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APPLICATION OF ENZYME-LINKED IMMUNOSORBENT ASSAY FOR AFLATOXIN CONTENT EVALUATION OF DIFFERENT POWDERED MILK AVALABLE IN ANAMBRA STATE, NIGERIA

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ABSTRACT

The application of enzyme-link immunosorbent assay for aflatoxin contamination of 20 different powdered milk samples consumed within Anambra State, Nigeria was carried out primarily to ascertain their health implications. The samples were subjected to Ridascreen ELISA competitive enzyme immunoassay for the quantitative determination of aflatoxins in the milk.All the reagents required for the enzyme immunoassay including statndards were contained in the test kit used. Preparatory samples stages were grinding, extraction, filtration/centrifugation followed by acid mixture digestion process with tablet of selenium catalyst. The proximate analyses of the samples were done via AOAC standard procedures. The results revealed relatively low moisture contents with a range of 1.64 to 4.72%, with sample T recording highest value of 4.72% . The highest value of ash content was observed for sample D (6.57%) while sample R had lowest value of 0.85%. The lowest amount of crude fat was recorded by sample F (2.60%) as against high value of 21.33% for sample B. Crude protein showed least amount in samples A (20.19%) with the highest amount found in sample H (23.67%). The quantification of aflatoxins in the 20 powdered milk samples using ELIZA method revealed relatively low concentration of aflatoxins in the samples and the trend observed had a range of 0.36ppm (sample B) to 0.81ppm (sample F). The average aflatoxin concentration was estimated to be 0.61ppm. Samples C, E and F had mean level of aflatoxin recorded as 0.71ppm while the mean amount implicated for samples A, B, G and K was 0.51ppm. Conclusively, the results of the proximate analysis of the milk samples revealed appreciable occurrence of crude protein and relatively low amount of aflatoxins. The level of aflatoxins in all the samples was found to occur below National Regulatory threshold value of 4ppm, hence all the samples analyzed can be adjudged to be safe for consumption with little precautionary measures for hygiene and preservation.

KEYWORDS:

Aflatoxin, Immunoassay, Enzyme, Crude Protein, Centrifugation, Test kit.

INTRODUCTION

Mycotoxins are naturally occurring toxins mainly produced by molds; the principal classes of mycotoxins include metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, and can be found in various agricultural commodities throughout the food chain [1]. Aflatoxins are toxins produced by fungi such as Aspergillus flavus nd Aspergillus parasiticus which can be found in the milk of cows that ate aflatoxin contaminated crops such as corn etc. Animals that consume aflatoxin contaminated food can transfer the toxins to milk, egg where man can be potentially eposed to health hazard. Presence of aflatoxins in the body system may result into nausea, vomiting, abdominal pain, convulsions and other acute liver related injury. Also, long time accumulation in the body system can lead to hepatocellular carcinoma (Lamanaka *et al*., 2007). Sequel to the above health implication of aflatoxin in the body system, the need to properly investigate milk samples for presence of aflatoxin cannot be overemphasized which necessitated and give credence to this research. The crops that are most prone to contamination are maize, soybeans, and peanuts. When these contaminated commodities are exported to other countries, they spread the contamination, thereby making aflatoxin a worldwide problem (Watson, 1998). When cows, sheep, goats, or other ruminant animals consume feed contaminated with aflatoxins, a metabolized form of aflatoxin will be produced and excreted in milk. The general attraction to the study of mycotoxins started in 1960, when more than 100,000 turkeys died in London, England, after consuming contaminated groundnut meal that was imported from Brazil (Blount, 1961). The diseases that resulted were called "Turkey X disease." *Aspergillus flavus* was later isolated from the groundnut meal, and the toxic agents were named aflatoxin (*A. flavus toxin*) accordingly (Splensly, 1963; Kensler et al., 2011). Subsequent studies resulted in the identification of aflatoxins as the toxicological agent (Hell *et al.*, 2000), which initiated mycotoxicology as a serious and complex problem of food safety.

MATERIALS AND METHOD

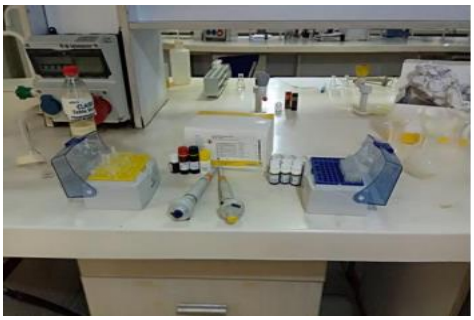
Sample collection

The samples used in this research work were twenty different types of powdered milk which include SMA gold, Lactogen, NAN, Complan, Fort milk, kerryGold, Milksi, Loya Milk, My Boy, Promex, Nunu, Lahda, Olympic Milk, Three crown, Cowbell, Peak milk, Infacare Milk, peak 1,2,3, Dano milk, Good Morning all purchased within Awka metropolis and were labeled samples A - T. We avoideed direct sunlight during all incubation steps, and incubated plates in the dark. We inserted a sufficient number of microtiter wells into the microwell holder for all standards and samples to be run. Using a multichannel pipette set at 200µl, aflatoxin conjugate was pipetted into the microwells to be used. Using the multichannel pipette set at 100µl, aflatoxin standards from 0.00ppb-20.0ppb in ascending order were pipetted into the first 6 microwells containing the conjugate. The multichannel pipette set at 100µl with the prepared samples (filtrates) were pipetted into the microwells starting from well. Followed by multichannel pipette set at 100µl, the aflatoxin standard of concentration 10.0ppb was pipetted into the last well. The thoroughly mixed solutions were transferred from each microwell into a new set of microwell. The microwells were incubated for 15 minutes on the bench and then discarded after the incubation period by washing with deionized water 5 consecutive times, and dried using an absorbent paper towel. The substrate was pipetted into each of the microwells and incubation was done for another 5 minutes. The microwells were arranged and placed in the ELISA Reader for aflatoxin determination. All reagent were brought to room temperature (20^0C-25^0C/ 68^0F-77^0F).

Ridascreen: aflatoxin total was carried out for the quantitative determination of aflatoxins in the samples. A microtiter plate spectrophotometer was used for quantification.

Digestion of samples for proximate analysis: 2g of sample was weighed into a filter paper and transferred with the filter paper into a neat, dried Kjeldahl flask. 25 ml of conc. sulfuric acid was added to the sample in the flask, followed by the addition of two tablets of selenium catalyst or copper catalyst. The mixture in the flask was heated gently in an inclined position until it was digested by the Kjeldahl filament. A clear liquid that is free of black or brown color indicates complete digestion of the mixture.

ELISA Machine for Aflatoxin Analysis.



Accessories for the Preparation of Samples and Test kits

Procedure for determination of protein: The digested mixture in a flask was allowed to cool to room temperature, and the content was diluted with about 200 ml of distilled water. The distillation apparatus (consisting of a 500-ml flask with the stopper carrying a dropping funnel and a splash head adapter as well as a vertical condenser to which a straight delivery tube is attached) was connected. A 50-ml boric acid solution was measured out into a 500-ml conical flask. A few drops of screened methyl red indicator were added and placed on the receiver so that the end of the delivery tubes dipped just below the level of the boric level. A measured quantity of anti-bumping granules was added to the distillation flask. 40 ml of NaOH was added through the dropping funnel to make the liquid distinctly alkaline. About 50 ml of distilled water was added through the funnel, and the mixture was shaken gently to ensure mixing of the contents. The mixture was boiled vigorously until about 200 ml had been distilled off. The distillate was titrated with 0.05M sulfuric acid to a dull blue color. This indicated the endpoint (Vml). Blank titration was also performed using all other reagents except the samples.

The ash, carbohydrate, moisture content, and crude protein were determined by adopting AOAC standard procedure. Carbohydrate can be determined when all the other parameters has been determined. i.e Carbohydrate(by diff) = 100-(moisture% + protein% + fat% +ash%)

RESULT

Table 1: The Proximate analysis on twenty different powdered milk.

Milk Brand	Moisture	Ash content	Crude Fat	Crude Protein
A	2.19±0.18	4.74±0.01	10.67±0.01	20.19±0.01
B	2.99±0.18	5.17±0.01	21.33±0.01	23.45±0.01
C	2.65±0.23	5.96 ±0.01	3.80±0.01	20.86±0.01
D	2.91±0.01	6.57±0.01	20.67±0.01	20.24±0.01
E	1.64±0.13	3.88±0.01	2.60 ±0.01	21.34±0.01
F	2.46±0.18	0.98±0.01	6.00 ±0.01	22.42 ±0.01
G	2.65±0.15	4.93 ±0.01	13.33±0.01	21.67±0.01
H	2.36±0.28	4.78±0.01	20.66±0.01	23.67±0.01
I	2.05±0.01	4.91±0.01	20.66±0.01	20.19±0.01
J	2.83±0.00	5.30±0.01	10.67±0.01	23.13±0.01

K	3.29 ±0.12	5.36±0.01	15.33 ±0.01	23.24±0.01
L	2.22±0.39	2.50 ±0.01	7.33±0.01	22.42±0.01
M	3.62±0.28	2.91±0.01	7.33±0.01	22.90±0.05
N	3.48±0.37	5.32±0.01	16.67±0.01	22.96±0.02
O	2.76±0.10	3.23±0.01	10.00±0.01	22.45±0.01
P	3.42±0.13	4.93±0.01	12.67±0.01	21.45±0.01
Q	3.28±0.01	2.91±0.01	7.32±0.02	22.72±0.34
R	2.67±0.01	0.85±0.01	7.83±0.01	22.36±0.04
S	2.43±0.01	2.85±0.01	8.90±0.01	22.82±0.03
T	4.72±0.09	3.41±0.01	8.67±0.01	21.66±0.01

The results are presented as Mean ± SD.

All the samples were analyzed for moisture, crude protein, crude fat and total ash content. High amount of moisture (4.72%) was observed in Complan Milk whereas low amount (1.64%) in Infacare Milk. In case of crude fat, high amount was observed in KerryGold, Loya Milk, Goodmorning and Olympic with values, 21.33±0.01, 20.67±0.01, 20.66±0.01 respectively. Low amount was observed in Milksi, Infacare Milk, Peak 1, 2, 3 and Lactogen with values, 3.80±0.01, 2.60±0.01, 6.00±0.01 and 7.83±0.01 respectively as shown in figure 1, 2 and Table 1.

Fig 1: The result of proximate evaluation of the samples

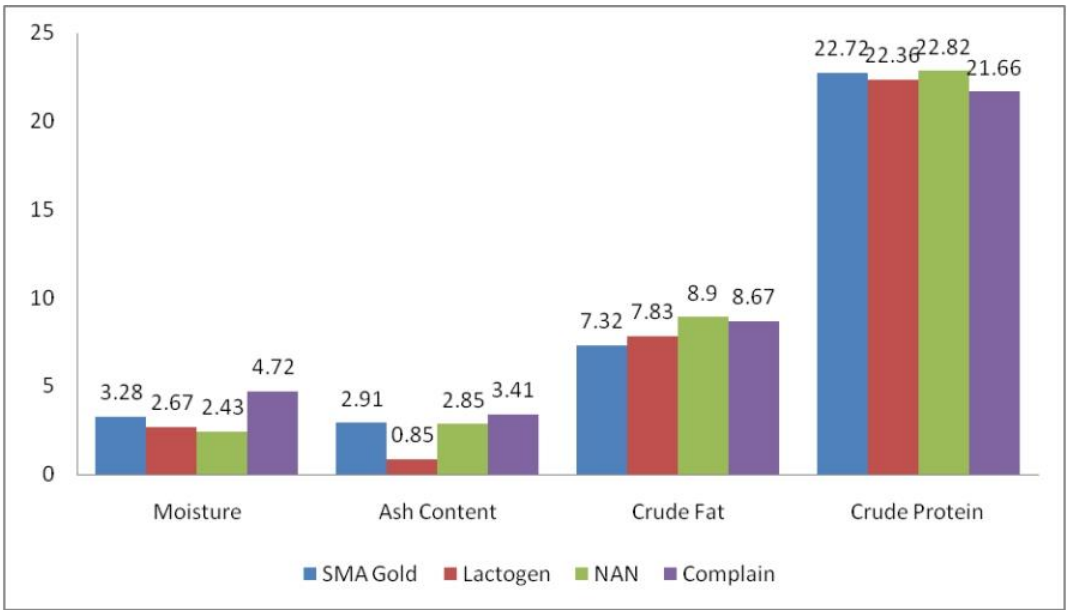


Fig 2: The result of proximate evaluation of the samples.

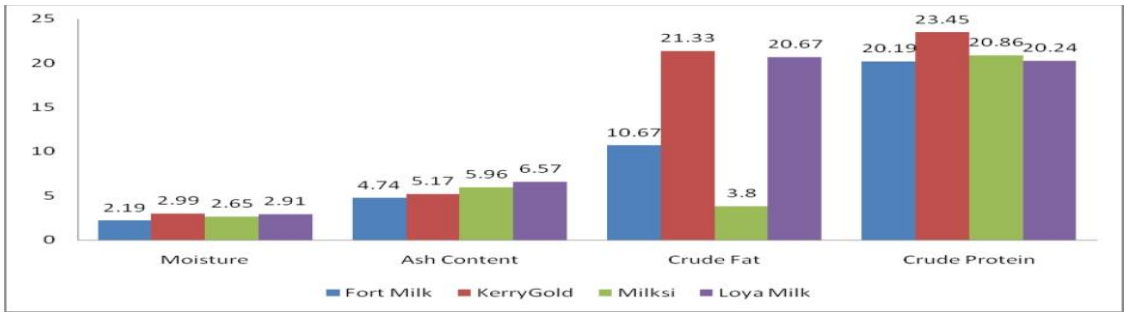


Fig 3: The result of proximate evaluation of the samples.

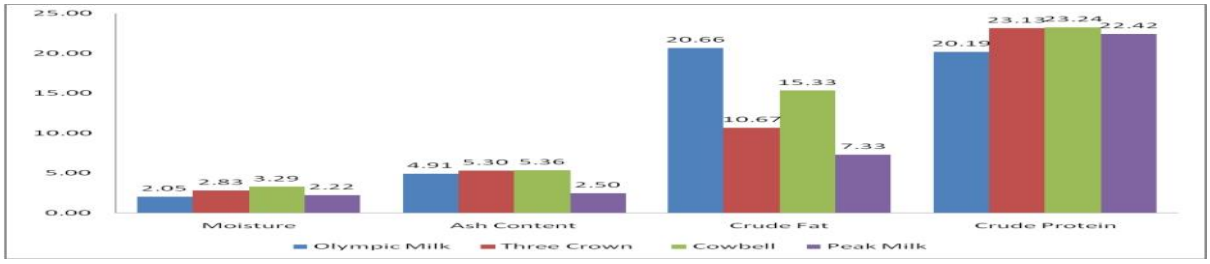
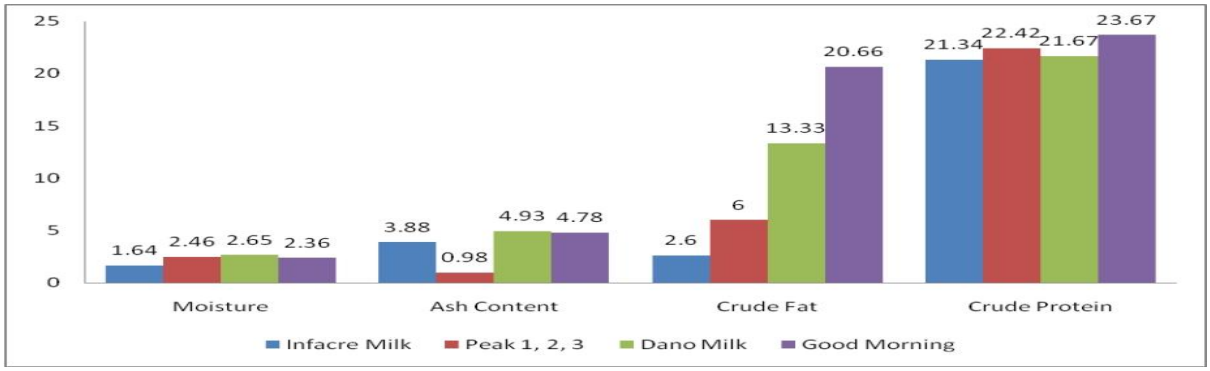


Fig 4: The result of proximate evaluation of the sample



Then aflatoxin content was quantified for all the samples using Enzyme-linked Immunosorbent Assay (ELIZA) method. All the samples analyzed had very low quantities of aflatoxin. None of the samples contained aflatoxin above the national regulatory threshold of 4ppm. This is shown in Table 2.

Table 2: Quantification of aflatoxin in twenty different powdered milk by ELIZA method.

Milk Brand	Aflatoxin content
A	0.52±0.03
B	0.36±0.09
C	0.72±0.06
D	0.62±0.04
E	0.70±0.02
F	0.81±0.03
G	0.51±0.06
H	0.63±0.03
I	0.56±0.04
J	0.56±0.06
K	0.50±0.08
L	0.59±0.03
M	0.58±0.03
N	0.60±0.09
O	0.65±0.13
P	0.65±0.05
Q	0.62±0.02
R	0.56±0.01
S	0.60±0.01

T	0.64±0.04

The results are presented as Mean ± SD.

The aflatoxin content of the milk brands analyzed are within the expected limit (≤ 4ppm). Moisture is an important factor affecting mold growth and aflatoxin production. Jarvis gives a water activity (a_w) value of 0.78 as the minimum and >0.93 as optimum for growth of *A.flavus*. Kiermeier published data on the effect of water content on production of aflatoxin in dried milk. The minimum water content where aflatoxin production occurred was about 52%. This is in agreement with our findings where Infacare Milk and Peak 1, 2, 3 with moisture content of 1.64±0.13 and 2.46±0.18 had the highest aflatoxin content values of 0.70ppm and 0.81ppm respectively. Proper nutritional conditions are also necessary for aflatoxin production. From the results, most of the samples high in crude fat favoured low yields of aflatoxin (Dienner and Davis, 1969). Lie and Marth (1986) found that appreciable amounts of AFB1 and AFG1 were produced when casein was used as a substrate.

CONCLUSION

The survival of a child is predicated on certain factors including nutrition. Globally, and in Africa specifically, poor nutrition contributes to more than a third of under-five death (UNICEF 2008& UNICEF 2010). The Nigeria Demographic and Health Survey (2008) indices show that among young children, malnutrition increases with age, which peaks among children of age bracket 6-8 months, and stunting which is highest among children of age bracket 12-17 months and children of age bracket 18-23 months. Aflatoxin M1 remains a mycotoxin that is yet to be investigated in most of developing countries including Africa. As long as conditions favourable for aflatoxin contamination in food and animal feed are present, AFM1 in milk and milk products will continue to be an issue that needs constant monitoring because of the serious effects it could cause on human health, particularly children. Developing countries compared to developed nations need to develop and implement regulations and control systems that would regulate AFM1 in milk and its products thus ensuring food quality and safety. The coming decade will definitively focus on development and application of new, quick and low cost technology for aflatoxin detection. This would be key to developing strategies that would improve prevention, promotion awareness with regards to fungi and aflatoxins contamination.

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