Journal of Science and Technology ***55*** *(2) (2017) 178-187*

DOI: 10.15625/0866-708X/55/2/8578

EFFECTS OF PARTIAL REPLACEMENT OF NITRITE BY ANNATTO (BIXA ORELLANA L.) SEED POWDER ON THE PROPERTIES OF PORK SAUSAGES

Tran Van Cuong[1, 3, *], Nguyen Thi Thoa[2, 3]

[1]*Faculty of Agriculture and Forestry, Tay Nguyen University, 567 Le Duan, Buon Ma Thuot, Daklak, Vietnam*

[2]*The Western Highland Agriculture and Forestry Science Institute, 53 Nguyen Luong Bang, Buon Ma Thuot, Daklak, Vietnam*

[3]*Department of Food Science and Technology, Chonnam National University, Gwangju 500-757, South Korea*

[*]Email: vcuong.edu.vn@gmail.com

Received; 30 July 2016, Accepted for publication: 31 December 2016

ABSTRACT

This study investigated the effect of powder from the outer layer of annatto (*Bixa orellana* L.) seeds on the physicochemical properties and the antioxidant and antimicrobial activities of pork sausages. In fact, six treatments of pork sausage were made including four different concentrations of annatto seed powder as follows: 0.025, 0.05, 0.1, 0.2 % (T1, T2, T3, and T4, respectively), and 100 % with nitrite (150 ppm, REF); and control (CTL) without nitrite. The Hunter color values of pork sausages were affected by the addition of annatto seed powder, especially by an increase of the redness and yellowness values; however, the lightness of the sausages decreased ($p < 0.05$). Thiobarbituric acid reactive substances (TBA) and peroxide values (POV) were analyzed. Treatments containing annatto seed showed lower TBA and POV than CTL samples ($p < 0.05$). Moreover, the addition of annatto seed powder enhanced the texture profile and physicochemical properties of sausages and were similar in value to REF. In conclusion, annatto seed powder might be a good source as a natural colorant and antioxidant for the replacement of nitrite in meat production, and therefore, enhance the quality characteristics of meat products.

Keywords: annatto seed powder, nitrite replacement, lipid oxidation, pork sausage, shelf-life.

1. INTRODUCTION

Nitrite is known as an indispensable additive in processed meat, especially for sausage products. The use of nitrite has been long and wildly used as a curing agent, which maintains color and antimicrobial properties, thereby enhancing the sensory characteristics, reducing spoilage and extending the shelf-life of meat products [1]. Recently, however, many authors have concluded that consumption of meat products containing nitrite can affect the health of consumers. Specifically, nitrite, can react with secondary or tertiary amines in meat to form carcinogenic, teratogenic and mutagenic N-nitroso compounds [2, 3]. It is, therefore imperative to minimize the nitrite content used in industrial manufacturing, but still meet the quality and perception requirements of the final product. In recent years, there have been many related studies on the replacement of nitrite in meat and meat product processing with natural compounds which have shown positive results.

Annatto (*Bixa orellana* L) seed has been used as a natural colorant, belonging to many traditional foods found in Asia. Annatto ranked second place in economic importance worldwide among all natural colorants [4]. It is widely used for the production of a variety of products, such as food, medicine and cosmetics. In addition, many studies have shown that it does not have any mutagenic, cancerous, or cytotoxic effects [5, 6]. Therefore, annatto seed powder is considered to be safe for human health. The color pigment was extracted from the outer coating layer of the seeds of the *Bixa orellana* L., including bixin (oil-soluble compounds) and nor-bixin (water-soluble compounds). Numerous studies have evaluated the antioxidant and antimicrobial activity from annatto seeds [7, 8, 9] while other studies have been carried out the extraction and recovery of bixin from annatto seed by various methods [4, 10, 11]. However, no studies have been conducted using annatto seed powder, which was prepared by the ball-milling method for partial replacement of nitrite in pork sausage products. Therefore, the aim of this study was to evaluate the effect of partial replacement of nitrite with annatto seed powder on the antioxidant capacity, texture and physicochemical properties of pork sausage.

2. MATERIALS AND METHODS

2.1. Instruments and method for preparation of annatto seed powder

Several instruments were used in this study, including the vacuum rotary evaporator (R-100, Buchi, Switzerland), Wise-bath ultrasonicator (Ultrasonic JAC 2010, 330 W, Korea), Freeze dryer (Ilshin freeze dryer, Korea), pH meter (MP-120, Mettler-Toledo, Switzerland), Spectrophotometer (UV-1601, Shimadzu, Australia), the Hunter color reader (CR -10, Minolta Co. Ltd., Japan), and Texture profile testing (Instron Universal Testing Machine, Model 3344, Canton, MA, USA).

Annatto seeds were purchased from the local market of Buonmathuot city, Daklak province. The fresh materials were sun-dried until the weight stopped fluctuating. Then, the solute was extracted using 50% ethanol in combination with ultrasonic assistance at the frequency of 40 kHz with 300 W of generation power (Ultrasonic JAC 2010, 330 W, Korea) at 30 ^{o}C for 30 min. The alcohol was removed from extracted solution using a rotary evaporator (R-100 rotary evaporator - Buchi, Switzerland) at 50 ^{o}C. Thereafter, the extract was kept at -70 ^{o}C prior to lyophilizing at -55 ^{o}C (Ilshin freeze dryer, Korea) until completely dry for about 5 days. Once completed dry, it was ground using a ball-milling grinder (Planetary Mono Ball-Milling, Pulverisette, Fritsch, Germany) for 12 h at 400 rpm, and was then used for the manufacturing of the pork sausages in this study.

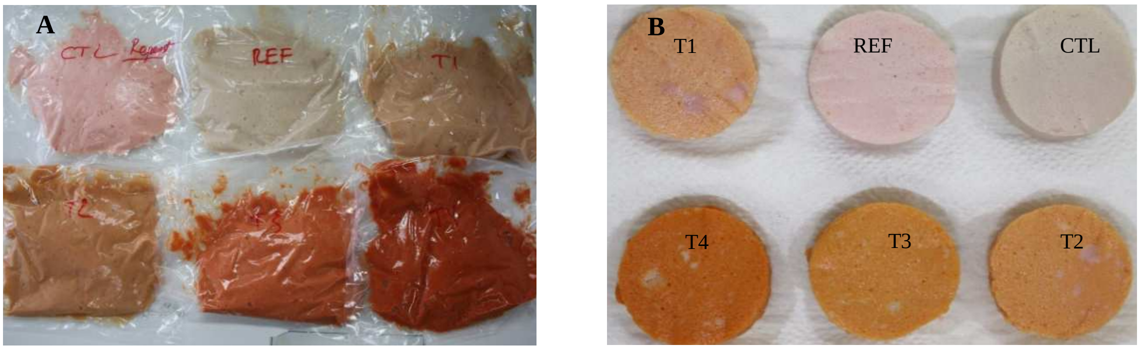

Figure 1. The color properties of sausages affected by different formulation before (A) and after (B) cooking.

2.2. Manufacturing of pork sausages and analytical methods

2.2.1 Manufacturing of pork sausages

Low fat sausages were prepared using general main ingredients, including 60% lean meat, 33% iced water, 5% back fat, and 0.4% STPP (sodium tripolyphosphate, a food standard). In total, six types of low fat sausages were prepared and the formulations are listed in Table 1.

The sausage samples were heated to 75 °C for 30 min (the internal temperature of sausages reached to 70 °C), and were indirectly cooled in an ice bath for 30 minutes at the end of the heating process, then stored at 4 ± 1 °C in a refrigerator prior to analysis of their physicochemical and quality properties.

Table 1. Formulation of pork sausages containing annatto seeds powder.

Ingredients (%)	Treatments					
	CTL	REF	T1	T2	T3	T4
Lean meat	60	60	60	60	60	60
Back fat	5	5	5	5	5	5
Iced water	33	33	33	33	33	33
Salt	1.3	1.3	1.3	1.3	1.3	1.3
STPP	0.4	0.4	0.4	0.4	0.4	0.4
Nitrite (ppm)	0	150	37.5	37.5	37.5	37.5
Annatto powder	0	0	0.025	0.05	0.1	0.2

2.2.2 pH and colors measurement

The pH values were measured using a pH-meter (MP-120, Mettler-Toledo, Switzerland), while the color of sausages was measured using a Hunter color reader (CR-10, Minolta Co. Ltd., Japan). Ten measurements were made at different positions on the surfaces of sausage samples, and the mean values were calculated. Hunter color values were reported as lightness (*L*), redness (*a**) and yellowness (*b**).

2.2.3. Physicochemical analyses, expressible moisture and cooking loss

Proximate analysis that included moisture, fat and protein content was measured according to AOAC (2000). Moisture (%) contents were measured using the dry oven method, fat contents (%) were measured using the Soxhlet extraction method, and protein contents (%) using the Kjeldahl method (PRO-NITRO S, J.P. Selecta, Spain).

The cooking yield (%) was measured by checking the weight loss after cooking (for 30 min at 75 ^{o}C), with values calculated using the following equation:

Cooking yield (CY, %) = Sample wt. (after cooking)/ Sample wt. (before cooking) × 100.

Expressible moisture (%) was measured according to the method of Jauregui et al. [12] with slight modification. Sausage samples were cut into curved shapes with approximate weights of 1.5 g, then covered with Whatmann filter paper #3 and put into 50 mL falcon tubes prior to centrifugation at 3000 rpm for 15 min.

2.2.4. Lipid oxidation analysis

The peroxide values (POV) were determined using the spectrophotometric method as described by Shantha and Decker [13] with some modification. The absorbance of the samples (after 20 minutes for reaction) were measured at 500 nm against a blank that contained all the reagents except the samples using a spectrophotometer (UV-1601, Shimadzu, Australia). The results of POV were expressed in milliequivalents of oxygen per kilogram of sausage (meq/kg). The secondary products of lipid oxidation during the storage period were determined as the thiobarbituric acid reactive substances (TBA) according to Shinnhuber and Yu [14]. The reactive substances were measured at 532 nm using a spectrophotometer (UV-1601, Shimadzu, Australia). TBARS values were calculated using the following equation:

TBA value (mg malondialdehyde/kg) = optical density (O.D.) × 9.48/sample weight (g).

2.2.5 Total volatile basic nitrogen (VBN) measurements

The total volatile basic nitrogen (VBN) content was determined according to the micro-diffusion method of Conway [15] with slight modification. Briefly, 1 g of the sample was homogenized with 9 mL double distilled water using the ultra-turrax homogenizer T25 (IKA Labortechnik, Germany) thereafter was filtered using whatman filter paper #1. The filtrate (1 mL) was then reacted with saturated potassium carbonate (K_2CO_3, 50 %, w/v) at 37 ^{o}C for 2 h. Boric acid (0.01 N) acted as an adsorbent. After reaction, the boric acid adsorbed volatile nitrogen was titrated with a standard HCl solution (0.001 N). Total volatile basic nitrogen (VBN) values were expressed in mg%.

2.2.6 Microbial analysis

Total plate counts (TPC) and violet red bile (VRB) agars were prepared for the determination of total viable counts and *Enterobacteriaceae*, respectively, according to the method of Park & Chin [16]. Pork sausage samples were aseptically homogenized, then 10 g of patty was mixed with 90 mL of steriled dd-water, and were serially diluted 10 times. In order, 0.1 mL of each dilution was inoculated on the TPC and VRB agars. They were incubated at 37 ^{o}C for 48 h, then the colonies on the petri dishes were counted, and the results of TPC and VRB were expressed as Log CFU/g.

Difco^{TM} Plate Count Agar (TPC) and Difco^{TM} Violet Red Bile Agar (VRB) were prepared for microbial testing. 23.5 g TPC (REF. 247940) was dissolved in 1 L double distilled water and then boiled for 3 minutes until completely dissolved (121 ^{o}C, 15 min). Finally, the agar cooled to 50 °C and poured into a petri dish. For the VRB medium (REF. 211695), approximately 41.5 g was add to 1 L distilled water and followed with similar preparation steps as the agar, and did not need to undergo sterilization.

2.2.7 Texture profile analysis

Instron Universal Testing Machine (Model 3344, Canton, MA, USA) was used for texture profile analysis. Sausage samples were prepared with 13 mm height and 12.5 mm diameter using a puncturing apparatus. Hardness (gf), springiness (cm), gumminess, chewiness, and cohesiveness were calculated according to the method of Bourne [17].

2.3. Data statistical analysis

Three replicates of the experiment were performed and data was analyzed using one-way analysis of variance (ANOVA). The significant differences were assessed by the Duncan post hoc test at p value < 0.05 using Statistical Package for the Social Sciences (IBM SPSS version 20.0, SPSS Inc., Chicago, IL, USA) software for Windows. Results were presented as the mean ± standard deviation (n = 3).

3. RESULTS AND DISCUSSION

3.1. Effects of annatto seed powder on pH and color values of sausages

The present study showed that the pH values of sausages were associated with the addition of annatto seed powder during refrigerated storage. The pH is one of the important parameters for sensory quality of food items. As shown in Table 2, pH values were slightly decreased from 6.06 to 6.00 with increasing annatto powder (0.025 to 0.2 %). However, the decrease in pH values did not change the sensory properties of the final product. In this study, the decrease in pH of meat product might be related to the pH value of annatto seeds powder. According to the previous study, the pH value of annatto powder is 5.73 (Cuong and Chin [18]), therefore, pH values were decreased when more annatto seed powder was added ($p < 0.05$).

The color properties of pork sausages are presented in Table 2. The lightness was significantly decreased by the addition of more annatto seed powder. The redness values ($a*$) were robust increased, while yellowness also increased compared to the control (CTL) or reference (REF) samples. It clearly shows that the color properties of pork sausages are associated to the addition of annatto seed powder. The color values of sausages were expressed by Hunter L (lightness), a* (redness), and b* (yellowness) values. The changes of Hunter color values of sausage samples with annatto powder before and after cooking are presented in Figure 1. Results showed the significant difference in redness among treatments. CTL sample was the lowest, followed by the REF sample, while redness increased with increasing level of annatto powder and was much higher than those of CTL and REF samples. In addition, yellowness was different among treatments, was lowest in the REF sample, while it increased with the addition of annatto seed powder. Therefore, it clearly indicated that the Hunter color values of pork sausage were directly affected by the addition of annatto seeds powder, which has a strong color and immediately changed the color of meat products. Many previous studies reported color changes in different types of meat as affected by various compounds from natural resources. On the other hand, several authors have reviewed the change of meat colors, which can be acceptable or unacceptable to consumers. In particular, for red meat products as such pork meat, the increased in redness might more satisfying to consumers [21].

Table 2. Effects of annatto seed powder on physicochemical properties of pork sausages.

Parameters	**Treatments**					
	CTL	REF	T1	T2	T3	T4
pH	6.08^{ab}	6.10^{a}	6.06^{bc}	6.04^{cd}	6.03^{d}	6.00^{e}
Lightness ($L*$)	75.19^{a}	73.47^{a}	66.94^{b}	64.84^{c}	61.70^{c}	57.72^{d}
Redness ($a*$)	4.85^{e}	11.58^{d}	20.65^{c}	22.34^{b}	22.32^{b}	24.90^{a}
Yellowness ($b*$)	9.56^{e}	4.48^{f}	20.18^{d}	24.63^{c}	26.72^{b}	29.59^{a}
TBA	0.18^{a}	0.06^{c}	0.09^{bc}	0.10^{b}	0.11^{b}	0.10^{b}
Peroxit (POV)	2.44^{a}	1.32^{b}	0.95^{c}	0.92^{c}	0.88^{c}	0.86^{c}
VBN	5.78^{a}	4.92^{b}	5.25^{ab}	5.26^{ab}	5.28^{ab}	5.37^{ab}
TPC	3.58^{a}	2.58^{b}	2.68^{b}	2.58^{b}	2.53^{b}	2.37^{b}
VRB	3.34^{a}	2.30^{b}	2.46^{b}	2.35^{b}	2.35^{b}	2.40^{b}

Means with different superscript letters (a-f) in the same row indicate significant differences among treatments at $p < 0.05$.

3.2. Effects on expressible moisture, cooking loss and physicochemical properties

Expressible moisture (EM, %), cooking loss (CL, %) and proximates analysis (moisture, protein, lipid) of pork sausages incorporated with annatto seed powder were presented in Table 3. As the results, the characteristics (EM and CL) of pork sausages were associated with the addition of annatto seed powder. EM and CL showed the increase when annatto seed powder was added. In fact, EM value was the lowest in CTL sample, while there was no difference between the nitrite sample and the supplemented annatto from 0.025 to 0.1%, while the 0.2% level that was supplemented with annatto had the highest EM values with 44.01%. Similar to the expressible moisture results, in terms of cooking loss, the CTL sample also showed the highest, while no significant difference was observed between REF and the other treatments with different levels of annatto powder. This can be explained by our previous observation [18] that the annatto powder after ball-milling has a good water holding capacity (approximately 40 %), thereby the higher level of annatto help to enhance the binding ability and water holding capacity of the product.

Proximate compositions including the moisture, protein, and lipid contents of sausages were analysed and presented in Table 3. As the results, there was no significant difference among treatments ($p > 0.05$). Therefore, the addition of annatto seed powder did not affect the physicochemical composition of pork sausages.

Table 3. Proximate analysis and cooking properties of low-fat pork sausages as affected by various level of annatto seeds powder.

Parameters		**Treatments**					
		CTL	REF	T1	T2	T3	T4
Expressible moisture (EM %)	Mean	36.82^{b}	40.73^{ab}	39.11^{ab}	40.14^{ab}	40.52^{ab}	44.01^{a}
	SD	3.51	0.93	1.82	4.48	4.97	2.85
Cooking loss (CL%)	Mean	13.84^{a}	6.07^{b}	6.35^{b}	6.09^{b}	6.34^{b}	5.61^{b}
	SD	2.61	2.46	3.13	3.04	3.54	0.53
Moisture (%)	Mean	67.52	70.05	68.56	68.43	69.21	69.00
	SD	1.56	0.75	1.04	0.93	1.18	0.78
Protein (%)	Mean	11.00	11.30	11.21	10.88	10.97	10.65
	SD	1.09	1.05	0.38	1.15	0.14	0.55
Lipid (%)	Mean	18.37	17.73	17.55	18.32	17.96	18.84
	SD	0.06	0.50	0.35	1.60	0.49	1.38

Means with different superscript letters (a-b) in the same row indicate significant differences among treatments at $p < 0.05$.

3.3. Lipid and protein oxidation

The addition of annatto seed powder significantly reduced the lipid oxidation of the sausage. The value of POV and TBA is the primary and secondary products of lipid oxidation, respectively. These are two important indicators for assessing the oxidation level of lipid or high lipid content products. As shown in Table 2, the blank sample was the highest, indicating those had more oxidation, while the REF sample or added annatto powder significantly reduced the TBA and POV values. This result could be explained as that the annatto powder with high antioxidant activity helps to prevent the primary and secondary oxygen reactions of lipids in the sausage samples. This result was similar to the previous observations. Figueirdo et al. [22] concluded that annatto powder helps to prevent lipid oxidation of pork during cold storage when compared to the blank sample. Other previous authors concluded that the use of naturally extracted compounds with high *in vitro* antioxidant activity also has the potential to prevent lipid oxidation in meat products [20].

The total volatile nitrogen (VBN) content is considered as one of the most important indicators of the freshness of meat and dairy products via the breakdown of protein form to volatile nitrogen. Normally, the VBN values may be due to the activity of the spoilage bacterial and endogenous enzyme system in meat products [23]. As shown in Table 2, CTL sample had the highest content of VBN, while the REF was the lowest, while there was no difference amongst samples of annatto seed powder supplementation and those values were lower than CTL and higher than REF samples. The highest value of VBN in CTL samples may be due to stronger protein breakdown than that of nitrite or annatto added samples.

3.4. Microbial analysis

The results of microbial counts (TPC and VRB) was present in Table 2. The CTL sample (without nitrite or annatto powder) had higher total bacterial counts (TPC) and *Enterobacteriaceae* (VRB) than the REF sample or annatto added samples. However, there was no difference (p> 0.05) among the nitrite or annatto added samples. These results conclude that the annatto seed powder has antibacterial activity. The current results are consistent with previous studies on the antimicrobial properties of annatto. Viuda-Martos et al. [9] observed that annatto extract was a better agent against *P. aeruginosa* than nisin. Venugopalan and Giridhar [24] reported that the alcoholic extracts from annatto seeds are resistant to *E. coli* and *B. cereus*. In addition, Yolmeh et al. [25] also observed that annatto has the potential to inhibit the growth of *E. coli* and bacteria on mayonnaise. Furthermore, some authors concluded that there is a correlation between the antioxidant and antimicrobial activities of natural phenolic compounds. This may be due to the biological compounds contained in the annatto extracts such as phenolic acid, carotenoids, flavonoids, etc., which act as toxins that inhibit or disrupt enzymes and microorganisms. Based on these results, it was conceived that phenolic compounds from annatto seeds not only showed very strong antioxidant activity but also possessed antimicrobial activity in pork meat.

3.5. Effect on texture profile of pork sausages

The texture profile of sausage was conducted using the Instron Testing Machine, which measures the hardness, springiness, gumminess, chewiness and cohesiveness. As shown in Table 4, the hardness of the CTL sample was lower than other samples, whereas, there was no significant difference among samples with annatto and nitrite added (REF). Moreover, the springiness, gumminess, chewiness and cohesiveness were not significantly different among treatments (p>0.05). Generally, the texture properties of the meat products such as hardness, springiness, gumminess, and chewiness have a major impact on consumer acceptance [26]. The hardness of the blank sample is lower than that of the other samples, which may be related to the partial oxidation of the protein at the same time due to the stronger activity of the endogenous enzymes and microbial systems in this sample compared with other samples. The results show that the addition of annatto powder to the meat products may have the effect of preventing oxidation, enhancing binding and improving the functionality and stability of protein as compared to the CTL.

Table 4. Texture properties of pork sausages as affected by various level of annatto seeds powder.

Parameters		**Treatments**					
		CTL	REF	T1	T2	T3	T4
Hardness (gf)	Mean	1678.4^{b}	1843.1^{a}	1796.6^{a}	1858.9^{a}	1856.7^{a}	1959.5^{a}
	SD	35.3	35. 3	31.8	14.0	29.5	31.5
Springiness (mm)	Mean	6.02	6.08	5.95	6.35	6.76	6.49
	SD	0.41	1.28	0.81	1.37	0.52	0.99
Gumminess	Mean	10.32	11.27	11.13	11.63	11.96	12.86
	SD	1.93	1.37	0.54	3.29	1.59	1.60
Chewiness	Mean	64.56	64.23	62.05	61.17	60.68	75.64
	SD	22.52	13.97	13.31	12.02	16.70	18.64
Cohesiveness	Mean	0.006	0.007	0.006	0.006	0.007	0.007
	SD	0.001	0.001	0.001	0.001	0.001	0.001

Means with different superscript letters (a-b) in the same row indicate significant differences among treatments at $p < 0.05$.

4. CONCLUSION

Taken together, annatto seed powder might be suitable for the replacement of nitrite in pork sausauge manufacture. Annatto seed powder improves the color and texture properties of sausages. Moreover, annatto added samples of sausage were more effective to retard lipid oxidation as well as inhibit the growth of microorganisms than the blank treatment.

Therefore, annatto seed powder could be a source of natural antioxidant and antimicrobial agents that could be used for the replacement of nitrite in the production of meat products without decreasing quality properties and also extending the shelf-life. In the future, more research should be conducted to assess the optimal level of annatto powder for industrial conditions and its impact to sensory properties as well as customer’s acceptance. Furthermore, related studies will be also focus on the role of annatto on oxidative reactions affecting lipids and proteins that would shed light on these complex mechanisms.

Acknowledgement. This manuscript was funded by BK21 plus program of Chonnam National University.

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