from *Camellia sinensis* leaf treated with different methods is shown in Table 2. The free sugar in the hot-water leachate consisted of glucose and sucrose. The free sugar content was significantly affected ($p < 0.05$) by the treatment method. The content of sucrose was the highest in the SHD-treated sample, followed by the SFHD- and HD-treated samples, but sucrose was not found in the FHD-treated sample. The content of glucose was the highest in the FHD-treated sample, followed by the HD-, SFHD-, SHD-treated samples. These effects of SHD and FHD may be explained by the inhibition and promotion of sucrose hydrolysis by steaming and freezing, respectively (Lee et al. 2008). This freezing effect appear to have been caused by tissue destruction developed by freezing, similar to the case of browning mentioned above. These results suggest that steaming and freezing treatments before drying affected the composition and content of free sugar of hot-water leachate from the powdered *C. sinensis* leaves. Steaming treatment was effective for sucrose maintenance

Table 2 Soluble solids and titratable acidity of hot-water leachate prepared from *Camellia sinensis* leaf treated with different methods

Characteristic	Treatment method ^a			
	HD	SHD	FHD	SFHD
Soluble solids (mg/100 mL)	320.00 ± 5.29 ^b	396.67 ± 7.57 ^a	295.33 ± 3.06 ^c	385.33 ± 11.37 ^a
Titratable acidity (mg/100 mL)	15.89 ± 0.71 ^c	21.73 ± 0.62 ^a	17.87 ± 0.41 ^b	21.46 ± 0.95 ^a
Sucrose (mg/100 mL)	12.58 ± 0.40 ^c	16.86 ± 0.08 ^a	ND	15.24 ± 0.55 ^b
Glucose (mg/100 mL)	15.05 ± 0.08 ^b	11.42 ± 0.04 ^d	23.05 ± 0.09 ^a	12.42 ± 0.17 ^c

Mean ± SD ($n = 3$) in a row followed by the same letter are not significantly different by Duncan's multiple range test ($p < 0.05$). ND, not detected

^aHD, treated with hot-air drying for 12 h at 50 °C; SHD, steam blanching for 3 min at 100 °C followed by HD; FHD, freezing for 15 days at − 20 °C followed by HD; SFHD, steam blanching for 3 min at 100 °C and freezing for 15 days at − 20 °C followed by HD

and freezing treatment was effective for promoting sucrose degradation.

Comparison of phenolic compounds

Phenolic compounds are the substrates in enzymatic browning reactions with many biological and functional activities for food quality and human health (Tomas-Barberan and Espin 2001). The representative phenolic substance of *C. sinensis* leaves is catechin, which has lower content in fermented tea than in non-fermented tea because the flavor and color of tea are formed by oxidation of the catechin (Jeong et al. 2009; Subramanian et al. 1999). The phenolic compound content of hot-water leachate prepared from *Camellia sinensis* leaf treated with different methods is shown in Fig. 3. The phenolic compound content was significantly affected ($p < 0.05$) by the treatment method. The phenolic compound content was the highest in the SHD- and SFHD-treated samples, followed by the HD- and FHD-treated samples. There was no significant difference in phenolic compounds between the SHD- and SFHD-treated samples. This result is contrary to the previously mentioned result for the browning index (Fig. 2), and it is thought that the consumption of the reaction substance was decreased due to oxidation inhibition during the heating treatment (Xiao et al. 2017). Therefore, the phenolic compound content of hot-water leachate from powdered *C. sinensis* leaves was increased by pre-drying heating treatment and decreased by freezing treatment, but the effect of freezing was negated by heating.

Comparison of sensory preferences

The sensory preferences, color, aroma, taste, and overall acceptability, of hot-water leachate prepared from the powdered *C. sinensis* leaves treated with different methods are listed in Table 3. All sensory preferences were

significantly affected ($p < 0.05$) by the treatment methods. Color preference was significantly better in the FHD-treated sample, and was significantly worse with steaming treatment, compared to that of other methods. The high color score in the sample from the FHD-treated leaves may be related to their greater brownness (Table 1 and Fig. 2). The higher degree of fermentation compared to that in the non-fermented tea raises the color preference (Choi et al. 2003). Thus the color preference of hot water leaching tea is judged to be higher in reddish brown than in green. The aroma preference was the highest in the HD-treated sample, the lowest in the SHD-treated sample. The taste score of the FHD-treated sample was significantly higher than that of the other treatment samples. The taste score of the SHD- and SFHD-treated samples was significantly lower than the others. The FHD-treated sample showed a significantly higher score in terms of overall acceptability, which was attributed to the high color, aroma, and taste scores in the FHD-treated sample. Therefore, the results suggest that the overall sensory acceptability of tea could be improved by freezing treatment.

Conclusion

The steaming of *C. sinensis* leaves inhibits the physico-chemical change and lowers the sensory preference of tea, whereas freezing changes the physicochemical characteristics through browning and component decomposition, but does improve the sensory preference according to browning promotion. The effect of freezing was controlled by the combination of freezing and steaming. This suggests that freeze treatments, rather than heat treatments such as steaming and roasting that have been used traditionally in tea preparation, have potential applications in the manufacturing and quality control of tea. Further research is

Table 3 Sensory preference of hot-water leachate prepared from *Camellia sinensis* leaf treated with different methods

Sensory property	Treatment method ^a			
	HD	SHD	FHD	SFHD
Color	6.05 ± 1.78 ^{ab}	5.00 ± 2.49 ^b	6.74 ± 1.33 ^a	5.21 ± 2.39 ^b
Aroma	5.58 ± 2.04 ^a	3.11 ± 2.00 ^c	4.79 ± 1.93 ^{ab}	3.58 ± 1.74 ^{bc}
Taste	4.74 ± 2.21 ^b	2.84 ± 2.39 ^c	6.21 ± 1.96 ^a	2.78 ± 1.70 ^c
Overall	6.11 ± 1.45 ^a	3.63 ± 1.89 ^b	6.37 ± 1.42 ^a	3.79 ± 1.36 ^b

Mean ± SD ($n = 3$) in a row followed by the same letter are not significantly different by Duncan's multiple range test ($p < 0.05$)

^aHD, treated with hot-air drying for 12 h at 50 °C; SHD, steam blanching for 3 min at 100 °C followed by HD; FHD, freezing for 15 days at − 20 °C followed by HD; SFHD, steam blanching for 3 min at 100 °C and freezing for 15 days at − 20 °C followed by HD

Fig. 3 Phenolic compound content of hot-water extracts prepared from *Camellia sinensis* leaf treated with different methods. Mean ± SD ($n = 3$) with a different letter above the bar are significantly different by Duncan's multiple range test ($p < 0.05$). HD, treated with hot-air drying for 12 h at 50 °C; SHD, steam blanching for 3 min at 100 °C followed by HD; FHD, freezing for 15 days at − 20 °C followed by HD; SFHD, steam blanching for 3 min at 100 °C and freezing for 15 days at − 20 °C followed by HD

needed on the specific application conditions and cost and action mechanisms of browning treatment.

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Compliance with ethical standards

Conflict of interests The authors declare no conflict of interest.

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