Determination of aflatoxins by HPLC and the identification of biosynthetic nor-1 gene of aflatoxins in poultry products by PCR assay
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ABSTRACT

Aflatoxins residues in different poultry products were determined using HPLC technique and the expression of biosynthetic nor-1 gene of these aflatoxins by PCR assay. 90 different chicken products were analyzed for presence of aflatoxins (AFB1, AFB2, AFG1 and AFG2) using highly accurate, robust, selective, sensitive and precise HPLC-FLD assay. Incidence of aflatoxins in examined samples showed that the level of AFB1, AFB2 and AFG2 were 10 % for each and of AFG1 was 16.7%. The highest aflatoxins levels of analyzed samples were found in the liver. Expression of nor-1 gene of aflatoxins in polluted poultry products by PCR assay. A Real time PCR (RT-PCR) technique was applied to detect the gene expression of structural nor-1, which catalyzed the first step in the biosynthetic pathway of aflatoxins. The obtained results showed a very strong relationship between the presences of aflatoxins biosynthetic genes as analyzed by multiplex PCR and aflatoxins determination by HPLC/FLD.

Keywords: Aflatoxins; HPLC; nor1 gene; PCR.

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1. INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi such as A.flavus, A.parasiticus, A.ochraceous, F.tritinctum, F.poae and penicillium spp.; aflatoxins, ochratoxins, patulin and T-2 are the most mycotoxins types causing problems and associated with calamity outbreaks through the field (Hassan et al., 2015).

Aflatoxins attracted special attention by their ability to contaminate hundred kinds of poultry and poultry products such as chicken liver, nuggets, wings and thigh. These toxins production ability had been reported in different spp. of genus Aspergillus fundamentally in section Flavigp. (Rodrigues et al., 2009).

Aflatoxins are produced by various spp. of Aspergillus, particularly A.parasiticus and A.flavus (Blesa et al., 2003; Kalcher et al., 2007 and Yu et al., 2008), about 60% of isolates were aflatoxins producer (Razzaghi-Abyaneh et al., 2006). The major aflatoxins are called aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2 (AFB1, AFB2, AFG1 and AFG2) and their two metabolites AFM1 and AFM2, but AFB1 is the most toxigenic one. AFB1 and AFB2 are produced
by *A. flavus*, while *A. parasiticus* produce the four aflatoxins (Dorner, 2004). Aflatoxins are extremely toxic, highly mutagenic and carcinogenic compound, chemically. They are a group of di-fura-roco-marine derivative. The cause why gene expression of aflatoxins producer and non-producer strains is obvious to be fundamental to follow the inculpated strains and its way in the environment. The most established assay for estimation of aflatoxins amounts in food and feed, is the HPLC technique depending on chemical and physical properties of mycotoxins provides an accurate, precise and specific method to determine the aflatoxins concentrations in polluted food and feed. Many researches explain biological, analytical and genetic methods (Hassan et al., 2015). Ochratoxin has four types (A, B, C and D) have been widely detected in food products. Ochratoxin A (OTA) is the most dangerous one. OTA is produced by *A. spp.*, *P. verrucosum* and *P. nordicum* (Frisvad and Samson 2000; Larsen et al. 2001; Abarca et al., 2001 and Castella et al., 2002). Ochratoxin is immunosuppressive, teratogenic and carcinogenic effects; it is considering fertility inhibitor and mutagenic as it can pass placenta (IARC, 1993 and WHO, 2002). OTA has been considered as the major cause responsible for the “Balkan Endemic Nephropath” in humans (Kuiper-Goodman & Scott, 1989).

The development of sensitive, accurate and rapid method for identification of incriminated aflatoxigenic species in foods and feed is main aim to avoid any potential health hazard and control mycotoxins (Valasek and Repa, 2005). Polymerase chain reaction (PCR) and real time Polymerase chain reaction (RT-PCR) have proved to be highly precise and rapid bimolecular techniques for genes expression or their transcripts participate in biosynthesis of mycotoxins by *A. flavus* and *A. parasiticus* (Fente et al. 2001; Somashekar et al., 2004 and Scherm et al., 2005). The aim of the present study was to quantity Aflatoxins in 90 different poultry products by HPLC assay and to detect structural *nor-1* gene expression by RT-PCR in contaminated samples. *Nor-1* gene is a key structural gene in biosynthetic pathway of aflatoxins it encodes an enzyme that catalyze the conversion of the first stable aflatoxin biosynthesis intermediate norsolorininic acid to averantin.

2. Materials and methods

*Materials:*

*Samples:*

A grand total of 90 different chicken products (chicken wings, chicken nuggets, chicken liver and chicken thigh) "15 of each". The samples were collected from different supermarkets, Elsharqia. The samples were placed in plastic bags then transferred in an ice box to the lab. without delay. The collected samples were analyzed for the presence of aflatoxins (AFB1, AFB2, AFG1 and AFG2)

*Material used for extraction of DNA:*

QIAamp DNeasy Plant Mini kit Catalogue no.69104: DNeasy Plant Kits provide a fast and easy way to purify DNA from plant and fungal tissue. Up to 100 mg of tissue can be processed using the DNeasy Plant Mini Kit.
Ethanol 96%: Applichem

PCR Master Mix used for conventional PCR:
Emerald Amp GT PCR mastermix (Takara) Code No. RR310A Contains: (Emerald Amp GT PCR mastermix (2x premix) and PCR grade water).

Oligonucleotide primers used in conventional PCR:
They have specific sequence and amplify a specific product as shown in Table (1).

Table 1: Percent of antibiotic residues positive beef samples.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aflR</td>
<td>AAC CGC ATC CAC AAT CTC AT</td>
<td>800 bp</td>
<td>Bintvihok, et al., 2016</td>
</tr>
</tbody>
</table>

DNA Molecular weight marker:
Gene ruler 100 bp DNA ladder (cat. no. SM0243) supplied from Fermentas.

Number of bands: 10
Size range: 100-1000 bp.

Material used for agarose gel electrophoresis:
Agarose 1.5% (Sambrook et al., 1989): A multi-purpose, high gel strength agarose suitable for a wide range of molecular biology techniques. As it has high gel strength and exclusion limits, multi ABgarose could effectively separate large DNA fragments with reduced running times. This in turn means less band diffusion, a problem often associated with long running times. It was prepared as follow: (1.5 g Agarose powder (ABgene) and 100mL TBE.

Ethedium bromide solution 10 mg / ml (Sambrook et al., 1989): 10 mg Ethedium bromide powder (Sigma) then add 1.0 ml sterile DDW, mix and stored covered at 4°C. It was added to melted agarose to reach a final concentration of 0.1-0.5 μg/ml.

Tris borate EDTA (TBE) electrophoresis buffer (1x) (WHO, 2002): 10.78 gm of Tris buffer, 5.5 gm Boric acid, Fluka) and 0.82 gm EDTAdiNA, Winlab). It was completed up to 1 liter with DDW, pH was checked up. If the pH was out of the range of 8-8.6, a new solution was prepared again. Any change in ion concentration would affect the DNA migration through the gel.

Methods:
Quantification of aflatoxins by HPLC:
Preparation of samples for residue study:
At the time of assay, partially thaw frozen tissues at room temperature (23oc) for 30 minutes and blend in a food processor for 20-30 seconds at high speed to obtain a uniform paste-like consistency.

Extraction and clean-up: (Brera et al., 2011):
1) Weight 5gm into a polypropylene tube.
2) Add 0.5 g of sodium chloride and 25 mL of methanol (80%).
3) Mix at high speed for 3 min. Filter the extract through filter paper.
4) Pipette 3 mL of filtrate and dilute with 3 mL of PBS. Mix thoroughly.
5) Centrifuge the diluted sample for 10 min at 10,000 rpm.
6) Apply 4 mL of the diluted sample to the conditioned immunoaffinity column (IAC) and wash with 1 mL of PBS. Elute mycotoxins in a 2-step procedure. First, apply 1.0 mL methanol to the IAC and let it flow through under gravity. Collect eluate in calibrated 5 mL volumetric flask. Wait 1 min and apply a second portion of 1.0 mL.
methanol. Pass air through the column to collect the remaining few drops.
7) Fill the 5 mL volumetric flask to the mark with DW, mix well, and store the sample at +4°C prior to analysis.

Validation of analytical method:
The method was validated according to ICH, 2005 guidelines

Limit of Detection (LOD) and Quantification (LOQ): They were calculated from Based on standard deviation of intercept (S) and slope (b) LOD = 3.3×S/b and LOQ = 10×S/b

System Suitability Test: Relative standard deviations of the retention time, tailing factor, number of theoretical plates, peak area, and capacity factor were measured to test system suitability

Accuracy and recovery: The standard additions at different concentrations are prepared by adding known quantities of AFs & OCA. Those samples are analyzed against standard solutions of same concentrations. The accuracy is then calculated from the test results as a percentage recovery.

Precision: It is determined using 5 replicates of each standard solutions and evaluating the relative standard deviation of repeatability (RSD %).

Selectivity and specificity: Verification of selectivity is conducted by evaluating the standard addition on blank matrix. Acceptance criteria: there is no interference between the pure standard and peaks of any impurities or extracted solvents.

Irradiation effects on mycotoxins reduction: (Aziz et al., 2004):

Chicken product samples of high positive results were packed into polyethylene pouches and irradiated, in three replications, with doses of 6, 8 and 10 KGY by using 60CO gamma rays (Gamma Cell mold 220 apparatus, NCRRT, Nasr City, Cairo, Egypt).

Expression of nor-1 gene of aflatoxins and ocrA gene of ochratoxin in polluted poultry products by a real time PCR (RT-PCR) technique:

Extraction of DNA (QIAamp DNeasy Plant Mini kit instructions):
1) Freeze 100 mg of the fungal tissue at – 80°C/ 24 hrs for later processing.
2) Add fungal material and a tungsten carbide bead to a 2 ml safe-lock tube. 400 μl Buffer AP1 and 4 μl RNase A stock solution (100 mg/ml) were added. Tubes were placed into the adaptor sets, which are fixed into the clamps of the TissueLyser. Disruption was performed in two 1–2 min. at high-speed (20–30 Hz) shaking steps.
3) The mixture was incubated for at 65°C/ 10 min and mixed 2 or 3 times during incubation by inverting tube.
4) Add 130 μl Buffer P3 to the lysate, mixed, and incubated for 5 min on ice.
5) The lysate was centrifuged for 5 min at 14,000 rpm.
6) The lysate was pipetted into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuged for 2 min at 14,000 rpm.
7) The flow-through fraction from step 16 was transferred into a new tube without disturbing the cell-debris pellet.
8) Add 1.5 volumes of Buffer AW1 to the cleared lysate, and mixed by pipetting.
9) Pipette 650 μl of the mixture from step 8 (including any precipitate that was formed) into the DNasey Mini spin column placed in a 2 ml collection tube and centrifuged for 1 min 8000 rpm and the flow-through was discarded.

10) Step 9 was repeated with the remaining sample. The flow-through and collection tube were discarded.

11) The DNasey Mini spin column was placed into a new 2 ml collection tube.

12) Add 500 μl Buffer AW2 was added to the DNasey Mini spin column, and centrifuged for 2 min at 14,000 rpm to dry the membrane.

13) The DNasey Mini spin column was transferred to a 1.5 ml or 2 ml microcentrifuge tube, and 50 μl Buffer AE were directly pipette onto the DNasey membrane. It was incubated for 5 min at room temperature (15–25°C), and then centrifuged for 1 min at 8000 rpm to elute.

14) Step 13 was repeated once.

Preparation of conventional PCR MasterMix:
(According to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit as shown in table (2).

Table2: Components of PCR mastermix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emerald Amp GT PCR mastermix (2x premix)</td>
<td>12.5 μl</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>4.5 μl</td>
</tr>
<tr>
<td>Forward primer (20 pmol)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Reverse primer (20 pmol)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>6 μl</td>
</tr>
</tbody>
</table>

Cycling conditions of the primers during ePCR:
Temperature and time conditions of the two primers during PCR are shown in Table (3).

Table3: Cycling conditions of the different primers during conventional PCR.

<table>
<thead>
<tr>
<th>No. of cycle</th>
<th>Final extension</th>
<th>Extension</th>
<th>Annealing</th>
<th>Secondary denaturati on</th>
<th>Primary denaturati on</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>95°C</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td>10 min.</td>
<td>1.40 min.</td>
<td>1.25 min.</td>
<td>50°C</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>94°C</td>
<td>5 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 sec.</td>
<td></td>
</tr>
</tbody>
</table>

DNA Molecular weight marker:
The ladder was mixed gently by pipetting up and down. 6 μl of the required ladder were directly loaded.

Agarose gel electrophoreses (Sambrook et al., 1989) with little modification:
Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70°C, then 0.5μg/ml ethedium bromide was added and mixed thoroughly.
The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization.
The comb was then removed, and the electrophoresis tank was filled with TBE buffer. Twenty μl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet.
The gel was photographed by a gel documentation system and the data was analyzed through computer software.

3. RESULTS

Quantification of aflatoxins by HPLC:
Intra-lab validation:
The results of mycotoxins assay validation were illustrated in table (1), showing that the used method with high accuracy and extremely precise method as the Intra-day precision (RSD %) not exceed 1.81% (< 2% that recommended by USP, 2017) and the inter-day precision not exceed 2.63% (< 6% that recommended by ICH, 2005).

There were high resolution of chromatograms, it is showed in the results as the retention time of Afla -G2, -G1, -B2, -B1 and OTA standards at 0.803, 1.002, 1.901, 2.713 and 7.803 min (fig. 1).

**Selectivity and specificity:** As shown in fig (1& 2), HPLC Chromatograms of mycotoxins pure standards and spiked matrix at different levels, showing no matrix interferences were observed on the chromatograms and no interfering peaks were obtained with the same retention times (RT) of mycotoxins peaks.

**Mycotoxins residues in different chicken products:**

Due to the toxicity and carcinogenicity impact of mycotoxins residues on human public health; our study includes detecting the residues on mycotoxins (Aflatoxins types; B1, B2, G1, G 2) and Ocratoxin A (OTA) in different chicken products samples.

The data represented in table (2) showing Incidence of mycotoxins in different chicken products samples showed that the level of Afla B1, Afla B2 and Afla G2 were 10 % for each and of Afla G1 and Ochra A were ranged from 10 to 16.7% for each.

**Mycotoxins residues in different chicken products after exposure to gamma irradiation:**

In all analyzed samples, there are a positive correlation between the increase of gamma irradiation dose applied to the samples and the level of reduction of total mycotoxins present in these samples. It is clear in table (9).

**Expression of the structure genes (nor-1) of aflatoxins by RT-PCR:**

The results showed that the DNA extract of the isolates *A.flavus* and *A.parasiticus* have the nor-1 gene in analyzed samples. Represented electrophoresis band pattern of Real time PCR products shown in (Fig 5). The RT-PCR results obtained with genomic DNAas a template indicated that all tested aflatoxigenic isolates expressed nor-1 gene with significant expression levels in chicken products. Positive correlation was noted between mean expression level of nor-1 and the amount of Aflatoxins production by isolates.

The observed differences in aflatoxins producing ability (Table 4) could be explained by differences in expression of aflatoxins biosynthetic and regulatory genes between toxigenic isolates from chicken products due to distinction in environmental circumstances. Transcriptional expression of the key aflatoxins biosynthetic gene nor-1 gene which encoding an enzyme catalyze the conversion of the first stable aflatoxins biosynthesis intermediate norsolorinic acid to averatin. *Nor-1* gene was investigated by Real time PCR and as shown in (fig. 5) have expressed the nor-1 gene in different levels.
Table 4: Validation sheet of mycotoxins.

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Type of sample</th>
<th>Positive samples</th>
<th>Mean ± SE (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afla B1</td>
<td>Nuggets</td>
<td>3</td>
<td>5.03 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3</td>
<td>16.3 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>Thigh</td>
<td>3</td>
<td>4.95 ± 0.84</td>
</tr>
<tr>
<td>Afla B2</td>
<td>Nuggets</td>
<td>3</td>
<td>1.2 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3</td>
<td>10.1 ± 0.99</td>
</tr>
<tr>
<td></td>
<td>Thigh</td>
<td>3</td>
<td>0.84 ± 0.11</td>
</tr>
<tr>
<td>Afla G1</td>
<td>Nuggets</td>
<td>5</td>
<td>0.28±0.04</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3</td>
<td>1.58 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>Thigh</td>
<td>3</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>Afla G2</td>
<td>Nuggets</td>
<td>3</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3</td>
<td>0.56 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>Thigh</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not detected

LOD (AFG2) = 0.014 ppb
Table 6: Incidence of mycotoxins residues in chicken products samples.

<table>
<thead>
<tr>
<th>Percentage (%)</th>
<th>Positive samples</th>
<th>Samples no.</th>
<th>Type of sample</th>
<th>Mycotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>30</td>
<td>Chicken wings</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken nuggets</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken liver</td>
<td>Afla B1</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken thigh</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>30</td>
<td>Chicken wings</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken nuggets</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken liver</td>
<td>Afla B2</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken thigh</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>30</td>
<td>Chicken wings</td>
<td></td>
</tr>
<tr>
<td>16.7</td>
<td>5</td>
<td>30</td>
<td>Chicken nuggets</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken liver</td>
<td>Afla G1</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken thigh</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>30</td>
<td>Chicken wings</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken nuggets</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken liver</td>
<td>Afla G2</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>30</td>
<td>Chicken thigh</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken wings</td>
<td></td>
</tr>
<tr>
<td>16.7</td>
<td>5</td>
<td>30</td>
<td>Chicken liver</td>
<td>Ochra A</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Chicken thigh</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Results of mycotoxins residues in positive chicken products samples.

<table>
<thead>
<tr>
<th>Range (µg/kg)</th>
<th>Mean ± SE (µg/kg)</th>
<th>No. of +ve samples</th>
<th>Type of sample</th>
<th>Type of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max.</td>
<td>Min.</td>
<td>ND</td>
<td>ND</td>
<td>Chicken wings</td>
</tr>
<tr>
<td>6.19</td>
<td>3.96</td>
<td>5.03 ± 0.65</td>
<td>3</td>
<td>Chicken nuggets</td>
</tr>
<tr>
<td>17.9</td>
<td>15</td>
<td>16.3 ± 0.85</td>
<td>3</td>
<td>Chicken liver</td>
</tr>
<tr>
<td>6.63</td>
<td>4.1</td>
<td>4.95 ± 0.84</td>
<td>3</td>
<td>Chicken thigh</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Chicken wings</td>
</tr>
<tr>
<td>1.87</td>
<td>0.8</td>
<td>1.2 ± 0.34</td>
<td>3</td>
<td>Chicken nuggets</td>
</tr>
<tr>
<td>11.8</td>
<td>8.4</td>
<td>10.1 ± 0.99</td>
<td>3</td>
<td>Chicken liver</td>
</tr>
<tr>
<td>1.06</td>
<td>0.68</td>
<td>0.84 ± 0.11</td>
<td>3</td>
<td>Chicken thigh</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Chicken wings</td>
</tr>
<tr>
<td>0.4</td>
<td>0.16</td>
<td>0.28 ± 0.04</td>
<td>5</td>
<td>Chicken nuggets</td>
</tr>
<tr>
<td>2.02</td>
<td>1.21</td>
<td>1.58 ± 0.24</td>
<td>3</td>
<td>Chicken liver</td>
</tr>
</tbody>
</table>
Determination of aflatoxins by HPLC and the identification of biosynthetic nor-1 gene by PCR assay

Table 8: Distribution of mycotoxins in different chicken products.

<table>
<thead>
<tr>
<th></th>
<th>Thigh</th>
<th>Nuggets</th>
<th>Liver</th>
<th>Mycotoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.63*</td>
<td>1.34*</td>
<td>5.37</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>0.61</td>
<td>0.45</td>
<td>1.53</td>
<td>SE</td>
</tr>
</tbody>
</table>

Table 9: Results of mycotoxins in different chicken products after exposure to gamma irradiation.

<table>
<thead>
<tr>
<th>Irradiation dose (kGy)</th>
<th>(Control conc.) ppb (Reduction %)</th>
<th>Sample type</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 (33.7%)</td>
<td>4.97 (19.7%) 5.97 (3.6%)</td>
<td>AFB1</td>
</tr>
<tr>
<td>13.4 (25%)</td>
<td>14.74 (17.5%) 16.8 (6%)</td>
<td></td>
</tr>
<tr>
<td>0.46 (26.6%)</td>
<td>0.51 (16.5%) 0.57 (7.5%)</td>
<td></td>
</tr>
<tr>
<td>1.13 (39.6%)</td>
<td>1.3 (20%) 1.74 (6.8%)</td>
<td></td>
</tr>
<tr>
<td>7.14 (39.5%)</td>
<td>9.12 (22%) 11.5 (6%)</td>
<td></td>
</tr>
<tr>
<td>0.55 (48.4%)</td>
<td>0.83 (22%) 0.95 (10%)</td>
<td></td>
</tr>
<tr>
<td>0.18 (55%)</td>
<td>0.24 (40%) 0.34 (15%)</td>
<td></td>
</tr>
<tr>
<td>0.81 (60%)</td>
<td>1.41 (30%) 1.72 (15%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 10: Total mycotoxins after gamma irradiation exposure.

<table>
<thead>
<tr>
<th>Irradiation dose (kGy)</th>
<th>(Control) conc. ppb ± SE</th>
<th>Mycotoxin type</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>5.99 ± 3.85 (27.22%)</td>
<td>6.74 ± 4.2 (18.1%)</td>
<td>7.78 ± 4.77 (5.47%)</td>
</tr>
<tr>
<td>2.94 ± 2.11 (40.12%)</td>
<td>3.75 ± 2.69 (23.63%)</td>
<td>4.73 ± 3.92 (3.6%)</td>
</tr>
<tr>
<td>0.34 ± 0.24 (63.04%)</td>
<td>0.6 ± 0.4 (34.78%)</td>
<td>0.78 ± 0.47 (15.22%)</td>
</tr>
<tr>
<td>0.04 ± 0.02 (90.24%)</td>
<td>0.22 ± 0.18 (46.34%)</td>
<td>0.36 ± 0.29 (12.2%)</td>
</tr>
<tr>
<td>0.28 ± 0.2 (73.83%)</td>
<td>0.57 ± 0.18 (46.73%)</td>
<td>0.81 ± 0.2 (24.3%)</td>
</tr>
<tr>
<td>2.05 ± 1.07 (37.88%)</td>
<td>2.53 ± 1.21 (23.33%)</td>
<td>3.07 ± 1.4 (6.97%)</td>
</tr>
</tbody>
</table>

Table 10: Total mycotoxins after gamma irradiation exposure.
Determination of aflatoxins by HPLC and the identification of biosynthetic *nor-1* gene by PCR assay

**Fig. 1.** Chromatogram of AF-G2, -G1, -B2 and -B1 standards at a conc. of (0.03, 0.075, 0.02 and 0.015 ppb; respectively).

**Fig. 2.** Chromatogram showing Afla-G2, -G1, -B2, -B1 and OTA spiked matrix at a conc. of (0.015, 0.06, 0.03, 0.03 and 0.02 ppb; respectively).

**Fig. 3.** Standard curve of AFB1.

**Fig. 4.** Standard curve of AFB2.
**4. DISCUSSION**

*Mycotoxins residues in different chicken products:*

Incidence of mycotoxins in different chicken products samples showed that the level of aflatoxin B1, aflatoxin B2 and aflatoxin G2 were 10% for each and of aflatoxin G1 and ochratoxin A were ranged from 10 to 16.7%. Our study showing that the highest mycotoxins residues (sum. of AFB1, AFB2, AFG1, AFG2 and OTA) were found in chicken liver (5.37 ± 1.53; 64%), followed by chicken thigh (1.63 ± 0.61; 20%) and chicken nuggets (1.34 ± 0.45; 16%) and finally chicken wings (not detected). Ochratoxin A residue was found in chicken liver (1.15 ± 0.12) higher than chicken nuggets (0.86 ± 0.09) followed by thigh samples (0.5 ± 0.07). The results refer to that the highest concentration of examined mycotoxins were present in liver as the liver is the harbor site of mycotoxins residues. These finding agree with of those obtained by (Dragan et al., 2011) in Serbia who recorded that the incidence of OTA in chicken liver, kidney and gizzard samples from chicken was 38.33%, 28.3% and 26.6%, respectively, with levels ranging from 0.14 to 3.9 ng/g in liver, 0.1 to 7.02 ng/g in kidneys and 0.25 to 9.94 ng/g in gizzard. In Czech Repulic, reported that ochratoxin residues was 0.12 ppb in chicken meat. Liver is the primary organ involved in mycotoxin detoxification, which is probably the reason why it was affected much more than the kidneys (Yang et al., 2013)

Our data are agree with those reported with Hassanin et al., 2016. They found that chicken burger (1.63 ppb), followed by chicken nuggets (1.04 ppb), luncheon (0.98 ppb) and frankfurter (0.82 ppb), chicken thigh (0.68 ppb), pannet (0.43ppb) and finally chicken wings (0.29 ppb). While the incidence is so lower than that reported in Egypt by Nahed et al., 2016 who reported that incidence of ochratoxin in chicken burger and luncheon was 70% and 80%, respectively. The residue of ochratoxin was very low in their study, where OTA residues were 0.277 and 0.243 ppb in chicken burger and luncheon, respectively. This could be explained by the fact that in our work we used HPLC-FLD which consider highly precise and accurate technique for determination of mycotoxins residues.

The obtained results were agreed with those achieved by Resanović, 2000 and Saeed et al., 2003 who declared that however aflatoxins residues found in liver, muscles, stomach, kidneys, adipose tissue and meat but liver is
the harbor site of aflatoxin residues. In the same line the results agreed with those obtained by Bennett and Klich, 2003, Miliţă et al., 2010, Herzallah, 2013, Hasanen et al., 2016 and Darwish et al., 2016 who reported that the highest concentration of AFB1 and total aflatoxins were in liver higher than in kidneys than in gizzard, while the lowest concentrations were in thigh and breast. The present results are completely disagreed with those achieved by Abo El-Yazeed et al., 2015 who detected that the residual concentrations were higher in breast than in liver.

Markov et al., 2013 from Croatia reported that mycotoxins were detected in 64% of 90 meat samples analyzed, and found that 10% of the samples were contaminated with AFB1, with a maximum AFB1 level of 3.0 mg/kg. Using different testing systems, Herzallah, 2009 found levels of total aflatoxins in imported and fresh meat samples collected during March ranged from 0.15 to 6.36 μg/kg. In a review study, Rodriguez-Amaya and Sabino, 2002 from Brazil found variable frequency of AFB1 contamination in chicken liver samples; with positivity at ~50% of samples tested, and maximum mean level 3.2 μg/kg.

Bintvihok et al., 2002 found that aflatoxin B1 and its metabolite residues were detected in liver (0.15 ppb), muscle (not detected) of domestic fowls after fed them with aflatoxin B1 (3 ppm) for seven days, samples were purified with a Sep-Pak florisil and quantified by HPLC.

Arulmozhi et al. 2002 reported that broiler chicks exposed to aflatoxin (100 ppb) for six weeks, aflatoxin residues detected on 45th day in liver, kidney and muscle by HPLC ( 6.6, 3.2 and 5.1 ng/g respectively)

Broiler chicks of 7, 14 and 28 days of age exposed to different dietary levels of AFB1 (1600, 3200 and 6400 μg/kg) for 7 days. Maximum AFB1 residue (6.97 and 3.27 ng/g in liver and muscle respectively) concentration was high in young age birds those kept on high AFB1 ration. After withdrawal of AF contaminated rations, residues clearance was slow and AFB1 was detectable in liver and muscles of birds for longer duration in younger birds fed with high AFB1 dietary levels (Zahid et al., 2010)

Yang et al., 2012 conducted an experiment where broilers fed daily with corn naturally contaminated with AFB1 and AFB2 (134 and 23.6 μg/kg respectively) and aflatoxins extracted by AflaStar™ R immunoaffinity columns and determined by HPLC/MS/MS technique. AFB1 residues in livers and breast muscles were at levels of 0.137 and 0.016 μg/kg, respectively.

Fan et al., 2013 conducted an experiment where broilers were fed with a moldy peanut meal naturally contaminated with aflatoxins (AFB1 330 μg/kg) for 42 days and aflatoxin residues in liver and muscle samples were detected by HPLC-FD with postcolumn photochemical derivation as samples subjected to immunoaffinity columns, where AFB1 in liver was 0.24 μg/kg, however no residues were observed in muscles.

Deng et al., 2010 reported that long term exposure of Tilapia fish with different levels of AFB1 (19, 85, 245, 638, 793 and 1641 μg/kg) for 20 weeks, resulted the aflatoxicosis in a dose- and duration-dependent manner,
also aflatoxin residues detected only in liver but not in edible flesh. A residue of 11 μg/kg in liver from 40 chickens having been fed 50 μg of ochratoxin per kg of feeding stuffs (Micco et al., 1987) also points in the same direction. A result of these estimates which is worth emphasizing is that the ochratoxin concentration in blood is clearly lower than that found in pigs. The highest levels were found in liver and kidney. Meat/muscle had relatively low levels.

Iqbal et al., 2014 from Pakistan, using reverse phase High Performance Liquid Chromatography (HPLC) with fluorescence detection, documented that 35% of chicken meat samples were positive for aflatoxins, with the maximum level of AFB1 and total aflatoxins found in the livers 2.98 ± 0.76 and 3.23 ± 0.82 μg/kg, respectively. El-Desouky et al., 2014 from Egypt, using immunoaffinity column with HPLC, reported the presence of AFB1 in 45, 32, and 25% of 60 chicken livers, gizzards, and hearts in their study samples, with an overall maximum level of 2.24 μg/kg. Most of these previous findings are in complete agreement with the findings of the present study, although with different tissues and species.

Our data are in the same line with Hassanin et al., 2016. They found that chicken burger (1.63 ppb), followed by chicken nuggets (1.04 ppb), luncheon (0.98 ppb) and frankfurter (0.82 ppb), chicken thigh (0.68 ppb), pannet (0.43 ppb) and finally chicken wings (0.29 ppb). While the incidence is so lower than that reported in Egypt by Nahed et al., 2016 who reported that incidence of ochratoxin in chicken burger and luncheon was 70% and 80%, respectively. The residue of ochratoxin was very low in their study, where OTA residues were 0.277 and 0.243 ppb in chicken burger and luncheon, respectively. This could be explained by the fact that in our work we used HPLC-FLD which consider highly precise and accurate technique for determination of mycotoxins residues.

Our results are disagree with Hanif et al., 2012 who found that The highest OTA levels were detected in serum > kidneys > liver of OTA treated groups of broilers after fed with two levels (500 and 1000 ppb) of ochratoxin A (OTA) and Tissue distribution in pigs, rats, chickens and goats generally follows the order kidney > liver > muscle > fat (Harwig et al., 1983), or in some recent studies kidney > muscle > liver > fat (Mortensen et al., 1983; Madsen et al., 1982), and completely disagreed with those achieved by Abo El-Yazeed et al., 2015 who detected that the residual concentrations were higher in breast than in liver.

Mycotoxins residues in different chicken products after exposure to gamma irradiation:

In all analyzed samples, there are a positive correlation between the increase of gamma irradiation dose applied to the samples and the level of reduction of total mycotoxins present in these samples, however, the maximum reduction percentage of mycotoxins were achieved at 10 kGy; it reaches 37.88 % for total mycotoxins, 27.22 % for Afla B1, 40.12 % for Afla B2, 63.04 % for Afla G1, 90.24 % for Afla G2 and 73.83 % for OTA. These results agree with Ghanem et al., 2008 who found that the dose of 10 kGy percentages of AFB1 degradation reached highest values at 58.6, 68.8, 84.6, 81.1 and 87.8% for peanuts,
peeled pistachios, unpeeled pistachios, corn and rice samples, respectively. %. Their results indicate the possibility of using gamma irradiation as a means of degradation of AFB1 in food and feed crops to levels lower than the maximum allowed levels. While Vita et al., 2013 investigated the effect of gamma irradiation dosages, ranging from 0 to 15 kGy and the reduction of mycotoxins concentration in almond samples. The maximum reduction was found at 15 kGy and it was 19.25%, 10.99%, 21.11%, 16.62%, and 23.9% for AFB1, AFB2, AFG1, AFG2 and OTA respectively. Results showed that gamma radiations even at 15 kGy, were not effective in completely destroying aflatoxins and ochratoxin A.

Some researchers reported a significant reduction and in many cases the complete elimination of mycotoxins in various foods; Jalili, et al., 2010 who studied Gamma ray was applied to reduce mycotoxins, i.e. ochratoxin A (OTA) and aflatoxins B1, B2, G1 and G2 (AFB1, AFB2, AFG1 and AFG2) in black pepper. The maximum reduction was found at 60 kGy which was 52%, 43%, 24%, 40% and 36% for OTA, AFB1, AFB2, AFG1 and AFG2, respectively. Results showed the gamma rays even at 60 kGy were not effective in completely destroying of ochratoxin and aflatoxins. These results were in agreement with the results obtained by Prado et al., 2003 who found that even at a dose of 30 kGy in peanuts, the percentage of reduction achieved was about 61% and the destruction rate was nearly stable after the dose of 15 kGy. Also, the results were in agreement with Farage et al., 1995 who found that an 83% reduction of aflatoxin after a 20-kGy dose of γ-irradiation of yellow corn and peanuts was achieved. On the contrary, Aziz and Youssef, 2002 found that the dose of 20 kGy was sufficient to destroy completely AFB1 in peanuts, yellow corn, and cottonseed meal.

Herzallah et al., 2008 mentioned that the degradation rate increased with increased irradiation dose. For example, the concentrations of the total aflatoxin were 860 and 630 μg/kg after irradiation doses of 5 and 25 kGy, respectively, compared with the control treatment of 965 μg/kg. The percentages of the reduction of aflatoxins achieved were 40.1 and 42.7% after a dose of irradiation of 25 kGy in T3 for the total and B1 aflatoxins, respectively.

Jalili et al., 2012 investigated the efficacy of gamma radiation (⁶⁰Co) for decontaminating ochratoxin A (OTA) and aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) residues in artificially contaminated black and white pepper samples. Mycotoxin levels were determined by high-performance liquid chromatography (HPLC) after immunoaffinity column (IAC) chromatography. Both the gamma irradiation dose and moisture content showed significant effects (P<0.05) on mycotoxin reduction. The maximum toxin reductions, found at 18% moisture content and 30 kGy, were 55.2%, 50.6%, 39.2%, 47.7% and 42.9% for OTA, AFB1, AFB2, AFG1 and AFG2, respectively.

Our results are in complete disagreement with findings obtained by Hooshm and Kloopenstein, 1995 and Feuell, 1966 found that different radiation doses of 5, 7.5, 10 or 20 kGy did not significantly affect aflatoxin
B1 when applied to spiked grain samples, and the residual toxins were measured using an enzyme linked immunosorbent assay (ELISA).

Expression of nor-1 gene of aflatoxins and ocrA gene of ochratoxin polluted poultry products by a real time PCR (RT-PCR) technique:
A multiplex polymerase chain reaction (PCR) strategy was established for rapid identification of mycotoxigenic fungi (Sadhasivam et al., 2017).

The RT-PCR results obtained with genomic DNA as a template indicated that all tested aflatoxigenic isolates expressed nor-1 gene with significant expression levels in chicken products. Positive correlation was noted between mean expression level of nor-1 and the amount of Aflatoxins production by isolates.

The observed differences in aflatoxins producing ability could be explained by differences in expression of aflatoxins biosynthetic and regulatory genes between toxigenic isolates from chicken products due to distinction in environmental circumstances. Transcriptional expression of the key aflatoxins biosynthetic gene nor-1 gene which encoding an enzyme catalyze the conversion of the first stable aflatoxins biosynthesis intermediate norsolorinic acid to averatin. Nor-1 gene was investigated by Real time PCR as the nor-1 gene expressed in different levels. These result was in agreement with that obtained by Yousefi et al., 2009; Iheanacho, 2012 and Passon et al., 2010 who found that A.oryzea and A.niger expressed the nor-1 gene and with Mayer et al., 2003 who found that A.Oryzea expressed expressed the nor-1 gene, and disagree with Mahmoud, 2015 found that A.flavus expressed (nor-1) and other strains expressed a regulatory alfR. Moreover, Cruz and Buttner, 2008 found that A.flavus have alfR gene and expressed it with different levels. While, Schermet al., 2005 stated that there are some other genes, alfR and alfQ present in toxigenic strains of A.flavus and A.parsiticus controlled in aflatoxin biosynthesis

5. REFERENCES


Determination of aflatoxins by HPLC and the identification of biosynthetic nor-1 gene by PCR assay


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Determination of aflatoxins by HPLC and the identification of biosynthetic nor-1 gene by PCR assay


Dina et al. (2019). BVMJ-36(2): 161-186


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