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The effect of different levels of ozone and ozonized water on biogenic amines in broiler chicken meat by HPLC

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Abstract

Biogenic amines (BA) are low molecular weight substances, formed mainly by decarboxylation of specific amino acids present in food through the action of enzymes during storage produced by some microorganisms, this fact can be used to relate BA as a bacteriological quality indicator. This study was aimed to evaluate the effects of an experimental ozone gaseous treatment and production of the biogenic amines' putrescine and cadaverine. Amines were extracted with perchloric acid, derivative with dansyl chloride, separated using a reversed-phase high-performance liquid chromatographic method, and detected by fluorescence. The results showed that during the 7 days, the amount of putrescine had increased in all four experimental treatments. The highest increase was related to Group 1. The Duncan post hoc test showed that the highest amount of Cadaverine after killing was related to Group 1 (control) with 88.85 mg/kg and the lowest value for Group 2 is 12.03 mg/kg. Also, the results of Duncan's post hoc test for comparing Cadaverine seven days after slaughter among experimental treatments showed that the highest amount of Cadaverine after slaughter was related to Group 1 (control) with 135.6 mg/kg and the lowest value for the group is 94.83 mg/kg. The results of the test to compare the pH of the treatments showed that the highest amount of pH is for Group 4 and the lowest value for Group 1 (control). The results of this study showed that with increasing ozone gas concentration and decreasing the concentration of water, the biogenic amines' putrescine and cadaverine in frozen poultry meat decreased. Putrescine and cadaverine levels appeared to be useful to control the effectiveness of the ozone treatment on meat quality and may be useful as a quality index to highlight the loss of poultry meat freshness.

1. Introduction

The worldwide poultry meat production and consumption have increased rapidly and, per capita consumption of poultry meat in many parts of the world will continue to grow. Competitive price, the absence of cultural and religious obstacles, and dietary and nutritional properties are the main factors explaining the poultry meat's attractiveness for consumers (Petracci and Cavani, 2012). Hygiene intervention in the poultry meat processes alone does not lead to safe products, owing to the constant flow of bacteria entering the processing plant and unavoidable cross-contamination. Eradication of pathogens in the livestock, or the rearing of animals that are "Specified Pathogen Free" (SPF), might make relevant contributions, but the SPF system is currently too expensive (Bolder, 1997). Decontamination of meat and poultry carcasses can help to reduce human foodborne infections and seems to be the only possibility to assure food safety. So many decontamination treatments of the poultry carcasses have been described, which can roughly be divided into three types: chemical, physical and combinations of the two. The problems are that not all of the treatments are applicable in the meat industry, and physical or chemical treatments of poultry carcasses are not allowed in Europe according to certain

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EU regulations, yet they approved in the USA. Decontamination treatment with ozone has been approved by the U.S. Food And Drug Administration (FDA)-Department of Health and Human Services, which has considered ozone as food additives, recognized as GRAS (Generally Recognized for Safe) substance, and has approved its use in the gas phase and water, as a scavenger in processing, storage and processing of food, according to the Good Practices Manufacturing (GMP) (Code of Federal Regulation-CFR document 21), as an antimicrobial agent. Ozone treatments for food decontamination are also permitted in Canada, but the gas concentrations do not exceed the minimum levels technologically necessary. Ozone (O_3) is a tri-atomic gaseous molecule consisting of three oxygen atoms. It is an allotrope of oxygen much less stable than the diatomic allotrope (O_2) characterized by strong oxidizing nature that makes it a useful tool for the inactivation of bacteria, fungi and viruses. Ozone first attacks the bacterial membrane at the glycoproteins, glycol lipids, or at certain amino acids such as tryptophan, and then also acts on the sulphydryl groups resulting in disruption of the normal cell. Bacterial death is rapid and often attributed to changes in cell permeability followed by lysis, and the bactericidal effect depends on several factors, such as temperatures, relative humidity, pH values and presence of organic matter (Moore *et al*., 2000). In the food industry ozone is used as a sanitizing agent capable of killing numerous microorganisms by oxidizing their cell membranes. It has been proposed as a chemical decontamination treatment of poultry carcasses because it is capable of experimentally extending the shelf life of perishable foods by reducing microbial activity (Moore *et al*., 2000). The use as a water-disinfecting agent has not been reported (Bolder, 1997) in a superficial disinfectant treatment of chilled poultry carcasses, and neither visual defect to the carcasses nor sensory off-flavors were shown (Rice *et al*., 2001). Sometimes ozone generators are used in food storage rooms to control the growth of microorganism and the shelf life of products. Ozone represents a public health hazard above concentrations of 100 μg/kg, especially when it is applied in production areas, because of high oxidizing potential, that can cause damage to mucus and respiratory tissues (Bodmer *et al.,* 1999), but the modern ozone generators can be better controlled the gas emissions in habitat. Dietary polyamines such as putrescine, spermidine and spermine are low molecular weight bases with an aliphatic structure (Ladero *et al.,* 2010). They have traditionally been classified within the group of biogenic amines (BAs). However, they started to be considered separately during the 1990s for their role in the growth and function of normal cells, and due to their mode of formation (Kalac, 2009). The polyamines are ubiquitous, widespread from bacteria to mammals (Kalac, 2009). They can be found in different foodstuff like wine, fruits and vegetables, cheese and meat in low concentration (Naila *et al.,* 2010). These components have positive and negative effects on human health; they can be useful for growth and wounds healing, and also harmful for those suffering from tumor as they accelerate the growth of tumors (Dadáková *et al.,* 2012). Different methods have been used for measuring the biogenic amines (BAs) and polyamines existing in foods. Gas chromatography (GC) (Almeida *et al.,* 2012), thin-layer chromatography (TLC) (Shakila *et al.,* 2001), capillary electrophoretic (CE) (Lange *et al.,* 2002) method, ion chromatography-mass spectrometry (Saccani *et al*., 2005), ultra-performance liquid chromatography (UPLC) (Dadáková *et al.,* 2012) and high-performance liquid chromatography (HPLC) are techniques that have been applied ever to determination BAs and polyamines (Önal, 2007). HPLC is the most widely used technique for its high sensitivity and wide range of linearity (Tamim *et al.,* 2002). GC is rarely applied for the determination of BAs. Although TCL is a good method that does not need complicated equipment; however, it is time-consuming with low sensitivity, so it has not been applied mostly. Additionally, CE is not a good technique in detecting BAs as they do not exhibit strong fluorescence, so they could not be detected directly in a sensitive manner. Among the mentioned methods, HPLC with pre-or post-column derivatization is by far the most frequently reported technique for BAs separation and quantification (Önal, 2007). Extraction of BAs is so important because most of them are linked to other components. Due to the complexity of the meat matrices (protein-rich food, often with high-fat content) and the low amount of BAs and polyamines in different matrices, extraction and purification of these components are necessary for analyzing. Indeed, acid extraction is mostly used for these components by hydrochloric acid, perchloric acid or trichloroacetic acid (TCA) (Özdestan and Üren, 2009). As mentioned, BAs and polyamines do not have chromophoric or fluorogenic properties, so they are determined by pre-column or post-column derivatization (Özdestan and Üren, 2009). Derivatization procedure is so crucial that should be done precisely. Some reagents that are mostly used for derivatization include dansyl chloride (Bashiry *et al.,* 2014), benzoyl chloride (Özdestan and Üren, 2009) and orto-phthaldialdehyde (OPA) (Triki *et al.,* 2012). Among these reagents, dansyl chloride is frequently used specially for spermidine and spermine. Smela *et al*. (2003) compared dansyl chloride and orto-phthaldialdehyde (OPA) and concluded that dansyl chloride is more suitable for derivatization of spermidine and spermine (Smela *et al.,* 2003). Also,

using Ozone has been successful in the elimination of *Salmonella* infection on commercial eggs (Parvin Ahangar *et al.,* 2020). This study aimed to evaluate the effects of an experimental ozone treatment during the storage of chilled poultry carcasses, and the correlation of BAs production as a quality index in poultry meat.

2. Material and methods

2.1 Preparation

Immediately after slaughter and chilling, 100 broilers, weight 2.5 kg, were collected in an EU authorized slaughterhouse. Lot control 1, (twenty-five carcasses), according to the usual methods, were stored in a refrigerated cell equipped with a thermograph set to 0-1°C. To obtain a major decontamination effect, being O_3 in the gaseous/aqueous phases effective against the majority of microorganisms and viruses at relatively low concentrations and short contact time (Bolder, 1997), Lot 2, 3 and 4 (seventy-five carcasses) was previously washed by ozonized water using a concentration of O_3 mL/L, before the gaseous sanitizing treatment. Then the carcasses of Lot 2, 3, 4 and control 1 were stored in a refrigerated cell, also equipped with a video thermograph set to 0-1°C. In the cell of Lot 2, 3 and 4 a generator of gaseous ozone (OZONET of OXITECH Srl) and a timer, to deliver ozone for 60 mins every 4 hrs, on the basis of preliminary bacterial reduction tests on carcasses surfaces, were installed [Kim, Murray]. Dimensions of both refrigerated cells were: height 3.60 cm, inches deep 3.24 cm and length 1.32 cm. Temperature values and ozone air levels inside the cells was monitored using a probe and a display, positioned on the outside of the cells, along the time of the experiment. At 0th and 7th days of storage three carcasses of each batch were collected, and 2 aliquots, consisting of the breast muscle (predominantly white fibers) and limbs (prevalence of red fibers), have been separately subjected to the determination of the pH for each sample. Then sensory and analysis of BAs was carried out on a pool of the two aliquots combined. 25 carcasses from broiler chickens as the control group and 75 remaining chickens were divided into three groups. Different concentrations of ozone and ozonized water were allocated to each treatment (Group 1 (control), Group 2 (2 ppm ozone water + 100 ppm ozonized gas), Group 3 (4 ppm ozone water + 50 ppm ozonized gas), Group 4 (8 ppm ozone water + 10 ppm ozonized gas). All treatments were carried out with different concentrations of ozone and ozonized water for 10 mins.

2.2 Analysis of biogenic amines

Reagents: Solvents and reagents were of analytical or High-Performance Liquid Chromatography (HPLC) grade. The biogenic amine standard of putrescine dansyl-hydrochloride (PUT) and Cadaverine Dansyl-Dihydrochloride (CAD) were obtained from Sigma (St. Louis, MO).

2.3 HPLC system

Analyses were carried out using an HPLC system (JASCO), equipped with a quaternary pump (JASCO 2089 plus), and a 20 µL loop, combined with a Jasco integrator, and an 821-Fp fluorescence detector. Detection of amines was accomplished at an excitation wavelength of 350 nm and an emission wavelength of 520 nm. The separation was performed on a reversed-phase C18 Luna column 5 µm 518 (Phenomenex Inc., Torrance CA) (250 mm length x 4.6 mm internal diameter, and 5 µm particle size) with a 3 × 4 mm Security Guardcartridge guard column) (Phenomenex Inc., Torrance CA).

2.4 Measurement of BAs content

Two grams of the sample was weighed into a 50 mL polypropylene conical tube (Beckton Dickinson and Co., Franklin Lakes, USA) and homogenized (Ultra-Turrax 25, IKA-Labortechnik, Staufen, Germany) in 10 mL of 0.4 M perchloric acid. The homogenized sample was centrifuged for 10 mins at 3,000 rpm (Union 5KR, Hanil Co., Incheon, Korea) and the supernatant was filtered through filter paper (Whatman No. 1, Whatman International Ltd., Maidstone, England). Approximately, 10 mL of 0.4 M perchloric acid was added to the remnant and mixed thoroughly in a vortex mixer (Vortex - Genie2, Scientific Industries, Inc., Bohemia, USA). This mixture was centrifuged for 10 mins at 3,000 rpm and the supernatant was filtered again through the same type of filter paper. Finally, the volume of filtrate collected from both steps was adjusted to 25 mL with 0.4 M perchloric acid. Sample extract (1 mL) was transferred into a 15 mL polypropylene conical tube (Beckton Dickinson and Co., Franklin Lakes, USA) and 50 µL of an internal standard (1,000 ppm 1, 7-diaminoheptane) was added. Two hundred microliters of 2 N sodium hydroxide, 300 µL of saturated sodium bicarbonate and 2 mL of dansyl chloride solution (10 mg dansyl chloride dissolved in 1 mL acetone) were added to a sample extract before incubation for 45 mins at 40°C in a water bath. After incubation, 100 µL of liquid ammonia was added to the reaction mixture for the removal of any residual dansyl chloride. After 30 mins at an ambient temperature, the volume of the reaction mixture was adjusted to 5 mL with acetonitrile. This reaction mixture was centrifuged for 5 mins at 2,500 × *g*. The supernatant was filtered with a 0.45 µm syringe filter with a PVDF Membrane (Acrodisc LC13 PVDF minispike, Pall Co., Ann Arbor, USA). Ten microliters

of a filtered sample were injected into the HPLC with a diode array detector (Agilent 1100, Agilent Techology Inc., Wilmington, USA) equipped with a Spherisorb ODS2 column (4.6 × 150 mm i.d., 5 µm, Waters, Milford, USA). Gradient elution program was used with a mixture of 0.1 M ammonium acetate as solvent A and acetonitrile as solvent B. Both solvents were vacuum filtered by a membrane filter (47 mm PTFE 0.45 µm, Pall Co., Ann Arbor, USA) and degassed with an ultra-sonicator (5210, Branson Ultrasonic Co., Danbury, USA). The flow rate was 1 mL/min. The gradient began at 50% solvent A and 50% solvent B and ended at 10% solvent A and 90% solvent B at 19 mins, respectively. Ten minutes of a waiting time before the next analysis was necessary for equilibrium. The column temperature was 40°C. The amounts of the dansyl derivatives of the biogenic amines were quantified by a measurement of the UV-absorption at 254 nm and the fluorescence at 550 nm (Min *et al.,* 2007).

2.5 Statistical analysis

Statistical analysis was performed using SAS 8.01 for Windows (SAS, 2000). One-way ANOVA was performed, and Duncan's multiple range test was used to analyze the significant differences among the mean values (Min *et al.,* 2007).

3. Results

Duncan's post hoc test to compare putrescine and cadaverine after slaughter showed that between the four treatments was statistically significant ($p<0.05$). The highest amount of putrescine and cadaverine after killing belonged respectively to Group 1 (control) with 89.89 mg kg and 88.85 mg/kg and the lowest amount putrescine and cadaverine belonged respectively to Group 2 (2 ppm ozone water + 100 ppm ozonized gas) 13.14 mg/kg and 13.03 mg/kg (Table 1).

Duncan's post hoc test to compare putrescine and cadaverine 7 days after slaughter (freezing) showed that between the four treatments was statistically significant ($p<0.05$). The highest amount of putrescine and cadaverine 7 days after slaughter belonged respectively to Group 1 (control) with 169.11 mg/kg and 135.09 mg/kg and the lowest amount putrescine and cadaverine belonged respectively to Group 2 (2 ppm ozone water + 100 ppm ozonized gas) 79.49 mg/kg and 94.83 mg/kg (Table 2).

Duncan post hoc test was not statistically significant for the comparison of pH among treatments on day of slaughter and 7 days after slaughter (freezing) (Table 1 and Table 2).

The correlation of putrescine and cadaverine groups after 7 days indicates that there is a high correlation between putrescine and cadaverine after slaughter and 7 days after the slaughter, respectively, 0.88 and 0.96 (Table 3).

4. Discussion

Consumption of poultry meat frequently causes food -borne diseases. In the food industry the presence of bacteria biofilms is a significant problem, because of the environmental persistence of bacteria and their resistance to desiccation, UV radiation and other antimicrobial treatments. Since the processes used for the cleaning and

Table 1. The levels of putrescine and cadaverine and pH in different experimental groups on pre-freezing days (mean±SE)

Factor	Group 1 (control)	Group 2 (2 ppm ozone water + 100 ppm ozonized gas)	Group 3 (4 ppm ozone water + 50 ppm ozonized gas)	Group 4 (8 ppm ozone water + 10 ppm ozonized gas)
Putrescine (mg/kg)	89.89±3.21[a]	13.14±1.24[b]	38.71±3.25[c]	79.4 ±3.21[d]
Cadaverine (mg/kg)	88.85± 5.82[a]	13.03± 1.10[b]	55.79 ± 3.40[c]	77.24 ± 4.28[d]
pH	6.82 ± 0.18	6.89± 0.20	6.78 ± 0.15	6.71± 0.16

Different letters in the same row represent significant differences ($p<0.05$).

Table 2. The levels of putrescine and cadaverine and pH in different experimental groups on 7 days after freezing (mean±SE)

Factor	Group 1 (control)	Group 2 (2 ppm ozone water + 100 ppm ozonized gas)	Group 3 (4 ppm ozone water + 50 ppm ozonized gas)	Group 4 (8 ppm ozone water + 10 ppm ozonized gas)
Putrescine (mg/kg)	169.11±3.02[a]	79.49±2.12[b]	89.64±3.07[c]	113.88±2.7[d]
Cadaverine (mg/kg)	135.09±4.08[a]	94.83±2.15[b]	109.95±3.56[c]	124.74±4.09[d]
pH	6.92±0.29	6.51±0.19	6.61±0.27	6.49±0.15

Different letters in the same row represent significant differences ($p<0.05$).

Table 3. Correlation coefficients between putrescine and cadaverine

	Treatment	Count	Correlation	Significant
Group 1	Putrescine (During slaughter and seven days later)	75	0.882	0
Group 2	Cadaverine (During slaughter and seven days later)	75	0.965	0

disinfection of equipment and poultry surfaces can reduce, but not always eliminate bacteria and pathogens contamination, in Europe, there is an increased interest in developing decontamination methods applicable to meat (Mercogliano *et al.,* 2014). Nowadays, researchers consider utilizing ozone as an efficient method for disinfection. This method decreases influences on organoleptic attributes significantly. In 1997, the US agriculture department has approved utilizing ozone as a method for recycling and reusing the water of fowls' chiller. Paralyzing microorganisms using ozone is a complicated process. In this process, ozone assails the membrane and its compounds of constituent namely unsaturated fats and cell constituents or enzymes and nucleic acids. Destruction of the cell membrane or decomposition of cell constituents causes microorganisms eradication (Mercogliano *et al.,* 2014). Decontamination ozone treatments can limit the surface contamination of poultry carcasses and can be a valuable aid to GMP in poultry slaughterhouses if an integrated approach to the problem of "decontamination" treatment is considered. In fact, the ozone treatment of the poultry carcasses inside chilled cells may show positive effects on the bacterial contamination and shelf life of the poultry meat (Bolder, 1997). For pathogens control in meat production, as decontamination treatment and auxiliary tools, EU Reg./2004/852 and EU Reg./2004/853 authorize only the use of ozonized potable water. Furthermore, Italian National Committee for Food Security (CNSA, 27/10/2011) experts have expressed a favorable opinion also regarding gaseous ozone treatment of chambers of seasoning and/or storage areas of cheeses in the absence of food. Several indicators have been proposed for the evaluation of meat quality (volatile bases, nucleotides break-down, volatile acidity, but all are limited (Veciana-Nogués *et al.,* 1997; Min *et al.,* 2007). Quality sensory analysis is, undoubtedly, widely accepted as the most rapid method to detect the quality of fresh meat, but it is subjective (Mietz, 1978). Furthermore, it would be desirable to identify parameters most effective to evaluate meat's shelf life, before the effects of the spoilage changes of sensory quality (Durlu-Özkaya *et al*., 2001). European expert group BIOHAZ recognized a strong correlation between the BAs presence in food and the quality of raw materials, in terms of freshness and biological safety. Biogenic amines, histamine, putrescine, tyramine, tryptamine, 2-pheniylethylamine and cadaverine, can be formed during storage of fresh meat (Mietz, 1978; Gloria *et al.,* 1999; Silva and Glória, 2002). Since they are metabolites of microbial activity, they are considered to be useful indices of the freshness of meat, in contrast, polyamines spermine and spermidine are naturally occurring amines in fresh meat (Wortberg and Woller, 1982; Tamim *et al*., 2002). Levels of all amines increase with increasing putrefaction except spermidine and spermine, which decrease during putrefaction of poultry carcasses. Biogenic amines histamine, tyramine, and phenylethylamine remain low for approximately 24 hrs, after which they rose sharply to high levels at 72 hrs (Wortberg and Woller, 1982). PUT and CAD also exhibited a common response pattern, but in poultry carcasses, these amines remain low through the first 24 hrs, and then rise to high levels, and they plateau at 48 hrs (Wortberg and Woller, 1982). In decontamination, treatments are important to respect the GMP optimizing the ozone concentrations and monitoring the storage conditions of poultry carcasses. In particular ozone, as storage applications, should be allowed to dissipate prior to workers entering the area treated. According to the experiment results, diamine putrescine is the major portion of amines is constituted in slaughtered chicken. Diamine putrescine and cadaverine show improved indexes of deterioration control utilizing ozone. A low quantity of biogenic amines in foodstuff is not considered a serious risk. However, high consumption of biogenic amines can cause toxic effects (Kordiovská *et al.,* 2006). As a result, biogenic amines are measured in the foodstuffs due to their potential toxicity. In addition, taking advantage of utilizing them as quality indexes of foodstuffs can be another reason (Křížek *et al.,* 2002). A general conclusion of this study shows using water and ozone gas for disinfection of the chicken carcasses during slaughtering leads to health promotion in the society. It is because of product durability increase and reduction of biogenic amines in carcasses causing the decrease of population and microbial effects.

5. Conclusion

The results of this study show the increase of ozone gas density and decrease of the ozone water density cause the reduction of putrescine biogenic amines and cadaverine. Furthermore, ozone is decomposed quickly and has no effect on the environment air due to the high inconsistency of the ozone in water and the presence of organic materials. As a result, ozone can be used as an efficient factor of disinfection in the water chilling system of the slaughterhouses due to having the least effect on the chicken carcass attributes. Eventually, the microbial quality level of the product is significantly improved.

Conflict of interest

The authors declare that there are no conflicts of interest.

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