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ORIGINAL ARTICLE

Presence of Nitrites, Nitrates, Nitrosamines in the Eggs of Intensively raised Layers in Abeokuta, Nigeria

Thomas, F. C. [1]*; **Aduloju, O. S.** [1]; **Okediran, B**[2]; **Ajibola, E. S.** [1] **and Maduagwu, E. N.**[3]

[1]Federal University of Agriculture Abeokuta; [2]University of Ilorin, Kwara State; [3]Covenant University, Ogun state.
*Corresponding author: Email: funmithomas2007@gmail.com; Tel No:+2348181962523

SUMMARY
In this study, the presence and concentrations of nitrosamines and their precursors (nitrates and nitrites) in raw table eggs obtained from four layer-farms in Abeokuta, were analysed. Nitrosamines are highly toxic and carcinogenic group of chemicals that have the potential to be formed in the body through a process called nitrosation. Nitrates and nitrites react under acidic and /or high temperature conditions with nitrosable substrates usually secondary amines to form several of hundreds of N-nitroso amine compounds known. A spectrophotometric method was used to assay for nitrosamines and their precursors in eggs (n=5 from each farm), entailing separate analysis of each egg yolk and albumen. The three compounds measured, were detected in all the samples. The nitrates and nitrites levels were significantly higher in the yolk than in the albumen ($P<0.05$). However, there was no significant correlation observed between the levels of nitrosamines and its precursors in the eggs ($P<0.05$). Findings from this study reveals that nitrosamines and their precursors are present in eggs from these layer farms. These compounds can be potentially toxic, thus constituting a hazard to human and animal health.

Key words: Carcinogenesis, eggs, layers, nitrates, nitrites, nitrosamines.

INTRODUCTION
N-nitrosamines are a group of extremely toxic and mostly carcinogenic compounds, whose potential carcinogenic effects have been studied extensively (Herrmann *et al* 2015; Lona-Ramirez *et al* 2016). They are chemical compounds with the general formula R_2N (-R_2) N=O, which can be produced from reactions between nitrites and secondary amines under strongly acidic conditions and high temperatures. They have been detected in a wide variety of consumer products like pesticide formulations, cigarettes, cosmetics, condoms, party balloons and other rubber products as well as various foods and beverages especially fried bacon, cured meat and beer (Frank and Berry C, 1981; Havery and Fazio, 1985) and in drinking water (Krasner *et al*, 2013). Among the over 200 nitrosamine compounds known to date, about 85% have

been shown to be carcinogenic in a variety of species (Maanen and Pachen 1997; Gangolli *et al*, 1994; Tenovuo 1986; Wolff and Wasserman 1972). Furthermore, nitrosamines and its precursors (nitrates and nitrites) have also been shown to be teratogenic and mutagenic (Cowdin *et al*, 2003; Brender *et al*, 2004; Manassaram *et al*, 2007).

Nitrates are widely distributed inorganic nitrogenous compounds present in foods and water (McKnight *et al*, 1999). They occur naturally in plants and are essentially non-toxic, but have come under increasing scrutiny due to the tendency to be reduced in the alimentary tract to nitrites through the action of gut bacteria ((Duncan *et al*, 1997). Nitrites are then consequently able to form nitrosamines in combination with amines (NH_2) (Bartsch and Montesano, 1984). Apart from the fact that nitrites have the ability to combine with certain amino groups to yield mutagenic and carcinogenic N-nitroso-compounds they are also able to convert the haemoglobin in the blood into methaemoglobin, which is unable to act as an oxygen carrier. If this change is sufficiently complete, victims may die of tissue anoxia (Rodkey, 1986).

Eggs are round or oval cell obtained from a number of different species most commonly birds and have been eaten by mankind for millennia. Most edible eggs consist of a protective oval egg shell, the albumen, the vitellus (egg yolk) and various thin membranes. Every part is edible, although the shell is generally discarded. Eggs are considered a good source of protein and choline (Palmer 1944, Mine 2007). Most commonly produced chicken eggs intended for human consumption are unfertilized. They are important in many modern branches of the food industry; and can be pickled, hard and soft boiled, scrambled, fried and refrigerated, and they could also be eaten raw.

They have been known to be subject to contamination with microorganisms or chemicals; Contamination by pathogenic bacteria; *Salmonella enteritidis* has been commonly reported (Jawale and Lee 2014; Upadhyaya *et al* 2015). Other egg-contaminating microorganisms that have been reported include high pathogenicity avian influenza-H5N1 (Bertran *et al*, 2015). Chemical residues including nitrates, perchlorate, thiocyanate, pesticide residues and other environmental chemicals have also been identified in eggs and other poultry products (Van Overmeire *et al* 2006; Olejnik and Szprengier-Juszkiewicz 2015; Esteve-Garcia and Garcia-Reuiero, 2005; Blount *et al*, 2008; USDA, 2008). In addition to these contaminants that have been detected in eggs, drug residues such as amantadine, sulphonamides, aminopyridines and tetracyclines have also been identified in eggs (Gajda and Posyniak 2015; Poźniak *et al* 2015; Lin *et al* 2015; Summa *et al* 2015; Adesiyun *et al* 2014).

A study was conducted recently to assess the teratogenic effects of nitrosamines on chicken egg embryo, where levels of nitrosamines were detected within egg albumen and yolk (Joshi, 2011). Currently, no reports have been made on the presence of detectable levels of nitrosamines, nitrates or nitrites in eggs in Abeokuta, Nigeria. Against the background of the knowledge that certain bacteria can reduce nitrates to nitrites (Stoltennow, 1998) thereby increasing the availability of the latter for nitrosation (formation of nitrosamines), and the fact that some drug residues (which have been seen in eggs) contain compounds which are nitrosable (secondary, tertiary and quaternary amines) under conditions of high temperature or action of microorganisms (that maybe present in eggs); this work aimed to detect the presence of nitrates, nitrites, as well as nitrosamines in raw table eggs obtained from commercial layer-poultry farms in Abeokuta, Ogun state,

Nigeria. An attempt to correlate the presence of the nitrogenous precursors of nitrosamines (nitrites and nitrates) to the level of nitrosamines was also made. The potential risk of nitrosamine poisoning, possibly following human consumption of eggs was also studied.

MATERIALS AND METHODS

Sample collection and preparations

Fresh table eggs (n=5 per farm) were obtained from four different farms within Abeokuta metropolis. Four farms were purposively selected for egg sample collection using nearness to study location as criteria. Other criteria for selection of sampled farms were presence of actively laying birds with commercial scale production and absence of history of disease outbreak on the farm for several months up to the time of sampling. Eggs collected (purchased as for retail) were preserved under room temperature until analysed. All analyses were carried out at the Physiology/ Biochemistry laboratory of the College of Veterinary Medicine, Federal University of Agriculture Abeokuta and were conducted under ethical considerations.

Egg yolk was carefully separated from albumen. Prior to analysis, sample treatments were carried out to facilitate the accurate measurement of nitrite levels. Albumen; an aliquot of the albumen from each egg (~ 2 mL each) was aspirated into clean dry glass test tubes into which 0.5 mL of $NaCO_3$ was added, followed by 1.5 mL of sulphanilic acid. The acid was used to precipitate the protein in the albumen leaving a clear supernatant which was decanted into a new test tube and subjected to further analyses.

For egg yolk sample, 10 g was weighed and mixed thoroughly with 100 mL of double distilled water. The emulsion produced was subsequently mixed thoroughly with 50 mL of petroleum ether in a separating funnel to de-fat the yolk. One (1) mL of the water soluble layer was collected after carefully decanting the fatty supernatant¸ separated samples were subsequently aliquoted and analysed for nitrites, nitrates and nitrosamines.

Nitrites, nitrates and nitrosamine analysis

Analytical grade chemicals were used throughout. Double distilled was used for solving. N-nitroso-dietlyamine (NDEA) stock was obtained from the stock in the Biochemical Toxicology unit of the Department of Biochemistry, University of Ibadan donated by Prof. R. Preussman of the Institute of Toxicology and Cancer Risk Factor, German Cancer Research Centre, Heidelberg, Germany.

Analyses of nitrites was carried out using according to the method of Montgomery and Dymock (1961) method as described in Ologboho *et al* (1996). The other two compounds were analysed indirectly after conversion to nitrites according to the same method. Nitrates were analysed indirectly after reduction to nitrites, through passage into a cadmium column. The column was prepared by using a 0.5 cm × 10.5 cm glass column. Prior to analysis, the column was activated by washing with 25 mL of dilute HCl (0.1 M) followed by 50 mL of distilled deionized water and finally with 25 mL of dilute ammonia buffer.

A standard curve for nitrites was obtained by plotting the optical densities of nitrites standards (serial dilutions of sodium nitrites from 1 to 0 µg/ml), while the standard curves for nitrates and nitrosamines was obtained by plotting the optical densities of standard concentrations of nitrates (sodium nitrate) and nitrosamines (NDEA) after reduction and photolysis respectively to nitrites.

For nitrites analysis, after sample preparations, each sample was thoroughly mixed and 1 mL aliquot was used to detect nitrites by addition of Naphthylethylene Diamine (NEDA) solution (for colour

generation), after which optical densities were determined using a spectrophotometer (Jenway® Spectrophotometer read at 550 nm). Optical Density (OD) obtained after spectrophotometry was read off the nitrites curve to determine the concentrations of nitrites (termed actual nitrites).
Nitrates were determined in another aliquot of the same sample (after reduction to nitrites through the Cadmium column) after which concentrations of nitrates were calculated from the formula;
(Concentration of derived nitrites – concentration of actual nitrites) X 3.5 (3.5 being the calculated conversion factor of OD differences from nitrates standard curve).

Nitrosamines were also analysed following ultraviolet degradation of the same to nitrites. Nitrosamine concentration was then calculated using the formula;

Concentration of nitrosamines = OD (after UV exposure) – OD (actual nitrite). The optical density derived thereof is read from a nitrosamine standard curve prepared using pure stock solution of N-nitroso dietlyamine (NDEA) diluted in serial concentrations.

Each parameter was assayed in duplicates per sample.

Statistical analysis
Results were computed as means and standard deviation. Means of each analyte across various farms were compared using Kruskal-Wallis test. Mann-Whitney test was used to compare means of measurements in yolk and albumen. The within farm correlation of nitrosamines and its precursors were evaluated using Spearman's correlation. P value was considered significant at <0.05.

RESULTS
The concentrations of nitrites, nitrates and nitrosamines detected in eggs from various farms are shown in Figures 1 and 2 (bars represent means of five eggs per farm).
Overall, of the three analytes assayed, nitrates were found in the highest concentrations in all but one farm set of samples. Nitrites were the next most abundant compounds while nitrosamines were detected in minute quantities. Levels of nitrates were significantly different among the farms as two farms (3 & 4) had a higher level of nitrates than in the other two farms (1& 2) ($P<0.05$). There were no significant correlations between concentrations of nitrosamines and its nitrogenous precursors. Concentrations of nitrosamines were found to be slightly higher in yolk than in the albumen although this was not significant.

DISCUSSION
Nitrites, nitrates and nitrosamines were detected in all the egg samples, occurring in both egg albumen and yolk from all the four farms. On the average, these compounds were higher in the yolk than in the albumen. Lee *et al* (1975), also reported a similar finding of higher concentrations in in yolk than albumen of nitrates and nitrites, in a study carried out to determine the influence of dietary intake of nitrates and nitrites on the concentrations of the same in egg yolk and albumen.
The finding of nitrates in eggs was also not surprising as nitrate levels in plants are quite high, and about 60-90% of poultry feed is made up of plant material, therefore, it would appear that the layer birds are able to secrete nitrates into eggs during formation (Blount *et al*, 2008). Besides, nitrates have also occurs in water especially groundwater (Santamaria 2006; Vermeer and Pachen 1998), which constitute a major source of water supply to poultry farms, thus it could possibly contribute to the source of nitrates detected in poultry eggs.
Since nitrates have been shown to be capable of being reduced to nitrites by bacteria in the gut (for example of laying

birds) during digestion, and can then be absorbed into circulation (Stoltennow, 1998), the detection of nitrites in eggs can thus be explained as probably from secretion into eggs from circulation of laying birds. It therefore follows that if nitrites can be secreted into eggs, nitrosamines may also be secreted into eggs in similar mode, or formed after the chemical combination of secreted substrates such as nitrites and nitrosable amines. Nitrosamines and their precursors have been shown to occur in feedstuff such as soya beans, groundnut and maize, therefore it is possible that these compounds are secreted into eggs directly from feed materials fed to the birds. On the average, nitrosamines appear to be elevated in the yolk relative to the albumen and this could be due to the water soluble nature of albumen relative to yolk that could provide a more conducive nitrates and nitrosamine to the former. However, there was an observable increase

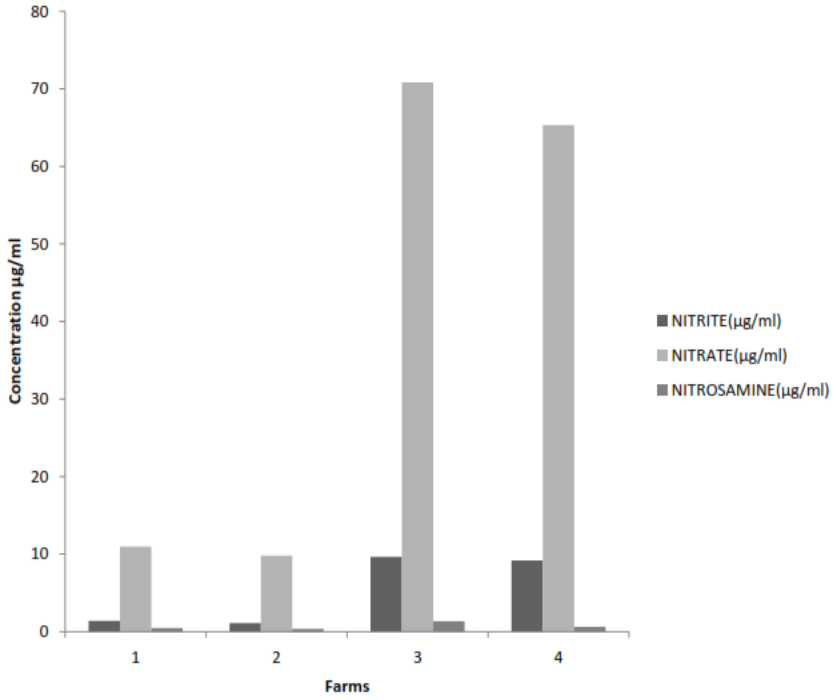

Figure 1: Concentrations of nitrates, nitrites and nitrosamine in egg-albumen (n=5 eggs/farm) from four different farms in Abeokuta

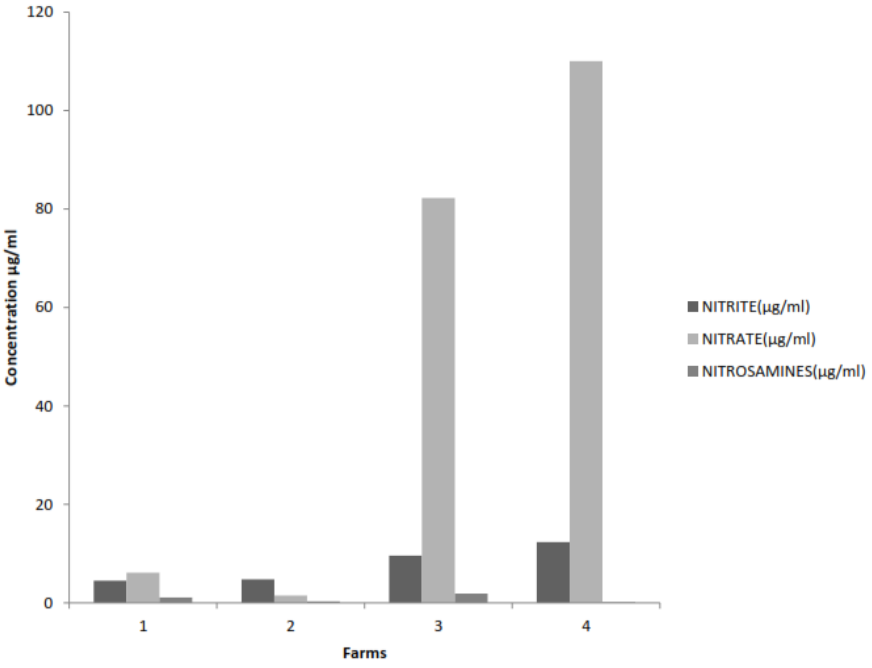

Figure 2: Concentrations of nitrates, nitrites and nitrosamine in egg-yolk (n=5 eggs/farm) from four different farms in Abeokuta

medium. The method of analysis used in this study was an indirect method, utilizing the measurement of nitrates after conversion of in nitrites (spectrophotometrically after the various treatments to convert all nitrates and

nitrosamines to nitrites). This indicates that the test is reliable in at least qualitatively delineating the presence of the latter two commands. More sensitive tests to specifically determine levels of nitrosamines, nitrates and nitrites entails the use of High performance liquid chromatography (HPLC), Gas chromatography/mass spectrometry, or differential pulse polaroraphy which has been used by several researchers (Somer *et al* 2016; Scheeren *et al* 2015; Moloney *et al* 2012) . It is therefore recommended that such advanced procedures be utilized to give more accurate measurements of these compounds in eggs and other feed stuff.

Iyengar *et al* (1987), reported the synthesis of nitrosamine in macrophage cell lines exposed to antigen of *Escherichia coli* in vitro, other bacteria have also been shown to be able to catalyse formation of nitrosamines from nitrates and nitrites, in the presence of proteins (yolk contains about 40% of the total protein of eggs) (Taylor, 2006). It can also be hypothesized that subclinical infections (systemic or localised) with microorganisms capable of synthesizing nitrosamines such as *E. coli*, may predispose to finding higher detectable amounts of these compounds in eggs from specific farms, although no investigations was carried out to confirm infections.

Furthermore, as nitrosamines have been long established to be present in many feedstuff (Crosby *et al* 1972; Scanlan and Issenberg 1975; Oliveira and Gloria 1995; Lakshmi *et al* 2006) which may be utilized in poultry feed production and ingestion of such feed might lead to the secretion of nitrosamines into eggs.

Freshly laid eggs (as purchased at the various poultry farms) were used in this experiment, and these were stored at room temperature thereby minimizing the interference of heat in the formation of nitrosamines. However, it was also probable that prolonged storage for eggs could potentially result in more bacteria deterioration and concomitant nitrosation and of amines obtained from breakdown of albumin through the action of bacteria and other microorganisms concurrently present within the eggs.

Based on the findings of this study, it could be concluded that nitrosamines and their precursors are present in fresh table eggs that are meant for human consumption in Abeokuta, Nigeria. This is of public health significance. Although there is a likelihood that preformed nitrosamines in eggs maybe degraded back to constituent amines and nitrates or nitrites under the influence of heat and light, there is also the possibility that precursor compounds of nitrosamines (nitrite, nitrates and amines) could under the influence of processing heat induce the formation of toxic nitrosamines. It is therefore recommended that further studies be carried out to determine presence and exposure levels of these nitrosamines in prepared (boiled or fried) eggs.

In addition exact chemical mechanisms/pathways, and predisposing management factors responsible for the dynamics of nitrosamines in eggs require further elucidation.

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