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Antioxidant and antibacterial effects of black pepper *(Piper nigrum L.)* essential oil in frozen raw pork sausage

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Abstract: The objective of this study was to evaluate the effect of 2000 ppm black pepper powder (BPP), 200 ppm sodium nitrite ($NaNO_2$), 200 ppm black pepper essential oil (BPEO), 400 ppm BPEO and 600 ppm BPEO in inhibiting lipid oxidation and bacterial growth in frozen raw pork sausage. The method used to evaluate the lipid antioxidant activity is Peroxide Value (PV), expressed as milliequivalent peroxide value (mPV). To evaluate antibacterial activity, Heterotrophic Plate Count (HPC) method was used, expressed in Colony Forming Units per gram (CFU/g). In the peroxide value analysis, the results showed that 2000 ppm BPP and 600 ppm BPEO have the strongest inhibition of lipid oxidation in frozen raw pork sausage. Data for the PV analysis was statistically treated but found not significantly different from each other. Further, the results for the bacterial analysis, showed that the inhibitory effect of the five treatments have no significant difference, yet in terms of log cycle reduction, 600 ppm BPEO and 2000 ppm BPP still has the strongest antibacterial inhibitory effect among the five treatments. These results help to promote BPP and BPEO to replace the synthetic $NaNO_2$ in preserving raw pork sausage.

Key words: Black pepper; Essential oil; Antioxidant; Antibacterial and raw pork sausage

1. Introduction

Spoilage in meat products are typically due to one of the two major causes: microbial growth or chemical deterioration like oxidative rancidity (Asimi et al., 2013). These two factors contribute to flavor changes, undesirable color, development of pungent or rancid odor and changes in textural characteristics in the meat. These are the qualities in which meat products are said to have "lost their freshness" and has become undesirable based on its sensory properties. For processed meat producers, this is a major setback because it discourages repeat purchases from costumers.

Meat products, particularly those with a high proportion of fat, are susceptible to lipid oxidation specially when exposed to oxygen or at elevated temperatures during processing, storage and distribution. All meat products utilize antioxidants to prevent oxidative changes (Kapoor et al., 2009). Some of the popularly used synthetic antioxidants for locally processed meat products are nitrites ($NaNO_2$) (Nonot et al., 2011; Palo et al., 2008), butylatedhydroxyanisole (BHA) / butylatedhydroxy-toluene (BHT), and tert-butyl hydroquinone (TBHQ) (Sebranek et al., 2005). However, the market for these synthetic antioxidants is said to be in a decline because of their negative implications in human health (Brewer, 2011). The demand for some natural antioxidants, on the other hand, is rising since consumers are opting for more organic or natural products that contain less or no synthetic preservatives. These antioxidants include herbal essential oils, tocopherols (vitamin E) and ascorbates (vitamin C) that gain easier consumer acceptance and legal requirements since these additives/ preservatives have no issues on either toxicity or carcinogenicity (Daniells, 2006). In a study by Sebranek, Sewalt, Robbins and Houser (2005), essential oil of Rosemary was utilized at concentrations ranging from 500ppm to 3000ppm to compare its antioxidant effect with BHA/BHT (Butylatedhydroxyanisole/Butylatedhydroxytoluene) in pork sausage. The results showed that the extract was equally effective as the BHA/BHT in refrigerated pork sausage and more effective than BHA/BHT in frozen pork sausage.

Some herbal essential volatile oils are also proven to have antibacterial effects (Dorman and Deans, 2004). With this dual purpose of natural additives, possessing both antioxidant and antibacterial activities, they can equally compete with the synthetic ones in food preservation of processed meat products. The major antioxidative plant phenolics can be divided into four general groups: phenolic acids, phenolic diterpenes, flavonoids, and volatile oils (Brewer, 2011). The antioxidant compounds present in black pepper fruit, which includes those that are found in its essential oil, are ascorbic acid, beta-carotene, camphene, carvacrol, eugenol, gammaterpinene, lauricacid, linalyl-acetate, methyl-eugenol, myrcene, myristicacid, myristicin, palmiticacid, piperine, terpinen-4-ol, and ubiquinone (Suhaj, 2006). The identified antioxidant compounds

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in black pepper essential oil are α, β-Pinene, α,β-Carophyllene, Camphene, Piperine, Limonene and Cymene as illustrated in Fig. 1 (Brewer, 2011).

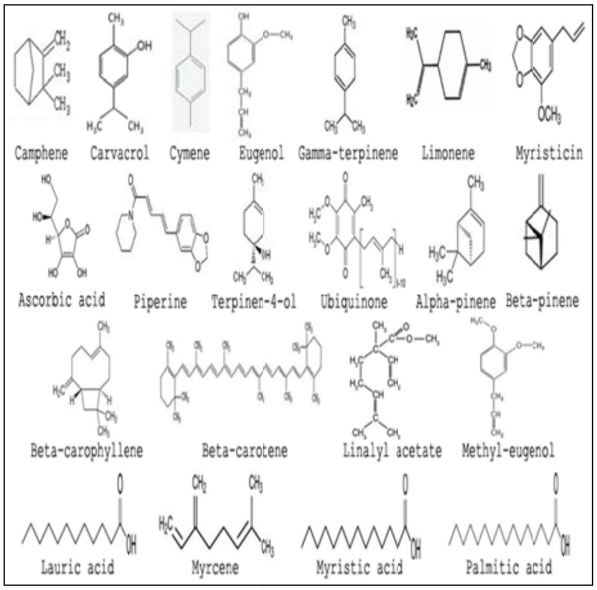

Fig.1: Molecular structure of antioxidant compounds present in black pepper essential oil (Brewer, 2011)

Black pepper is locally abundant as a raw material for essential oil extraction and processing. Although essential oil production for this spice is not yet a locally developed industry, but it has very good potential for mass production and commercialization. The black pepper essential oil (BPEO) is made from the unripe red fruit of the black pepper plant. The sun-dried peppercorns are used for the extraction of the oil, using steam distillation, which produces a yield of nearly 2%. The product is said to be 100% pure black pepper essential oil. Brewer (2011) found that black pepper essential oil have stronger antioxidant activity than BHA/BHT. Ravindran and Kallupurackal (2000) indicated that black pepper has antioxidant activity due to tocopherol and polyphenol contents. The potential of black pepper essential oil in replacing sodium nitrite for its antioxidant and antibacterial properties may be promising for meat producers (Suhaj, 2006).

The following are the mechanisms of antioxidants in delaying autoxidation: 1) scavenging species that initiate peroxidation, 2) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, 3) quenching O_2 preventing formation of peroxides, 4) breaking the autoxidative chain reaction, and/or 5) reducing localized O_2 concentrations.

Brewer (2011) mentioned that the most effective antioxidants are those that interrupt the free radical chain reaction. Usually containing aromatic or phenolic rings, these antioxidants donate H to the free radicals formed during oxidation becoming a radical themselves. These radical intermediates are stabilized by the resonance delocalization of the electron within the aromatic ring and formation of quinine structures. In addition, many of the phenolics lack positions suitable for molecular oxygen attack. Both synthetic antioxidants (BHA, BHT and propylgallate) and natural botanicals contain phenolics (flavonoids) that function in this manner. Botanical extracts with antioxidant activity generally quench free radical oxygen with phenolic compounds as well.

A study by Dorman and Deans (2004) had assessed several volatile oils, including that of black pepper, in their antibacterial activity against 25 different genera of bacteria. The volatile oils exhibited considerable inhibitory effects against the entire organism under test while their major components demonstrated various degrees of growth inhibition. But the black pepper essential oil belongs to the category with a weak antibacterial inhibitory effect.

The antimicrobial activity of black pepper is due to the presence of essential oil (2%), whose aroma is dominated by monoterpenes hydrocarbons: sabinene, β-pinene and limonene. Furthermore, terpinene, α-pinene, myrcene, and monoterpene derivatives like borneol, carvone, carvacrol, 1, 8-cineol and linalool are also present (Kapoor, 2009) (Fig. 2).

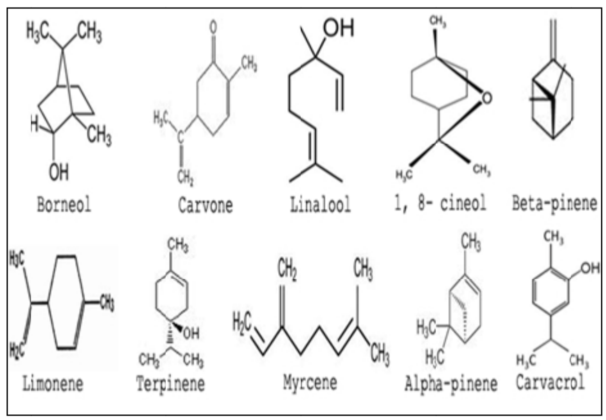

Fig. 2: Molecular structure of compounds responsible for antibacterial activity (Brewers, 2011)

Black Pepper Essential Oil contains minerals like iron, calcium, manganese, phosphorus, selenium, dietary fiber and potassium. The vitamins present in this essential oil include vitamin K and vitamin A (Ravindran, 2000).

Herb and spice extracts and oleoresins are Generally Recognized as Safe (GRAS). Some are considered to be indirect additives; as such, solvents permitted for the extraction process and solvent residues allowed are specified. Some extracts, concentrates, and resins are regulated by the USFDA "Dietary Supplement Health and Education Act of 1994" and are considered to be one (or more) of several defined dietary ingredients such as, vitamin A, mineral, herb or other botanical, amino acid or dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract, or combination of any ingredient described in clause (A), (B), (C), (D), or (E) and is excluded from regulation as a food additive. Extracts, concentrates, and resins are also regulated under the Food Labeling Regulation, Amendments; Food Regulation Uniform Compliance Date; and New Dietary Ingredient Premarket Notification Final Rule (1997). If they are added to cause flavor or color changes, they are regulated as such and specific quantities allowable for use in various classifications under which an extract, concentrate or resin could be covered, allowable use levels vary widely (Brewer, 2011). However, in the Philippines these ingredients are not yet regulated and no existing standards exist for use in the Philippine FDA. Furthermore, no commercially processed and distributed meat products utilizing essential oils as a food additive/preservative is currently being marketed.

The aim of the study was to compare the antioxidant and antimicrobial effects of 200 ppm sodium nitrite ($NaNO_2$), 2000 ppm black pepper powder (BPP) and black pepper essential oil (BPEO) at concentrations of 200, 400, and 600 ppm. The testing methods used were Peroxide Value (PV) Test to determine antioxidant activity expressed in milliequivalent peroxide value (mPV) and Heterotrophic Plate Count (HPC) in colony forming units per gram sample (CFU/g) to determine its effect in the inhibition of bacterial growth.

2. Materials and methods

2.1. Materials and sample preparations

The sample products used in this study were frozen raw pork sausage mixtures. The fat content of the product is approximately 35% and lean meat content is approximately 65%. The ground fat and lean meat were mixed with seasonings and spices in the selected pork sausage recipe, except for the black pepper powder. The bulk mixture was then divided into the five sample base formulations: 1) 2000 ppm BPP, 2) 200ppm $NaNO_2$, 3) 200ppm BPEO, 4) 400 ppm BPEO, and 5)600 ppm BPEO. The treatments were initially mixed into wine infused with star of anise to ensure even incorporation to the raw ground pork mixture. After making each base formulation, these were packed in sausage casings, labelled and stored at freezing temperature.

2.2. Antioxidant property assessment

The Peroxide value (PV) analysis was used to determine the extent of oxidation of the product through measuring peroxides present in the samples. Samples were stored in freezing temperatures for 50 days and oil was extracted from the stored samples every 10 days. Oils from the samples were extracted using the Soxhlet extraction method (Kolhe, Borole and Pate, 2011). Approximately two (2) grams of oil from each formulation were extracted and used for the PV analysis.

2.3. Antibacterial property assessment

The heterotrophic plate count method was used to determine the number of aerobic and facultative anaerobic bacteria present in the sample. The test was done every ten days after the sample formulations were completed for about 50 days. A 20 gram sample was used from each formulation. The samples were homogenized and serially diluted to 10^{-6} up to 10^{-8} concentrations for incubation at 27-33 °C. Results were counted and recorded after 48 hours of incubation.

2.4. Statistical analysis

Data gathered from the peroxide value analysis in 6 sampling periods and heterotrophic plate counts from 5 sampling periods were subjected to one way ANOVA to determine if there is significant difference in the antioxidant and antibacterial effects of the five different treatments.

3. Discussion of results

3.1. Color of treated sample

Based on the ocular assessment of the treated samples, as presented in Fig. 3 below, the only treatment that developed an undesirable pale brownish color after 50 days of frozen storage is the sample treated with 200 ppm $NaNO_2$. The other four treatments resulted in an attractive pink color for the sausage samples. Retention of desirable pink color is related to the maintenance of the oxidation state of the iron in the red myoglobin found in the muscle tissues of meat. Browning is an indication of the conversion of the iron in the heme of the red myoglobin from a +2 to a +3 oxidation state. Although color changes don't necessarily affect flavor but the product's general acceptability and appeal to consumers can be compromised.

Heme proteins and their related products as well as transition metal ions have been implicated in meat lipid oxidation. Because of heat processing, heme compounds in untreated meats are rapidly oxidized and produce ferrous and ferric ions. In cured meats nitric oxide, produced from nitrite, reacts with myoglobin and also combines with Fe^{2+} ions and thus suppresses meat flavour deterioration (Shahidi, 2002).

Tipsrisukond et al. (1998) as cited by Ravindran and Kallupurackal (2000) found out that extracts of ground black pepper are superior in reducing lipid oxidation of cooked ground pork. This browning reaction is also observed from frozen bacon products that have undergone long frozen storage and it was also observed in the frozen native pork sausage formulation treated with $NaNO_2$ in this study.

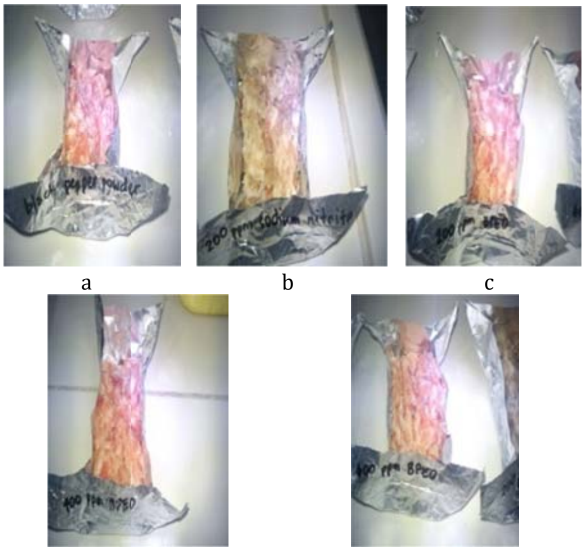

Fig. 3: Pictorial presentation of the five samples: (a). 2000 ppm BPP, (b). 200 ppm $NaNO_2$, (c). 200 ppm BPEO, (d). 400 ppm BPEO, (e). 600 ppm BPE

Oxidation reactions that were initially planned for observation in this study was only in the fat component of the sample, however, results clearly indicate that prevention of myoglobin oxidation was also observed from samples treated with BPP and BPEO. This observation suggests that the antioxidant property of the essential oil does not only affect the fat portion of the meat product, but also the lean portion of the meat product as manifested by the retention of the natural pinkish tint of the pork sausage samples.

3.2. Results of antioxidant property assessment

There was an increasing trend for the generation of peroxides in the five treatments. Treatment with 200ppm $NaNO_2$ showed the highest amount of peroxides generated after 50 days of storage while treatments with 2000ppm BPP and 600 ppm BPEO showed the lowest amount of formed peroxides in the extracted fat samples.

Table 1: Average progression of peroxide formation in mPV in six sampling periods

Treatments	Day 0 (mPV)	Day 10 (mPV)	Day 20 (mPV)	Day 30 (mPV)	Day 40 (mPV)	Day 50 (mPV)
2000ppm BPP	2.68	6.21	12.57	28.55	37.19	55.71
200ppm $NaNO_2$	3.27	15.28	22.56	49.26	60.88	99.09
200ppm BPEO	3.86	10.15	16.19	34.72	48.54	75.69
400ppm BPEO	4.17	9.11	12.08	29.53	42.39	64.24
600ppm BPEO	4.05	7.62	10.91	25.67	35.92	54.09

Acceptable maximum value is 10 mPV/kg fat (Codex Alimentarius Stan 211-1999, 2015)

It appears that the 200 ppm $NaNO_2$ and 200 ppm BPEO exhibited the weakest antioxidant activity and the 2000 ppm BPP and 600 ppm BPEO showing the strongest antioxidant activity among the 5 treatments. In day 10 of the analysis, treatment with 200 ppm $NaNO_2$ have already exceeded the acceptable maximum value of peroxides at 10 mPV/kg fat as indicated by Codex Alimentarius Standards 211-1999 (2015). However, sensory evaluation of the samples indicated that no perceptible off odor nor flavor was detected as the minimum amount of peroxides known to be detectable by sensory evaluation is at 40 mPV/kg fat (National Renderer's Association, 2008). Most samples exceeded the acceptable maximum peroxide value at 20 days of storage with 600 ppm BPEO treatment exhibiting the lowest peroxide value. This can further be observed from the graphically presented data in Fig. 4.

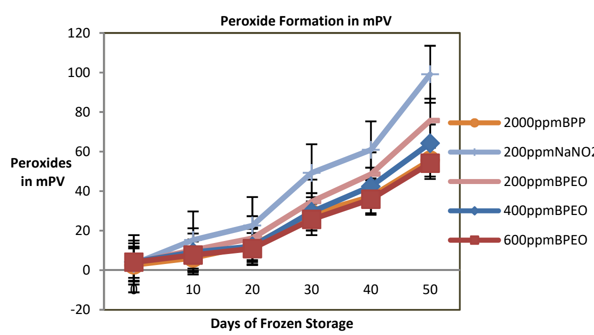

Fig. 4: Graphical presentation of the progression of peroxide formation in the fat of the raw pork sausage

Table 2 below shows the results of the statistical analysis conducted on the peroxide value analysis for six sampling periods.

Table 2: Results of one way ANOVA test for PV analysis.

Source	df	SS	MS	F	P-value
Treatments	4	1431.961	357.990	0.5331	0.7126*
Error	25	16789.067	671.563		
Total	29	18221.027			

*Not significant at α=0.05

Statistical analysis of the PV data indicated that even if 2000 ppm BPP and 600 ppm BPEO exhibited the highest inhibition for peroxide generation the effect of the treatments were still not significantly different from each other at α=0.05.

3.3. Results of antibacterial property assessment

In Table 3 is the presentation of the results for the heterotrophic plate count determination. The monitoring for this parameter extended to 49 days or 1 and ½ months. Based on the results of the 5 different treatments it was observed that there was a general increasing trend in the heterotrophic microbial count in the raw pork sausage samples even at frozen temperature storage.

In all five treatments a 0-3 log cycle increase in the aerobic bacterial population was observed. Two hundred ppm $NaNO_2$, 200 ppm BPEO and 400 ppm BPEO showed the highest increase of 3 log cycles. Treatment with 2000 ppm BPP exhibited 2 log cycles increase in the population. Treatment with 600 ppm BPEO exhibited the least increase in microbial growth at 0 log cycle.

This can be further observed in the graphical presentation of the heterotrophic plate count as shown in Fig. 5.

Table 3: Average heterotrophic plate count in colony forming units per gram (CFU/g) for five sampling periods

Treatments	Day 9	Day 19	Day 29	Day 39	Day 49
2000ppmBPP	$38x10^7$	$20x10^6$	$86x10^6$	$12x10^8$	$19x10^9$
200ppm$NaNO_2$	$33x10^6$	$49x10^6$	$10x10^8$	$86x10^6$	$25x10^9$
200ppm BPEO	$26x10^6$	$66x10^6$	$83x10^6$	$72x10^6$	$25x10^9$
400ppm BPEO	$98x10^6$	$68x10^6$	$40x10^6$	$17x10^7$	$25x10^9$
600ppm BPEO	$12x10^7$	$15x10^7$	$38x10^6$	$21x10^7$	$18x10^7$

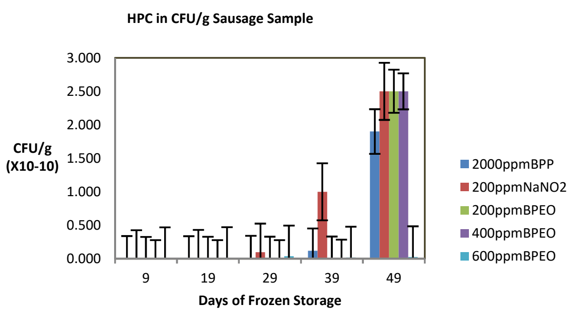

Fig. 5: Graphical presentation of the heterotrophic plate count of the raw pork sausage.

In Table 4 is the result of the statistical evaluation of the heteroptrophic plate count determination in five sampling periods. Statistical evaluation by One-Way ANOVA showed that the anti-microbial effect of the various treatments was not statistically significant from each other at α=0.05.

4. Conclusion

The findings of this study indicated that 2000 ppm BPP and 600 ppm BPEO are better additives than 200 ppm $NaNO_2$ in pork sausage to inhibit lipid oxidation and bacterial growth. For BPEO, the higher the concentration, the better its inhibitory effects on lipid oxidation and bacterial growth, $NaNO_2$ appeared to be a weaker antioxidant and antibacterial agent for frozen raw pork sausages used in this study.

Table 4: Results of One Way ANOVA test for HPC.

Source	df	SS	MS	F	P-value
Treatments	4	$9.34 X 10^{13}$	$2.33 X 10^{13}$	0.2653	0.8968*
Error	20	$1.76 X 10^{15}$	$8.80 X 10^{13}$		
Total	24	$1.85 X 10^{15}$			

*Not significant at α=0.05

5. Recommendations

A further confirmatory test on the antioxidant and antibacterial properties of black pepper powder and its essential oil is recommended where other parameters for determining oxidation and bacterial growth will be used. For instance, in determining the inhibitory effects on lipid oxidation, products other than peroxides may be determined (such as thiobarbituric acids and aldehydes) and color changes may also be objectively assessed. In determining antibacterial property, another media may also be used to determine inhibition on the growth of specific bacteria such as E. coli, Salmonella and Listeria.

Organoleptic properties of the sausage product should also be evaluated to know if it will be acceptable to the market. Variables such as color, aroma and texture are factors that can be affected by different treatments and concentrations of black pepper powder and black pepper essential oils. Black pepper powder appears to be a better choice because it is less costly than black pepper essential oil due to the extraction process, but sensory qualities mentioned above should be considered to open the possibility of using both BPP and BPEO in combination.

Finally, the optimum concentrations of black pepper powder and black pepper essential oil to be used should be determined to get the best sensory qualities of a pork sausage formulation.

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