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Effects of pasteurization on the physicochemical, sensory properties and microbiological quality of beetroot (*Beta vulgaris L.*) wine during storage

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ABSTRACT

Introduction: In the beverage sector, pasteurization is combined with other means of preservation, such as concentration and acidification, to extend product shelf life by inactivating all nonspore-forming pathogenic bacteria and the majority of vegetative spoilage microorganisms, as well as inhibiting or stopping microbial and enzyme activity. The main objective of this study was to focus on the effect of pasteurization and storage on the physical, antioxidant, and microbiological properties of red beetroot (*Beta vulgaris l.*) wine. **Method**: After fermentation for 10 days, red beetroot wine was either pasteurized at 70°C for 10 minutes or unpasteurized before storage at 4^0C for different times (1 month, 2 months). The physicochemical properties and microbiological quality of beetroot wine were analyzed to study the effect of pasteurization and storage on the properties of beetroot wine. **Results**: Physical properties such as pH and total dissolved solids content were significantly affected by pasteurization (higher pH and higher total soluble solids content in the pasteurized samples than in the unpasteurized samples), but they were not affected by storage time in this project. After fermentation, the total phenolic content of red beetroot wine was lost, but it remained unchanged after pasteurization. Vitamin C in the wine sample was lost only when the wine was stored. Pasteurization and 2 months of storage caused a significant reduction in the antioxidant capacity of red beetroot wine. The sensory quality of beetroot wine was not affected by pasteurization, but it was affected by storage. The microorganism quality of the wine was acceptable for consumption. The pasteurization process is effective in increasing the shelf life of red beetroot wine but does not significantly affect its nutritional and sensory values.

Key words: Red beetroot wine, pasteurization, storage, total phenolic content, antioxidant capacity, vitamin C

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INTRODUCTION

Red beetroot (*Beta vulgaris L.*) is a member of *the Chenopodioideae* family. It contains high sugar and nitrate contents and is used as a natural food to boost energy in athletes[1]. It has a deep red color feature derived from betalain. Betalains are composed of red–violet betacyanins (e.g., betanin and isobetanin) and yellow betaxanthins (e.g., vulgaxanthin I and II), which are classified as water-soluble nitrogenous pigments[2]. Sener et al., 2007[3] reported that red beetroot is a low-fat vegetable but contains many micronutrients, such as potassium, magnesium, folic acid, iron, zinc, calcium, phosphorus, sodium, niacin, biotin, B6 and soluble fiber, which together with biologically accessible antioxidants promote health effects. Wootton-Beard and Ryan, 2011[4] found that beetroot juice had a greater total phenolic content (TPC) than other juices. The TPC levels in beetroot juice were 1450 mg GA/L, tomato juice was 695 mg GA/L, and carrot juice was 474 mg GA/L.

Pasteurization is a low-order heat treatment that is performed at a temperature below the boiling point of water[5]. In the beverage sector, it is combined with other means of preservation, such as concentration and acidification, to extend product shelf life by inactivating all nonspore-forming pathogenic bacteria and the majority of vegetative spoilage microorganisms, as well as inhibiting or stopping microbial and enzyme activity. Because of the low-order heat treatment, pasteurization may be more effective than sterilization in minimizing nutrition loss and organoleptic changes in heat-treated products. In addition, some enzymes that breakdown vitamin C and polyphenols are also inactivated by pyrolysis. A study conducted by Bhattacherjee et al., 2011[6] showed that vitamin C in Amla juice (Indian gooseberry juice) was affected by pasteurization temperature. Bianchi et al., 2021[7] found that the betalain content of beetroot juice was affected by pasteurization (85^0C for 3 mins), but its antioxidant activity was not. On the other hand,

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the phenolic compounds of Rose apple cider were reported to be significantly affected by both pasteurization and storage[8]. Pasteurization is also used by winemakers to avoid malolactic fermentation and to eliminate browning activity[9]. Therefore, this study was carried out to investigate the effect of pasteurization and storage on physicochemical, sensory, and microbiological characteristics.

MATERIALS AND METHODS

Must preparation

Fresh red beetroot (*Beta vulgaris L.*) was purchased from a local supermarket in Ho Chi Minh City, Vietnam, in the rainy season. The beetroot was selected based on firmness, size, color, and absence of physical damage. After being purchased, the red beetroot was processed in the Food Technology Laboratory of International University- Vietnam National University Ho Chi Minh City, Vietnam. The material was washed and peeled off before being pulped with deionized water (the ratio 1:4 w/v) by a blender (Philips HR2118, Indonesia) to collect red beetroot juice.

Wine fermentation

The wine fermentation followed the procedure of Otegbayo et al., 2020[10] with modification. The soluble solid content of the filtered beetroot juice was 8 ^{0}Brix, which was adjusted to reach 22 $\pm$1 ^{0}Brix by adding sucrose and deionized water, while the pH of the juice was not adjusted (pH = 4.5). The fermentation step was performed in sterilized containers. The freeze-dried yeast, *Saccharomyces cerevisiae* Lalvin EC-1118 (Canada), was mixed with sucrose and deionized water at a ratio of 1:1:5 (w/w/v). The solution was incubated for 15 minutes to activate the yeast. The activated yeast was inoculated into filtered beetroot juice to reach a yeast cell count of 1 x 10^7 CFU/ml at 20°C for 10 days. After 10 days, the crude wine was clarified by using a centrifuge (Hettich Universal 1406, Refrigerated, Germany) at 4500 RPM at 20°C for 15 min to remove the yeast and the suspended solid.

Pasteurization and storage

The clarified wine was either heated at 70°C for 10 min using a thermostatic water bath (Memmert WNE7) or unheated. The temperature of the wine samples during pasteurization was monitored using a thermometer inside the wine bottles. The pasteurized and unpasteurized wine samples were stored at 4°C for 0, 1, and 2 months before analysis. All data were used in triplicate for each treatment.

Physical measurement

Total soluble solids (TSS) and pH values of samples were measured with a refractometer (Atago rx 5000 alpha) and a pH meter (HANNA instruments HI 2216), respectively. The alcohol content was determined by using distillation, which was based on the method of Martins et al., 2020[11] with some modifications. In the experiment, 20 ml of sample (V1) was poured into a distillation flask and heated to 78^{0}C. The volume of collected alcohol was measured (V2), and the alcohol content was determined by the following equation.

$$Alcohol\ content = \frac{V_2}{V_1}$$

Total phenolic content (TPC)

The extraction of the phenolic compound, which is based on the method of Vinson et al., 2001[12] with some modifications, was carried out first before measuring the total phenolic content. To perform the extraction, 2 ml of each sample was mixed with 8 ml of 1.2 M HCl in 50% methanol (v/v) at 60°C in the dark for 2 hours with occasional shaking. The extracted solution was immediately centrifuged at 4500 RPM at 4°C for 15 mins after incubation. The supernatant was stored in a freezer until analysis.

Determination of the total phenolic content was performed according to Lamuela-Raventós et al., 2018[13], using the Folin–Ciocalteu colorimetric method. One milliliter of the sample was mixed with 1 ml of Folin-Ciocalteu's reagent (10 times diluted with water) before adding 5 ml of 10% disodium carbonate solution. The mixture was then incubated at room temperature for 2 hours after being diluted with 8.4 ml of deionized water. The absorbance was measured at 760 nm by a spectrophotometer (Genesys 10S UV Vis). Distilled water was used as the blank, and gallic acid was used as a calibration solution. The total phenolic content was calculated as milligrams of gallic acid equivalents per liter of sample (mg GAE/L). The results were expressed as the relative TPC. The relative TPC was the percentage of TPC in wine samples compared to TPC in the raw red beetroot.

% Relative TPC = (TPC wine/TPC juice) * 100

Vitamin C content

The content of vitamin C was quantified by titration with iodine solution, which was based on the method described by Babashahi-Kouhanesta et al., 2014[14]. To prepare the iodine solution, 5 g of potassium iodide (KI) was mixed with 0.27 g of potassium iodate

(KIO3) in 200 ml of distilled water before adding 30 ml of 3 M sulfuric acid. The mixture was then diluted with 500 ml of distilled water. The diluted sample (25ml) was poured together with 5 - 6 drops of 1% starch solution into the volumetric flask for titration. The mixture was titrated with an iodine solution until a dark blue color was obtained. The content of vitamin C in the samples (mg ascorbic acid/100 ml) was calculated according to the following formula.

mg ascorbic acid/100 ml = C_{iodine}*$V_{corected\ iodine}$*3*$M_{ascorbic\ acid}$ * 1000/Vsample * DF *100

where:

C_{iodine}: concentration of iodine solution (mol/l)

$V_{corrected\ iodine}$: volume of iodine solution used (l)

$M_{ascorbic\ acid}$: molar mass of ascorbic acid (g/mol)

V_{sample}: volume of samples (ml)

DF: dilution factor

DPPH Radical Scavenging Activity

Extraction for the DPPH assay was based on the procedure of Ruzlan et al., 2010 [15] with adjustments. One milliliter of sample was extracted with 4 ml of 70% methanol solution (v/v) at 60°C in darkness for 2 hours with occasional shaking. The extracts were then centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatants were kept at -20°C until use. The DPPH assay was measured by the modified method described by Ruzlan et al., 2010 [15]. Then, 0.1 ml of supernatant was mixed with 4.9 ml of 0.05 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent in absolute methanol. The tubes were incubated at room temperature in the dark for 30 min before being measured by a spectrophotometer (Genesys 10S UV Vis) at 517 nm wavelength. A total of 4.9 mL of 0.05 mM DPPH solution and 0.1 mL of 70% methanol without any sample was used as the control solution.

The percentage of the free radical scavenging effect was calculated as follows:

DPPH scavenging effect (%) = $\left(1 - \frac{A}{A_0}\right) \times 100$

where A_0 is the absorbance of the control solution and A is the absorbance of the sample solution at 517 nm.

Microbiological analysis

Based on the method of Choo et al., 2018 [16] with some modifications, the red beetroot wine samples were serially diluted to 10^{-3} with buffered peptone water. They were plated into plate count agar (PCA) for aerobic mesophilic counts and into dichloramphenicol rose bengal agar (DRBC) for yeast and mold counts with the spread plate technique. Plates were incubated for aerobic mesophilic bacteria at 37°C for 24 h and for yeasts and molds at 25°C for 5 days.

Sensory analysis

The sensory properties were analyzed to measure the acceptance of the wine samples. In this study, 30 untrained panelists evaluated the samples using a 5-point hedonic scale (1 - dislike extremely, 2 - dislike slightly, 3 - neither like nor dislike, 4 - like slightly, 5 - like extremely). The qualified sample (15ml) was served in and labeled with a 3-digit code in Latin square order. The panelists scored each sensory attribute (color, aroma, taste, mouthfeel, and overall) based on the 5-point hedonic scale at room temperature of 25 ± 1°C. The panelists did not taste the unpasteurized samples but evaluated other attributes.

Statistical analysis

All experimental results were analyzed using Minitab software (Version 21, IBM Corp., USA). The data were analyzed statistically by ANOVA followed by multiple range comparisons using Fisher pairwise comparisons (P<0.05). All numerical data are expressed as means ± standard deviation (SD) of triplicate measurements.

RESULTS

Physical properties

The physical characteristics of the samples are shown in Table 1. The pH value of the samples was in the range of 3.78 - 3.96. Pasteurization caused a higher pH value of the wine, while storage time did not affect it in the study. When wine was not stored, the pH values of pasteurized and unpasteurized samples were 3.89 and 3.78, respectively. After 2 months of storage, the values were 3.96 and 3.86 for pasteurized and unpasteurized wine, respectively.

Regarding the TSS content, pasteurization caused higher TSS content than that of the unpasteurized samples during 2 months of storage, which ranged from 7.88 to 9.04. Two months of storage did not affect the TSS of either unpasteurized or pasteurized samples.

The alcohol content of the product in this experiment ranged from 11.74% to 12.11%. Contrary to pH and TSS, alcohol content was not affected by thermal treatment. The alcohol content was unchanged during the shelf life for both unpasteurized and pasteurized samples except for the 2-month-stored unpasteurized samples.

Bioactive compounds

To characterize changes in the bioactive compounds of red beetroot wine after pasteurization and storage, vitamin C, antioxidant activity, and total phenolic content were analyzed (Table 2 and Figure 1).

Table 1: Physical properties of pasteurized and unpasteurized red beetroot wines during storage.

Storage time (month)	pH		Total soluble solids content (oBrix)		Alcohol content (%)	
	Unpasteurized	Pasteurized	Unpasteurized	Pasteurized	Unpasteurized	Pasteurized
0	$3.78 \pm 0.03^{a,x}$	$3.89 \pm 0.06^{a,y}$	$7.89 \pm 0.04^{a,x}$	$9.04 \pm 0.03^{a,y}$	$12.05 \pm 0.12^{a,x}$	$12.11 \pm 0.11^{a,x}$
1	$3.82 \pm 0.03^{a,x}$	$3.91 \pm 0.05^{a,y}$	$7.89 \pm 0.01^{a,x}$	$9.02 \pm 0.04^{a,y}$	$12.04 \pm 0.04^{a,x}$	$11.96 \pm 0.14^{a,x}$
2	$3.86 \pm 0.06^{a,x}$	$3.96 \pm 0.04^{a,y}$	$7.88 \pm 0.01^{a,x}$	$8.99 \pm 0.01^{a,y}$	$11.83 \pm 0.17^{b,x}$	$11.74 \pm 0.20^{a,x}$

Different letters (a, b) indicate significant differences ($p < 0.05$) in the same column. Different letters (x, y) indicate significant differences ($p < 0.05$) in the same row.

The vitamin C content of the samples ranged from 1.14 to 1.85 mg AA/100 ml. The vitamin C content in the beetroot wine samples was significantly reduced after 2 months of storage. During storage, the vitamin C content of the pasteurized wine was lost significantly after 2 months of storage from 1.85 to 1.39 mg AA/100 ml (25% loss), while that of unpasteurized samples was reduced by 36%. The samples without pasteurization show insignificantly lower vitamin C content than the pasteurized wine when stored at 4°C.

To evaluate the free radical scavenging ability, DPPH assays was performed. The DPPH assay was carried out to examine the radical scavenging activity of red beetroot wines, which is based on the hydrogen-donating capacity of the antioxidant components found in wine [17]. The radical scavenging activity of the fermented samples was found to be within the range of 34.03% - 35.27% (Table 2).

It can be seen that pasteurization affected the DPPH scavenging activity of beetroot wine. Before storage, the radical scavenging activity (%) of pasteurized samples was 34.5%, but it was 35.27% for unpasteurized samples. The reduction in antioxidant capacity was significant only when the red beetroot wine samples were stored for 2 months. The values were 34.03% and 34.79% for pasteurized and unpasteurized wine, respectively.

After fermentation, the loss of TPC in red beetroot wine was approximately 17%, but pasteurization at 70°C for 10 min did not affect the TPC during storage. In particular, approximately 82.97% and 80.52% relative TPCs were recorded for pasteurized and unpasteurized samples without storage, respectively. Storage caused a further loss of TPC by 25%, but there was no further reduction when the storage time increased. The relative TPC of pasteurized beetroot wine was 80.52%, but after 1 month of storage, it was reduced to 74.9%, and after 2 months of storage, it was 73.22%.

Microbial analysis

The microbiological quality of the wine samples was evaluated by 2 indicators, including the total aerobic mesophilic bacteria and the yeast/mold count (Table 3).

The microbiological result indicates that the red beetroot wine had a total of aerobic mesophilic bacteria after pasteurization, and after 2 months of storage at 4°C, it was less than $1.0x10^2$ CFU/ml. The yeast count of unpasteurized beetroot wine after fermentation reached 8.2×10^6 CFU/ml, which increased to 1.0×10^7 and 5.3×10^7 CFU/ml after storage for 1 and 2 months, respectively. The pasteurization significantly reduced the yeast amount in wine ($8.2x10^6$ CFU/ml for the control sample and $2.8x10^3$ CFU/ml for the heat-treated sample). Storage also significantly affected the yeast amount in the pasteurized wine. The number of yeast increased to 7.5×10^3 and 3.9×10^4 CFU/ml from $2.8x10^3$ CFU/ml after 1 month and 2 months of storage, respectively.

Sensory quality

Sensory analysis is a kind of analytical method to evaluate the quality of wine. The sensory data are shown in Figures 2, 3 and 4. To ensure the panelists' safety, the panelists did not evaluate the taste attributes of the unpasteurized red beetroot wine.

There was no significant difference between the pasteurized and unpasteurized wine in terms of sensory properties for all attributes (Figure 2). The score ranged from 3.77 to 4.23. The color attribute can be considered an indicator of the pasteurization process. However, the color-liking scores of both pasteurized and unpasteurized samples are not significantly different.

While pasteurization did not affect the sensory attributes of red beetroot wine in sensory analysis, storage time had a significant impact on sensory quality

Table 2: Bioactive compounds (vitamin C & antioxidant capacity) of the pasteurized and unpasteurized red beetroot wines during storage.

Parameters	Pasteurized			Unpasteurized		
	Storage time					
	0 month	1 month	2 months	0 month	1 month	2 months
Vitamin C (mg AA/100 ml)	1.85 ± 0.12^{a}	1.74 ± 0.12^{ab}	1.39 ± 0.10^{cd}	1.80 ± 0.153^{ab}	1.53 ± 0.07^{bc}	1.14 ± 0.12^{d}
DPPH radical scavenging activity (%)	$34.50 \pm 0.27^{c,d}$	$34.32 \pm 0.12^{d,e}$	34.03 ± 0.22^{e}	35.27 ± 0.13^{a}	$35.01 \pm 0.40^{a,b}$	$34.79 \pm 0.29^{b,c}$

Different lowercase letters (a-e) in the same row indicate significant differences (P < 0.05). AA = ascorbic acid

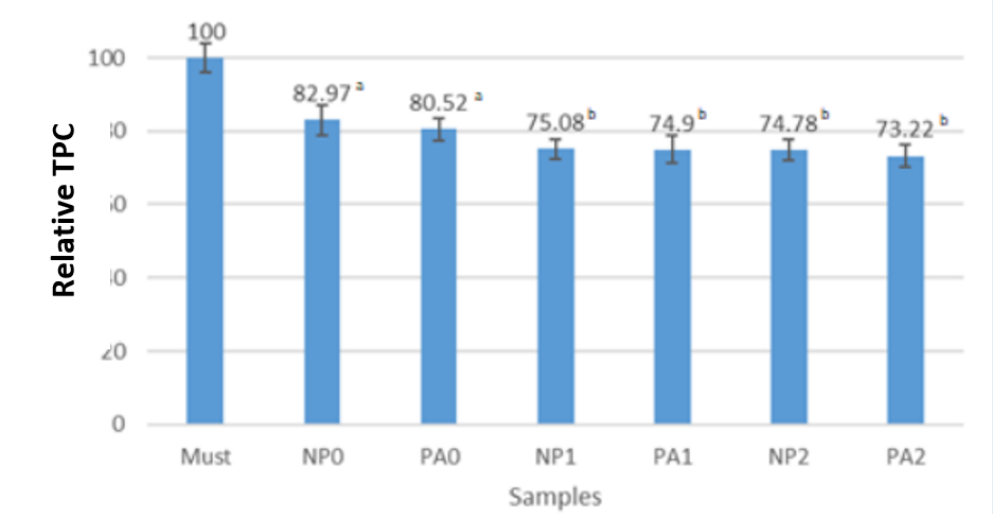

Figure 1: ***Relative total phenolic content of the pasteurized and unpasteurized red beetroot wines during storage .*** *Bars with the same letter are not significantly different (p > 0.05; Fisher's comparison test). NP0 = unpasteurized sample without storage. PA0 = pasteurized samples without storage. NP1 = unpasteurized samples stored for 1 month. PA1 = pasteurized samples stored for 1 month. NP2 = unpasteurized samples stored for 2 months. PA2 = pasteurized samples stored for 2 months.*

Table 3: Microbiological quality of the pasteurized and unpasteurized red beetroot wines during storage.

Storage time (month)	Microbiological qualities (CFU/ml)			
	Unpasteurized		Pasteurized	
	Aerobic bacteria	Yeast and mold	Aerobic bacteria	Yeast and mold
0	N.D	8.2x106 (yeast)	N.D	2.8x103 (yeast)
1	N.D	1.0x107 (yeast)	N.D	7.5x103 (yeast)
2	N.D	5.3x107 (yeast)	N.D	3.9x104 (yeast)

CFU = colonies forming unit, N.D. = not determined

(Figure 3 and Figure 4). The overall acceptability score of unpasteurized samples was 4.13 when it was not stored yet (Figure 3). However, the score was reduced to 3.7 after 2 months of storage at 4°C. In the case of unpasteurized samples, there was no significant difference between 1-month storage and 2-month storage except for the appearance attribute.

Pasteurized data shows that storage considerably affected sensory quality (Figure 4). The overall acceptability score of the control wine was 4.13, but it fell to 3.4 after 2 months of storage. Appearance and aroma were 2 attributes that were significantly affected by storage time.

DISCUSSION

Physical properties such as pH and total soluble solids content were significantly affected by pasteurization (higher pH and higher TSS in pasteurized samples than in unpasteurized samples), but they were not affected by storage time. The pH value is similar to the data reported by Martin et al., 2020[11]. The higher pH in pasteurized samples can be explained by inhibiting malolactic fermentation, while some organic acids, such as lactic acid, tartaric acid, and malic acid, can be produced in unpasteurized wine. pH value of wine plays an important role in the taste of wine. Low pH wine will taste tart and crisp, while higher pH wines will taste weak and flabby[10]. Most winemakers prefer a pH range of 3.0 - 3.5[18]. Our pH value was higher than the preferred pH value, but the sensory data and microbiological data showed a good result at our pH values. However, more research should be done to investigate a better shelf life of red beetroot wine. The TSS value has the same trend as the pH value, which indicate that pasteurized samples caused higher TSS value. This may be explained by the continuous fermentation process in unpasteurized wine, in which microorganisms consume more sugar. The TSS result in the study is consistent with the data of Rabie et al., 2015[19], which studied the effect of pasteurization on the physalic juice. The alcohol content was not affected by either thermal treatment or storage. This means that sugar was consumed to produce more acid than ethanol in the unpasteurized samples. Techakanon et al., 2020[8] explained that alcohol reduction could come from acid generated by some spoilage bacteria that was not inactivated by pasteurization.

The vitamin C content in red beetroot wine in this study was lower than the amount of vitamin C (4.62 mg AA/100 ml) reported by Otegbayo et al., 2020[10] but equivalent to 1.87 mg AA/100 ml investigated by Martins et al., 2020[11]. Many studies indicated that vitamin C reduction during storage is due to an oxidative mechanism resulting from the presence of not only oxygen but also exposure to light, heat peroxides, and enzymes such as ascorbate oxidase and peroxidase atmospheric oxygen[20,21]. It is surprising that pasteurization did not improve vitamin C loss. Heat treatment may not be good enough to inactivate ascorbic acid-degrading enzymes.

The DPPH radical scavenging of red beetroot wine in the study was lower than the result of grape wine recorded by Radovanović et al., 2010[22] but higher than the value of the Korean rice wine reported by Hong et al., 2009[23].

Many studies indicated that beetroot contained a high amount of total phenolic substances. Kovarovič et al., 2017[24] reported that the total phenolic content (TPC) in beetroot ranged from 820.10 mg GA/kg to 1280.56 mg GA/kg, while it was 1450 mg GA/l in the study of Wootton-Beard et al., 2011[4]. Therefore, TPC can be considered an indicator to evaluate effect of pasteurization. In this study, the TPC was not affected by pasteurization but was affected by storage. Peroxidase may cause phenolic compound loss after fermentation[19]. The enzyme was inactivated after pasteurization. The TPC of the red beetroot wine in this study was 1138.34 mg GA/l for unpasteurized samples and 1104.71 mg GA/l for pasteurized samples, which was lower than that of red grape wine (1600 mg GA/l) but higher than blackcurrant wine (1000 mg GA/l) and higher than black current and strawberry wine (695 mg GA/l)[25]

The microbiological data in the study met the acceptable level of microbiological criteria for wine in QCVN 6-3:2010/BYT, which is less than 10^3 CFU/ml of sample. The low aerobic mesophilic bacteria count was linked to factors such as low pH, the presence of alcohol, and the presence of antioxidants.

Regarding the sensory result, it is interesting that after pasteurization, the liking score for the color attribute of red beetroot wine was not significantly different from that of the unpasteurized samples. In general, the thermal treatment did not cause any change in panelists' preference when compared to the untreated ones. However, storage did affect the sensory properties significantly.

CONCLUSION

The result from this work demonstrate that the pasteurization process had a significant effect on the physiochemical and microbiological quality of red beetroot wine but not on sensory quality. Two months of storage affected the chemical, microbiological and sensory properties. Pasteurization at 70^0C

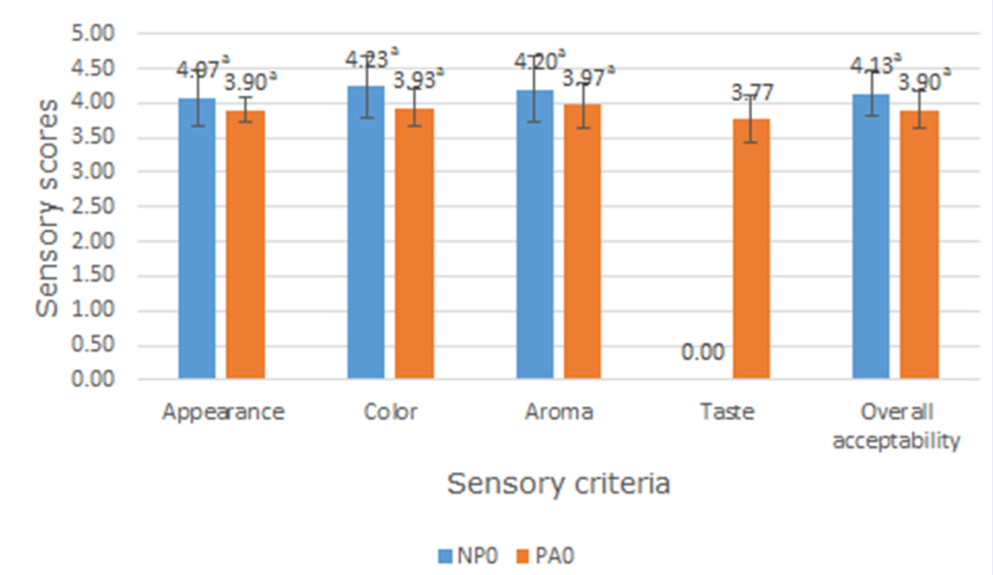

Figure 2: **Comparison of sensory scores between pasteurized and unpasteurized samples.** Bars with the same letter are not significantly different ($p < 0.05$; Fisher's comparison test). NP0 = sample was not pasteurized and not stored. PA0 = sample was pasteurized and not stored.

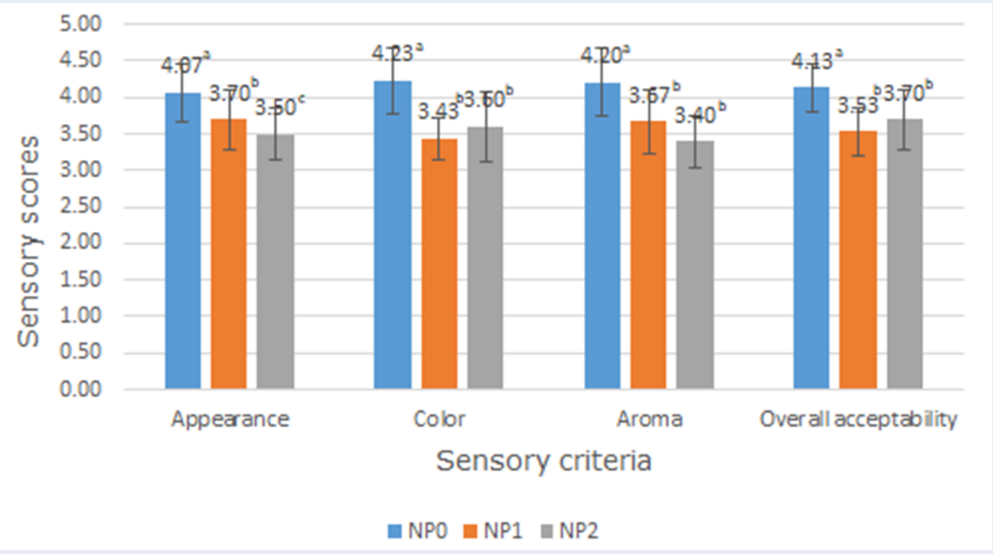

Figure 3: **Comparison of sensory scores of unpasteurized samples throughout storage.** Bars with the same letter are not significantly different ($p < 0.05$; Fisher's comparison test). NP0 = sample was not pasteurized and not stored. NP1 = sample was not pasteurized and stored for 1 month. NP2 = sample was not pasteurized and stored for 2 months.

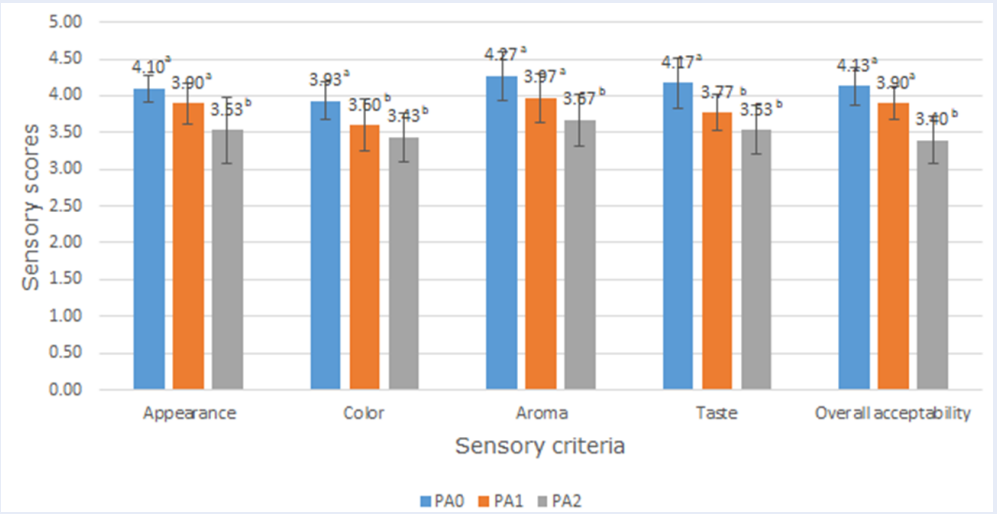

Figure 4: **Comparison of sensory scores of pasteurized samples throughout storage.** Bars with the same letter are not significantly different ($p < 0.05$; Fisher's comparison test). PA0 = sample was pasteurized and not stored. PA1 = sample pasteurized and stored for 1 month. PA2 = sample pasteurized and stored for 2 months.

in 10 mins decreased the amount of yeast significantly during storage, but the number of yeast was still higher than the limit of 1.0 x 10^2 CFU/ml in QCVN 6-3:2010/BYT. Further research is recommended to study better pasteurization conditions (temperature and time duration) to meet the standard and better preservation methods to stabilize the color attributes of wine.

LIST OF ABBREVIATIONS

TSS: total soluble solids
TPC: Total phenolic content
DPPH: 2,2-diphenyl-1-picrylhydrazyl
TA: Titratable acidity
GAE: Gallic Acid

COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

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