Research Article
Combined Effect of Monoxide Hemoglobin and Sodium Nitrite on Physicochemical, Microbiological and Sensory Properties of Pork Sausage

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Abstract: Sodium nitrite (NaNO$_2$) faces a challenge in the meat industry because of its carcinogenicity. The studies about nitrite substitutes have attracted a considerable interest for many years. This study aims at the combined effects of monoxide hemoglobin (COHb) and NaNO$_2$ on physicochemical, microbiological and sensory properties of pork sausages. The addition of COHb led to the reductions in $L*$, $b*$, TBA, VBN and aerobic plate count as well as the increase in $a*$ (p<0.05). The samples with 0.005% NaNO$_2$ and 0.2% COHb addition had equal even better sensory color, flavor, mouthfeel and slice traits than those with 0.015% NaNO$_2$ addition during storage. Moreover, such sample had less than 30 cfu/100 g *Escherichia coli* and no detectable *Staphylococcus aureus* during 29 days storage. Therefore, COHb showed a potential in the manufacture of sausages.

Keywords: Microbiological properties, monoxide hemoglobin (COHb), physicochemical properties, pork sausage, sensory properties, sodium nitrite (NaNO$_2$)

INTRODUCTION

Color is an important properties of meat and meat product because consumers usually make a decision of acceptance or rejection based on its color (Garcia *et al*., 2010). The present methods that improve the color of meat and meat product usually involve the utilization of nitrites and/or nitrates because of their multifunctional role (Filiatrault *et al*., 2013):

- Contributing the development of a characteristic pink color
- Having an antioxidative activity
- Being responsible for the production of a characteristic flavor of cured meats
- Inhibiting the growth of food spoilage bacteria and most especially, the growth of *Clostridium botulinum*, which produces the potential fatal toxin, botulin (Honikel, 2008). However, some reports have also indicated that the presence of nitrites and/or nitrates in meat products is potential health risks (Honikel, 2008). Nitrites can generate met-hemoglobin, which is a recognizable sign of nitrite toxicity in humans (Chow and Hong, 2002); and react with certain amines in foods to produce carcinogenic N-nitrosocompounds such as nitrosamines (Zhang *et al*., 2013). Therefore, utilization of nitrites and/or nitrates in the meat product faces a tough challenge.

A strategy that overcomes these shortcomings is to seek for a suitable replacer of nitrites and/or nitrates. Blood is one of the important byproducts during livestock and poultry processing, which is rich in protein, heme iron and other nutrients. Therefore, it is one of the most important candidates as an alternative of nitrites and/or nitrates (Zhou *et al*., 2012). However, blood is not stable and imparts color of the product to which it is added (Mielnik and Slinde, 1983). Stability of blood can be enhanced when hemoglobin (Hb) is converted to nitrosohemoglobin (NOHb) (Zhang *et al*., 2013). However, NOHb is light and heat-sensitive (Wang *et al*., 2010). The corresponding researches still continue.

Recently, there is a considerable interest in the utilization of carbon monoxide (CO) in Modified Atmosphere Packaging (MAP) for keeping fresh meat attractive color (Singh *et al*., 2011). Here, CO reacts with deoxymyoglobin (Mb) or/and oxymyoglobin (O$_2$Mb) to generate stable monoxide myoglobin (COMb) (Mancini and Hunt, 2005) and inhibits the outgrowth of microorganisms (Venturini *et al*., 2010), thus prolonging the shelf life of fresh meat. Moreover, some reports to date have shown no direct safety issue about the consumption of CO-treated meat (Wilkinson *et al*., 2006).

Similar to Mb, Hb can also bind with CO to generate stable monoxide hemoglobin (COHb). The treatment of CO was reported to enhance color stability of porcine blood during storage (Fontes *et al*., 2010). Moreover, the CO-treated blood has an attractive color

and thus showing great potential in the manufacture of sausage. The COHb-treated pork sausages was reported to have higher a* values but lower L* values than the non COHb-treated ones (Zhou *et al.*, 2012). However, the color of pork sausage treated with COHb alone was not uniform: its outer portion was relatively dark. Additionally, there is no report on the effects of COHb on other quality characteristics of meat products. We report the combined effects of COHb and $NaNO_2$ on physicochemical, microbiological and sensory characteristics of pork sausage in this study.

METASYNTHESIS

Materials: Trichloroacetic acid and thiobarbituric acid were purchased from Shanghai Zhongshi Chemicals Corporation (Shanghai, China). Sodium citrate was purchased from Suzhou Chemical Reagent Co., Ltd. (Suzhou, China). Sulfuric acid, formic acid, HCl and NaOH were purchased from Shanghai Chemical Reagents Company (Shanghai, China). Magnesium oxide was purchased from Tianjin Bodi Chemical Corporation (Tianjin, China). Fine iodized salt (≥99.5%, food grade) was purchased from Anhui Salt Industry General Corporation (Hefei, China). Monosodium glutamate (food grade) was purchased from Shanghai Totole Food Co., Ltd. (Shanghai, China). Phosphate (food grade) was purchased from a local Hypermarket of the Carrefour Group. White pepper was purchased from Jinjiang Ayibo Food Industry and Trade Co. Ltd. (Jinjiang, China). Ginger powder was purchased from Shanghai Haomeijia Food Co., Ltd. (Shanghai, China).

In addition, frozen pork legs were purchased from a local Hypermarket of the Carrefour Group. The proper amount of frozen pork legs was thawed for 24 h at 2-4°C. Subsequently, the thawed legs were removed of skin, bone, visible fat and connective tissue and then ground twice with a meat grinder (YEEKAI, MGB-120, Guangdong, China) fitted with a plate of 6 mm diameter holes. The proximate compositions of the ground meat were moisture 75.19±0.19%, protein 22.04±0.13%, fat 1.14±0.05% and ash 1.14 ±0.04% (all in triplicates).

Methods:
Preparation of monoxide hemoglobin (COHb): COHb was prepared according to the procedure described by Zhou *et al.* (2012). Specifically, 8.0 g sodium citrate (0.8%, m/v) was added to 1000 mL fresh porcine blood. The mixture was stirred for 1 min. Subsequently, the mixture was filtered with gauze. The filtrate was centrifuged with a CT15RT refrigerated centrifuge (Shanghai Tianmei Biochemical Engineering Equipment Co., Ltd., Shanghai, China) at the speed of 1,660 g for 10 min at 4°C. 335 mL Red Blood Cell (RBC) was obtained. The RBC was washed with 650 mL 0.9% (m/v) NaCl solution twice.

CO was prepared by a reaction of concentrated sulfuric acid and formic acid at 85°C. The resulting CO

was purified with a solution of 10% (m/v) NaOH and then introduced to saturate RBC with stirring at room temperature. Subsequently, the mixture was dehydrated by a freeze-drying process (ALPHA1-4LSC freeze dryer; Martin Christ, Osterode am Harz, Germany). The product was characterized by an analysis of Vis spectroscopy (not shown). Three characteristic peaks (411, 529 and 555 nm) were found. The product was proved to be COHb.

Sausage preparation: The frozen trimmed lean tissue of pork legs was thawed for 24 h at 2-4°C and mixed with fresh pork backfat at a ratio of 19 to 1. The resulting mixture was ground with a chopper (YEEKAI, MGB-120, Guangdong, China). Six treatments were prepared according to the following formulation: A: control, without $NaNO_2$ or COHb, B: 0.015% $NaNO_2$, C: 0.1% COHb, D: 0.2% COHb, E: 0.005% $NaNO_2$+0.2% COHb, F: 0.01% $NaNO_2$+0.2% COHb. In addition, other additives were also added: 5% water, 2.5% sodium chloride, 0.015% monosodium glutamate, 0.02% ginger powder, 0.15% phosphates and 0.02% white pepper powder. The additives were added based on meat mixture. The meat mixture was stuffed into polyethylene casing (ø: 30 mm) and then stored at 4°C for 24 h. Each sample was approximately 20 g. The raw sausages were heated to 85 ± 1°C in a water bath for 40 min. Cooled with tap water, the cooked sausages without packaging were stored in a refrigerator (4°C) and analyzed at days of 1, 8, 15, 22 and 29.

Color measurement: Color measurement was carried out according to the protocol described by Zhou *et al.* (2012). The color of pork sausage was measured with an automatic colorimeter WB-2000 IXA (Beijing Kangguang Instrument Co., Ltd, China). Specifically, the sausage samples were cut to cylinder (2.0-cm diameter, 1.0-cm high) and determined immediately. Six measurements for each of three replicates were expressed as L^* value, a^* value and b^* value. The overall lightness or darkness was determined by L^* value (0 = black, 100 = white). Red (positive = a+) and green (negative = a-) intensity represented the a^* scale and yellow (positive = b+) and blue (negative = b-) intensity represented the b^* scale.

Measurement of TBA: TBA values were performed according to the procedure described by John *et al.* (2005) with some modifications (in triplicate). Specifically, 5 g meat sample was taken and mixed with 25 mL solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 mol/L HCl. The mixture was heated for 10 min in a boiling water bath (99±1°C) to develop a pink azo compound, cooled in tap water and then centrifuged (Model CT14RD, Shanghai Techcomp Ltd., China) at 2010 g for 25 min. The absorbance of the supernatant was measured with a spectrophotometer (model 722E, Spectrum of Shanghai,

China) at 532 nm against a blank that contained all the reagents minus the meat. The malonaldehyde (MDA) concentration was calculated using an extinction coefficient of 1.56×10^5/M/cm for the pink TBA-MDA pigment.

The MDA concentration was converted to TBA number (mg MDA/kg meat sample) as follows:
TBA No. (ppm) = sample $A_{532} \times 2.77$.

Measurement of VBN: VBN values were analyzed according to the procedure described in National Standard of China (GB/T5009.44-2003) with some modifications (in triplicate). To 10 g meat sample, 100 ml distilled water was added. The sample was homogenized for 2-5 min at 21,000 g and then placed for 30 min. The homogenate was filtered with filter paper for analysis.

5 mL filtrate was put into a distillation flask. To the filtrate, 5 mL magnesium oxide suspension (10 g/L) was added. The mixture was distilled using the micro-Kjeldahl distillation apparatus (Auto K9840 Analyzer, Kjeltec, Beijing Changheng Rongchuang Technology Co., Ltd.). The distillate was collected for 3 min into 10 mL boric acid (20 g/L) containing five-six drops of mixed indicator (2 g/L methyl red indicator alcohol solution and 1 g/L methylene blue indicator aqueous solution). The solution was titrated by 0.01 mol/L HCl. The VBN in the sample in terms of milligrams of VBN per 100 g sausage can be given according to the following equation:

$$X = 28000(V_1 - V_2) \times c \div m$$

where,

X = VBN content of the sample (mg/l00 g)
V_1 = The consumption volume of HCl standard solution for titration of the sample (mL)
V_2 = The consumption volume of HCl standard solution for titration of the blank (mL)
C = The actual concentration of HCl standard solution (mol/L)
m = The mass of the sample (g)

Microbiological analysis: Aerobic plate count, *E. coli* count and *S. aureus* count of two treatments (the control and the 0.005% $NaNO_2$ and 0.2% COHb sample) were determined according to the protocol described by national food safety standard of China (GB 4789.2-2010). Specifically, 25 g samples stored for 1, 8, 15, 22 and 29 days were taken aseptically, transferred to sterile plastic pouches containing 225 mL 0.85% NaCl solution and then homogenized for 2 min at room temperature. Appropriate dilutions of samples (the samples stored for 1, 8 and 15 days were diluted to 100 times volume of the original volume, while the ones stored for 22 and 29 days were diluted to 1000 times volume of the original volume) were spread on triplicate agar plates and then

incubated at 36±1°C for 48 h under anaerobic conditions. Aerobic plate count, *E. coli* count and *S. aureus* count were determined on Plate Count Agar (Beijing Aoboxing Biotechnology Co. Ltd., Beijing, China), *E. coli* chromogenic agar (Beijing Aoboxing Biotechnology Co. Ltd., Beijing, China) and *S. aureus* chromogenic agar (Beijing Aoboxing Biotechnology Co. Ltd., Beijing, China), respectively. Results were expressed as $\log^{10}$ numbers of colony forming units/gram (cfu/g). Three samples for each treatment were analyzed.

Sensory evaluation: A sensory panel consisted of 9 food science and engineering-majored master graduate student, of Hefei University of Technology. The criteria for the panel were: 4 males and 5 females, age between 18 and 22, normal or superior visual and taste acuity. The panelists had grasped the knowledge of food sensory evaluation and were willing to evaluate the color, flavor, mouthfeel and slice traits of the COHb-treated pork sausages and also those throughout refrigerated storage. Before conducting the experiment, the panelists were trained for the development of a sensory memory with respect to each descriptive term throughout refrigerated storage. The method described by Wang and Zhao (2008) was used to prepare the list of descriptive terms, the pork sausage samples being presented in pairs for the panelists to list the differences and similarities. By consensus among the panel, the following scales were used: color (10-8 = red and bright, 7-5 = grey or slight red but still acceptable, 4-1 = excessive red and dark, not acceptable); flavor (10-8 = flavor, 7-5 = moderately off-odor but still acceptable, 4-1 = strongly off-odor and not acceptable); mouthfeel (10-8 = good, 7-5 = moderate, 4-1 = poor) and slice traits (10-8 = solid and smooth; 7-5 = slightly softening along peripheral and slight fissures, 4-1 = strongly softening). The pork sausage samples were stored under refrigeration (4±1°C) until the moment of evaluation to the panelists. The testing procedure was performed in the room where is used for sensory evaluation. Three samples for each treatment were coded with random numbers. The pork sausage samples in polyethylene casing during after 1, 8, 15, 22 and 29 days of refrigerated storage were submitted to each panelist and then opened and evaluated individually. All the sensory evaluations were carried out at room temperature and under natural light.

Statistics analysis: Statistical analysis was performed by Excel 2003 (Microsoft official Excel 2003 for Windows). The data were expressed as mean±standard deviation (SD). Analysis of Variance (ANOVA) was introduced to determine the significance of samples at $p < 0.05$ level. When a significant effect was obtained, Duncan's multiple range tests were used to compare sample means.

RESULTS AND DISCUSSION

Color: The addition of COHb resulted in the reductions of L^* values (p<0.05) (Fig. 1). The samples that had more COHb added had lower L^* values (p<0.05). The changes in L^* values resulted possibly from the changes of Hb during storage. Mielnik and Slinde (1983) observed that blood imparted the dark color of the sausages to which it was added. Slinde (1987) speculated that color of black salami sausage was ascribed to the dissociation of heme from Hb. Therefore, it was possible that some Hb presenting in the added COHb dissociated to set free heme, thereby leading to the reduction of L^* values. The addition of $NaNO_2$ also decreased L^* values (p<0.05). In comparison to the samples treated with 0.2% COHb alone, the treatment with a combination of COHb and $NaNO_2$ had no significant influence on L^* values (p>0.05). The addition of COHb or $NaNO_2$ caused the increase of a^* values (p<0.05). The effects of COHb on

a^* values depended on its levels: the samples that had more COHb added tended to have higher a^* values. Zhou *et al*. (2012) observed that both COHb and COMb were the chemical components of the pigments extracted from the 0.4% COHb non-cooked sausages. Therefore, they speculated that the changes in a^* values of the COHb-treated samples resulted possibly from COHb or/and COMb formed by a transfer of CO from Hb to Mb. In addition, the combined treatments had higher a^* values than the single ones, indicating that COHb and $NaNO_2$ affected synergistically a^* values. The combined effects of COHb and $NaNO_2$ on a* values might be ascribed to the formation of NOHb since $NaNO_2$ can react with Hb to generate NOHb (Zhang *et al*., 2013). The change tendency of b^* values was similar to that of L^* values, that is, the addition of COHb, $NaNO_2$ or a combination of COHb and $NaNO_2$ decreased b^* values, significantly (p<0.05). The samples that had more COHb added tended to have lower b^* values. Also, COHb and $NaNO_2$ did not affect

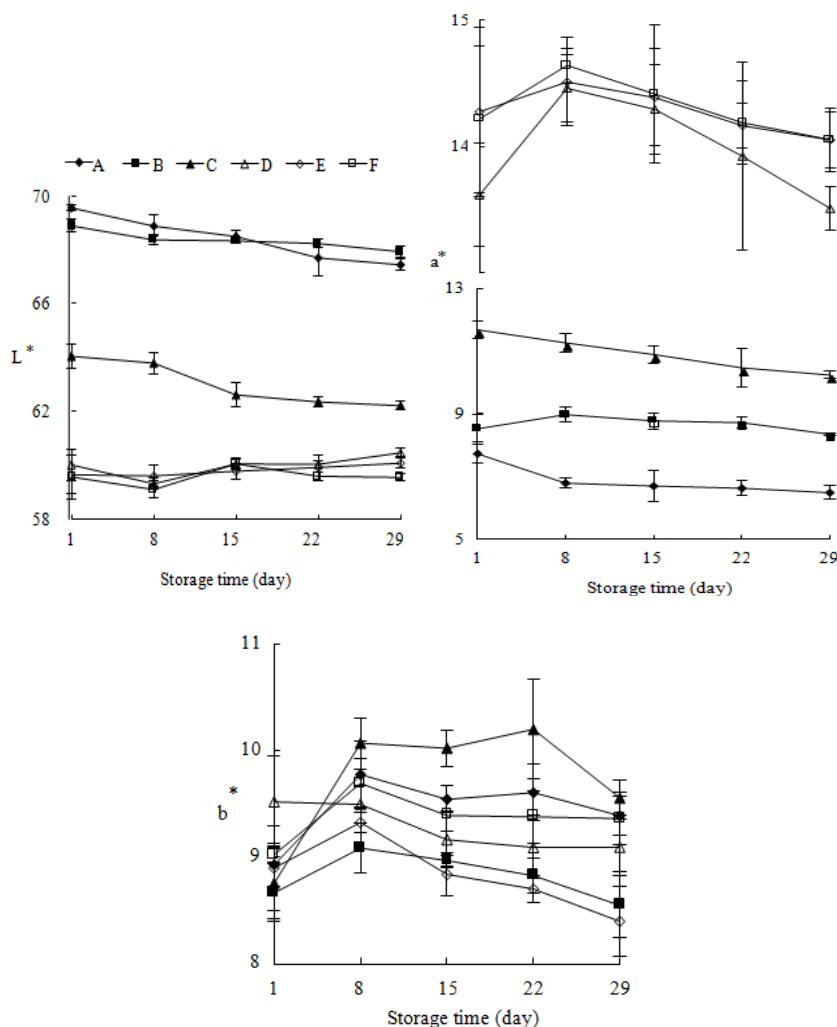Fig. 1: The changes in color of various treatments during storage; A: control, without $NaNO_2$ or CO-Hb; B: 0.015% $NaNO_2$; C: 0.1% CO-Hb; D: 0.2% CO-Hb; E: 0.005% $NaNO_2$ +0.2% CO-Hb; F: 0.01% NaNO2+0.2% CO-Hb; Error bars refer to the standard deviation obtained from three analyses
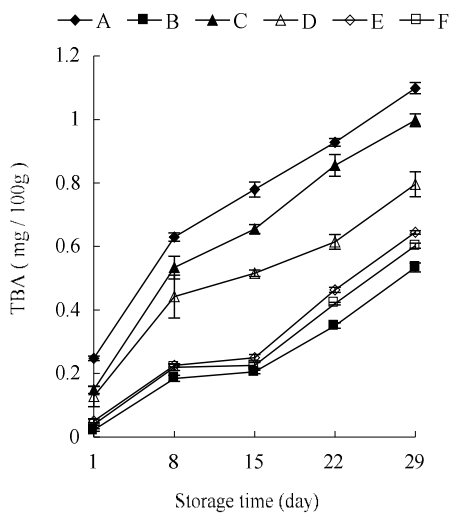
Fig. 2: The changes in TBA of various treatments during storage; A: control, without $NaNO_2$ or CO-Hb; B: 0.015% $NaNO_2$; C: 0.1% CO-Hb; D: 0.2% CO-Hb; E: 0.005% $NaNO_2$+0.2% CO-Hb; F: 0.01% NaNO2 + 0.2% CO-Hb; Error bars refer to the standard deviation obtained from three analyses
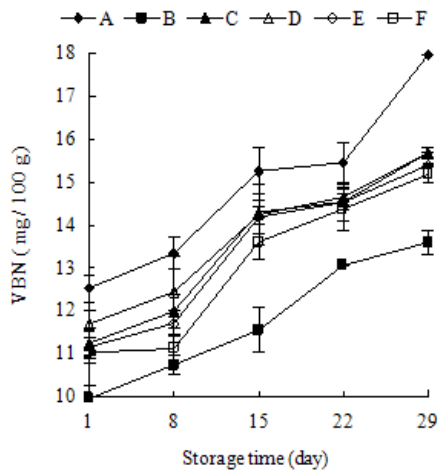

Fig. 3: The changes in TBA of various treatments during storage; A: control, without $NaNO_2$ or CO-Hb; B: 0.015% $NaNO_2$; C: 0.1% CO-Hb, D: 0.2% CO-Hb; E: 0.005% $NaNO_2$ + 0.2% CO-Hb; F: 0.01% NaNO2 + 0.2% CO-Hb; Error bars refer to the standard deviation obtained from three analyses

synergistically $b*$ values. The results indicated that the samples treated with COHb, or a combination of COHb and $NaNO_2$ were more reddish, dark and yellowish in comparison to the controls.

$L*$ values, $a*$ values and $b*$ values decreased significantly during storage ($p < 0.05$) in all cases. These results revealed the following facts:

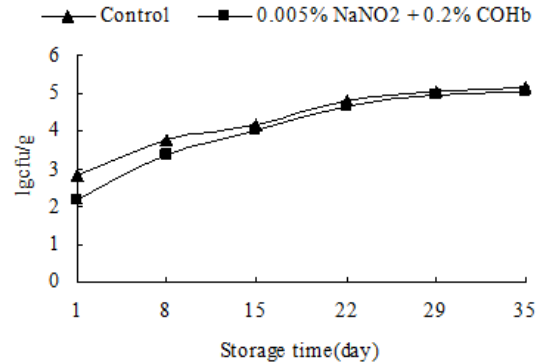- Decomposition and/or oxidation of pigments in the sausages had occurred

Fig. 4: The changes in aerobic plate count of the different treatments during storage; Error bars refer to the standard deviation obtained from three analyses

- The samples became darker and less reddish during storage. Additionally, for the different treatments, the changes in $a*$ and $L*$ values had different speed. The samples treated with COHb were slower than the controls, but faster than the ones treated with $NaNO_2$ or a combination of COHb and $NaNO_2$. These results indicated that the samples treated with $NaNO_2$ or a combination of COHb and $NaNO_2$ enhanced color stability.

TBA: Figure 2 summarized the changes in TBA values during storage. TBA value is an indicator of lipid oxidation (Tan *et al.*, 2007). As expected, TBA values increased significantly ($p < 0.05$) during storage in all cases, showing that lipid oxidation occurred. The addition of COHb led to the significant reductions of TBA value in the presence or absence of $NaNO_2$ ($p < 0.05$). Such behaviors of COHb are similar to those of NOHb (Shi and Kong, 2009). The samples that had more COHb added tended to have lower TBA values, but the differences of TBA values were not so high to be significant ($p > 0.05$). Also, the addition of $NaNO_2$ caused the significant reduction of TBA value ($p < 0.05$), which was in agreement with the prior reports (Shi and Kong, 2009). The present results showed that the treatments with $NaNO_2$, COHb, or a combination of $NaNO_2$ and COHb could protect lipid from oxidation.

VBN: It was observed that VBN values of all the treatments increased over the storage time (Fig. 3). VBN value is an indication of protein degradation (Tan *et al.*, 2007). Therefore, the results revealed that protein degradation had occurred during storage. The controls had higher VBN values than the treatments with $NaNO_2$, COHb, or a combination of $NaNO_2$ and COHb ($p < 0.05$). The present results indicated that the treatments with $NaNO_2$, COHb, or a combination of $NaNO_2$ and COHb could delay protein degradation. NOHb is also reported to decrease the VBN values meat batters (Zhang *et al.*,

2013). In addition, all the samples in this study did not exceed 20 mg/100 g, which is the value that is often described as the level necessary to detect meat spoilage (Tan *et al.*, 2007).

Microbiological analysis: The results of microbiological analysis were illustrated in Fig. 4. As expected, aerobic plate count increased with storage time. The 0.005% $NaNO_2$ and 0.2% COHb samples had significantly lower aerobic plate counts than the controls during storage (p<0.05). These results showed that the combined treatment was effective in inhibiting aerobic growth. *E. coli* of all the samples in this study did not exceed 30 cfu/100 g during storage, which is the value that is often described as the level necessary to detect microbiological safety for sausage product (national food safety standard of China, GB 4789.2-2010). Differed from the controls, the samples that had 0.005% $NaNO_2$ and 0.2% COHb added were not detected in *S. aureus* when the storage time was extended to 29 days,

indicating that a combination of $NaNO_2$ and COHb was effective in inhibiting *S. aureus* growth.

Sensory characteristics: It is well known that $NaNO_2$ can develop a characteristic pink color and is responsible for the production of characteristic flavor of cured meats (Filiatrault *et al.*, 2013). It was found that the addition of $NaNO_2$ increased significantly sensory color scores in the present study (p<0.05) (Fig. 5). The addition of $NaNO_2$ enhanced flavor, mouthfeel and slice traits scores, significantly (p<0.05). Similarly, the addition of COHb also resulted in the significant increase of sensory color, flavor, mouthfeel and slice traits scores (p<0.05). In comparison to the samples that had 0.1% COHb added, the ones that had 0.2% COHb added had higher sensory color, but lower flavor, mouthfeel and slice traits scores. In the presence of COHb, the addition of $NaNO_2$ had no significant influence of flavor (p>0.05), but increased sensory
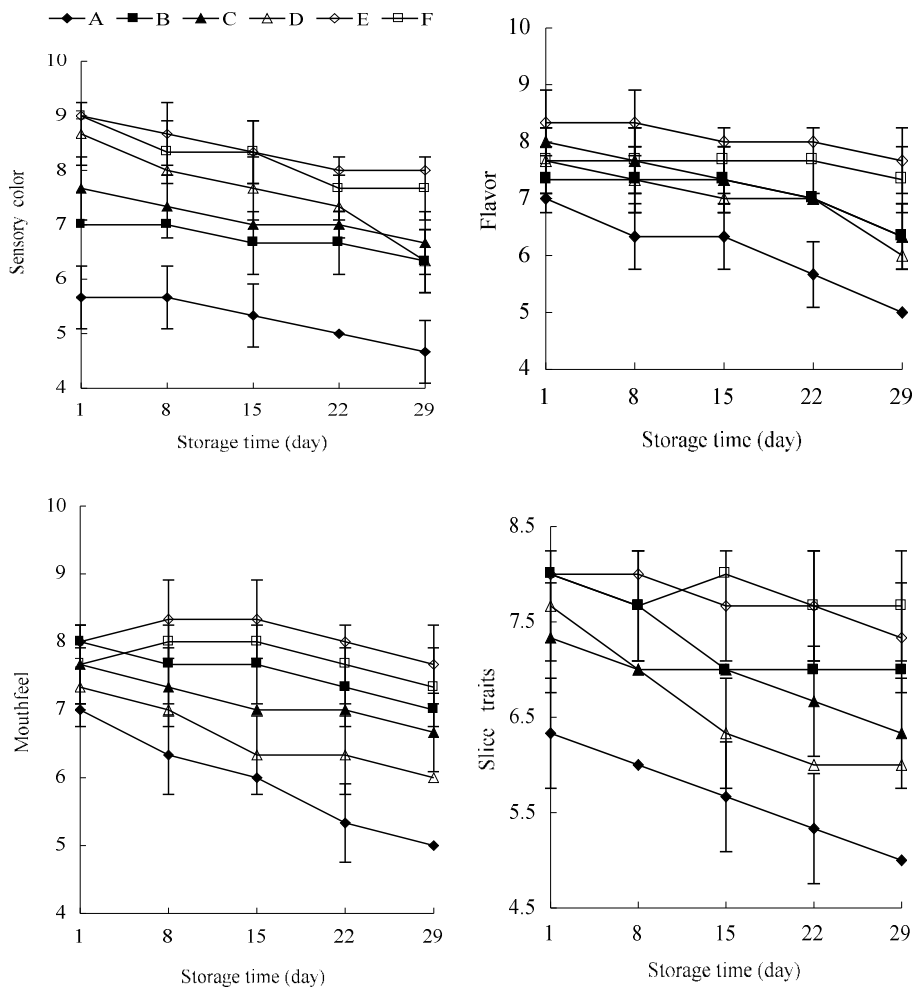

Fig. 5: The changes in sensory characteristics of various treatments during storage; A: Control, without $NaNO_2$ or CO-Hb; B: 0.015% $NaNO_2$; C: 0.1% CO-Hb; D: 0.2% CO-Hb; E: 0.005% $NaNO_2$+0.2% CO-Hb; F: 0.01% NaNO2+0.2% CO-Hb; Error bars refer to the standard deviation obtained from three analyses

color, mouthfeel and slice traits, significantly ($p<0.05$). In comparison to the NaNO$_2$ samples, the 0.005 NaNO$_2$ and 0.2% COHb samples had equal even better sensory color, flavor, mouthfeel and slice traits scores.

CONCLUSION

The samples that had 0.005% NaNO$_2$ and 0.2% COHb added had equal even better sensory color, flavor, mouthfeel and slice traits scores, but lower TBA values, VBN values, aerobic plate count than the ones that had 0.015% NaNO$_2$ added during storage. Moreover, such the samples had no detectable *S. Aureus*, less than 30 cfu/100 g *E. coli* and acceptable sensory characteristics during 29 days storage. Therefore, COHb exhibits a potential in the precess of sausages.

ACKNOWLEDGMENT

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