Natural Co-Occurrence of Aflatoxins, Cyclopiazonic Acid, and their Production by Aspergillus Flavus Isolates from Corn Grown in Egypt

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Abstract

Mycotoxins are metabolites of fungi that can adversely affect animal and human health. Mycotoxins can be produced in corn during storage or processing, but are most frequently associated with fungal infection that occur before harvest. Sixty corn samples were purchased from retail markets from Cairo and Giza Governorates in summer and winter seasons from Egypt. The samples were examined for dominants fungi and mycotoxins Aflatoxins (AFS) and cyclopiazonic acid (CPA) and their Aspergillus flavus production. AFS and CPA were determined by high performance liquid chromatography (HPLC). The results indicated that, the dominants fungi genera were Aspergillus, Fusarium, Penicillium and Alternaria in all corn samples in summer and winter seasons. From 239 of Aspergillus genus isolates 63.7% was identified as A. flavus, they were tested for AFS and CPA production. The results were showed that only 96 out from 151 A. flavus isolates (63.6%) were positive to AFS and CPA toxins production. The results indicated that 64.6 % from A. flavus isolates were produced both of AFS and CPA. The averages of AFS and CPA production were 9.4 and 12.56 mg/L in YES medium respectively. For natural co-occurrence of AFS and CPA in corn samples, the results indicated that 9 samples out from 30 (30.0%) and 7/30 (23.3%) from corn grain samples collected from Cairo Governorate in winter season were contaminated with both of AFS (B₁, B₂, G₁ and G₂ ) and CPA , respectively. On the other hand, in summer season 11 samples out from 30 (36.3%) and 8/30 (26.6%) were contaminated with both of AFS (B₁, B₂, G₁ and G₂ ) and CPA, respectively. The results also indicated that 7 out from 30 (23.3%) and 6/30(20 %) of corn samples collected from Giza Governorate were contaminated with AFS and CPA respectively in winter season. While, 10 samples out from 30 (33.3 %) and 10/30 (33.3 %) samples were contaminated with AFS and CPA in Giza Governorate in summer season respectively. These represent 23.3% and 33.3 % AFS positive samples for corn grain samples collected from Giza Governorate in both season. The concentration of both AFS (B₁, B₂, G₁ and G₂) and CPA were ranged from (0.93 -45.5, 0.10- 19.0, 0.20-23.0 and
0.22-18.0) and 1.2-56.0μg/kg in the corn samples were collected from Cairo and Giza Governorates in summer and winter seasons respectively. Our study try to resolve this problem by modification a novel incubating the CPA spots detection with indirect apparel color reagent which giving stable spots color on TLC an obvious detection and an accurate quantitative determination by using the densitometric procedure. This is the first report of natural co-occurrence of AFs and CPA and there fungi production in Egypt.

**Keywords:** Aflatoxins; Cyclopiazonic Acid; Fungi; *Aspergillus Flavus*; HPLC; TLC

**Abbreviations:** AFS: Aflatoxins; CPA: Cyclopiazonic Acid; HPLC: High Performance Liquid Chromatography; TLC: Thin Layer Chromatography.

**Introduction**

Mycotoxins are secondary metabolites produced by specific filamentous fungi that contaminate agricultural commodities. They are toxic to humans and animals, cause significant reductions in crop yield and cause economic losses [1,2]. Their occurrence in various countries has been well documented [3]. A number of fungal species associated with corn, belonging mainly to the genera Aspergillus, Fusarium and Penicillium, have been reported to produce Mycotoxin [4]. *Aspergillus flavus* and *Aspergillus parasiticus* are important contaminants of certain foods and animal feeds because of their ability to produce Aflatoxins [5]. When these fungi invade and grow in commodities such as peanuts, corn and cottonseed, the resulting contamination with Aflatoxins often makes the commodities unfit for consumption [6]. Maize is ranked second to wheat among the world cereal crops and is often invaded before harvest by *A. flavus* and *A. parasiticus* that produce mycotoxins [8]. Aflatoxins are considered the most carcinogenic, mutagenic, teratogenic, cytotoxicity and genotoxicity, substances found naturally in foods and feeds [7-9]. These metabolites cause liver damage to humans and to most experimental animal species tested [10]. Consumption of mycotoxin-contaminated foods has been associated with several cases of human poisoning, or mycotoxicosis, sometimes resulting in death [3]. AF outbreaks affecting a large geographical area and causing over 123 deaths were reported in Kenya in 2004 and 2005. Epidemiological studies from this case showed a relationship between the outbreak and the local methods of harvesting, storing and preparing maize. Contamination of maize with AF was found up to 1000 ng/g [11]. Recently, have demonstrated that consumption of maize is an important source of aflatoxin exposure in young children in West Africa. AF have been found as contaminants in agricultural and food products especially in cereals and cereal products and animal feeds [12-19]. In the European Union, the maximum tolerable levels for AFB1 and total AF in cereals for human consumption are 2 and 4 ng/g, respectively and 20 ng/g for total AF in poultry feeds [20,21]. Cyclopiazonic acid (CPA) is another toxic secondary metabolite produced by *A. flavus* [2]. It causes necrosis of liver or gastrointestinal tissue and necrotic changes in skeletal muscle and kidney. However, there are suggestions that the teratogenic potential of CPA is low [22]. Isolates of *A. flavus* have been reported to co-produce Aflatoxins and CPA, as well as other toxins in different amounts [23]. The present study was carried out to determine Aflatoxins, CPA and their *A. flavus* isolates production from Egyptian corn.

**Materials and Methods**

**Survey Samples Study**

Sixty samples (½ kg for corn) of local corn were collected from different markets in Cairo and Giza Governorates during winter and summer season.

**Isolation of Fungi Associated With Local Corn**

Fungi associated with corn were isolated according to methods of Lichtwardth, et al. [24,25]. All corn samples were immersed in sodium hypochlorite solution (3%) as a sterilizer, for 3 minutes, rinsed 3 times in sterile distilled water then dried between sterile filter paper. Isolation of fungi was made by randomly taking 100 disinfested grains from samples of each location and directly plated in 20 Petri-dishes (5 grains per a dish) on Czapek's agar medium, then incubated at 25°C for 4 day's. Then, the percentage of the infected grains were determined and the fungal colonies that developed from the infected grains were counted, isolated, purified and maintained on a slant potato dextrose agar (PDA) medium for identification trials.
Identification of the Fungal Isolates

All the isolated fungi were identified by studying the cultural characteristics, as well as the microsocial structures on Gzapek’s agar medium, according to the procedures of Numbers of the species of Aspergillus were classified according to the key published by Raper and Fennell, while for species of other isolated fungi the “Manual of clinical mycology” by Count, et al. was used [26-29].

Production of AFs and CPA by Isolated A. flavus Strain

The media used for examining mycotoxin production of the strains were YES medium (2% yeast extract and 15% sucrose (Merck) /liter distilled water) YES (Difco) [30].

Preparation of AFs and CPA Standards

Chemical and reagents: Acetonitrile, acetone, ethanol, diethyl ether, hexane, methanol and chloroform were supplied by Merck (Darmstadt, Germany) HPLC-grade. Stock standard solutions of Aflatoxins with concentrations of 100 µg/ml were prepared in benzene-acetonitrile (98:2, v/v), wrapped in aluminum foil to prevent gradual break down of Aflatoxins under UV light and kept under protected conditions at 20 °C. All other inorganic chemicals and organic solvents were of reagent grade or higher.

Aflatoxin standard: Preparation of aflatoxin standard was carried out according to the association of Official Analytical Chemists, AOAC [31]. The crystals of Aflatoxins B1, B2, G1 and G2 were diluted using benzene-acetonitrile (98: 2 v/v) to obtain a concentration of 8 to 10 µg/ml (stock solution).

Cyclopiazonic acid standard

Stock solution
Pure solid CPA (Sigma) was dissolved in methanol (liquid chromatography grade) at 1 mg /ml and stored at 0 °C in the dark.

Working solution
Stock solution was diluted with methanol (liquid chromatography grade) with a CPA concentration of 40, 100, 500 ng/ml and 10, 100, 200 µg/ml.

Extraction of both Mycotoxins ( AFs & CPA) According the Method of Gqaleni, et al. [2]

Sixty samples were extracted in a warring blender with methanol/dichloromethane (DCM) (1:1) and blended for 2 min. The mixture was filtered and the filtrate was evaporated to dryness. The residue was partitioned between 200 ml n-hexane and methanol (1:1), the n-hexane layer was discarded and the methanol layer was evaporated to dryness. The residue was partitioned between 200 ml di-chloromethane (DCM) and distilled water (1:1) and the DCM layer was extracted three times with saturated sodium hydrogen carbonate solution (100 ml). The (DCM) extract layer was concentrated, and containing aflatoxins, was rotary evaporated and concentrated under a gentle stream of nitrogen. The aqueous layer was carefully acidified to pH 2 (with 0.5 N hydrochloric acid) and extracted three times with (DCM) (100 ml). The (DCM) extract was concentrated and contained of cyclopiazonic acid.

Derivatization for Aflatoxins

Added 200ul hexane to residue from dry film and added 50 µl Trifluoroacetic acid (TFA), cap vial, and their mixed by vortex vigorously for 30 s. Let mixture stand 5 min. By pipet was added 1.950 ml Water- acetonitril (9 +1) vortex mix vigorously for 30 s, and at let layers separate 10 min. filter through 0.45 µm microfilter into 5 ml screw –cap vial for subsequent HPLC analyses AOAC [31].

TLC Plates Aluminium Sheets

TLC plates aluminium sheets (20 x 20 cm) precoated with silica gel G 60 without Fluorescence indicator, No 1.05553, Layer thickness 0.2 mm, were obtained from Merck, 64271 Darmstadt, Germany.

Thin Layer Chromatography (TLC) for Detection and Separation of Mycotoxins:

The TLC procedure for detection and separation of mycotoxins according to Vaamonde et al. as follow: Mycotoxin (AFs and CPA) detection were performed using thin layer chromatography on silica gel G60 plates with and without oxalic acid impregnation for individual of CPA and AFs separation respectively. For CPA the plates were activated for 2 h at 110°C. To produce oxalic acid-impregnated TLC plates, precoated plates were dipped in a 2% methanolic solution of oxalic acid for 2 min and air dried overnight. The TLC plates were developed in toluene-Ethylacetate-90% formic acid (6:3:1, v/v) for Aflatoxins but toluene-ethyl acetate-90% formic acid (5:4:1, v/v) for cyclopiazonic acid. CPA in daylight after spraying treatment of the plates with Erlich’s reagent (1 g of 4-dimethylaminobenzaldehyde in 75 ml ethanol and 25 ml concentrated HCl), with subsequent development of blue spots. The plate was dried at room temperature until the excess of solvent disappeared. Aflatoxins were visualized under long wave UV light (366 nm). The suitable concentration used for TLC was 0.5 µg/ml.
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aflatoxin B₁ and G₁ while that of B₂ and G₂ was more diluted (about 0.1 µg/ml). Dilution was carried out using benzene-acetonitril mixture (98:2, v/v) in preparation of TLC.

**Determination of AFs A by HPLC**

High performance liquid chromatography (HPLC) was used to Aflatoxins determination. A mobile phase consists of Water: Acetonitril: Methanol (240:120:40) [32,9,33]. The system equipped with (Waters 600) delivery system. HPLC column a reverse phase analytical column packed with C18 material (Spherisorb 5 µm ODS2, 15cm×4.6nm). The detection was performed using The fluorescence detector was operated at an excitation wave length of 360 nm and an emission wave length of 440 nm. The separation was performed at 40°C temperature at a flow rate of 1.0 ml/ min. Data were integrated and recorded using a Millennium Chromatography Manger Software 2010 (Waters, Milford MA 01757).

**Determination of CPA by HPLC**

Mobile phase solvents consists of

**Solvent A:** Methanol: water (85:15, v/v)

**Solvent B:** 0 Methanol: 4 mM zinc sulfate (85:15, v/v)

Solvent A and B were used in a linear gradient from mobile phase A to mobile phase B in 10 min at a flow rate of 1 ml/min.

**Apparatus**

High performance liquid chromatography (HPLC) was used to CPA determination. The system equipped with (Waters 600) delivery system. HPLC column Reverse phase hyper clone 5µ ODS C18 column (2.5mmX30cm); The detection was performed using U.V detector (waters 486) at 279 nm wavelength, oven temperature 40 °C, using linear program of 25 min period and 1 ml / min constant solvent flow rate. Data were integrated and recorded using a Millennium Chromatography Manger Software 2010 (Waters, Milford MA 01757). A calibration curve correlating peak-area and concentration was constructed for quantification purposes, using toxin standards. Confirmation of CPA in the sample was achieved using (UV) detector.

**Results and Discussion**

**Isolation and Identification of Fungi Associate with Corn.**

**Fungi isolation on Czapek’s Agar Medium:** Data in Table 1 represent the fungi isolated on Czapek’s agar medium from corn purchased from retail markets in Cairo and Giza Governorates during the winter and summer seasons, respectively. The results in Table 1 showed that the percentage of fungal infected grains in both seasons winter and summer were 97, 100, 100, and 98% in local corn grains were obtained from retail markets in Cairo and Giza Governorates, respectively. The highest rate of infected was found in corn samples (100%) in winter season which stored in shops in Giza Governorate while, in summer season the sample were infected 100 % in corn grains were collected from Cairo Governorate, this may be due to the bad storage conditions in these Governorates. Followed by samples (98%) from shops in Giza Governorate, while the lowest infection rate was recorded from the corn samples, (97%) in the winter season from Cairo Governorate.

| Commodities Governorates Seasons | No of samples | % Infections | Total fungal Counts | % Isolated Fungi |
|----------------------------------|---------------|--------------|---------------------|-----------------|
|                                  |               |              |                     | Aspergillus     |
|                                  |               |              |                     | Fusarium        |
|                                  |               |              |                     | Penicillum       |
|                                  |               |              |                     | Alternaria       |
|                                  |               |              |                     | Rhizopus        |
| Corn                             |               |              |                     |                 |
| Cairo                            |               |              |                     |                 |
| Winter                           | 30            | 97           | 110                 | 52              |
| Summer                           | 30            | 100          | 190                 | 63              |
| Giza                             |               |              |                     |                 |
| Winter                           | 30            | 100          | 130                 | 53              |
| Summer                           | 30            | 98           | 163                 | 57              |

Table 1: Fungi isolated of corn samples collected from Cairo and Giza governorates.

Concerning the different fungal genera which were found on and in corn grain, sample the genera of Aspergillus, Penicillum, Fusarium, and Alternaria, were found in all samples were collected from Cairo and Giza Governorates. However, the Rhizopus genus was not recorded in both seasons in corn grain samples. The frequency of occurrence of Aspergillus, Penicillum, Fusarium, and Alternaria were 50, 35.5, 14.5, and 10 % on
Natural Occurrence of Mycotoxic Fungi Isolated from Corn

Mycotoxins are a poison produced by an active growing mold. Molds may be activity growing without Mycotoxin formation. Therefore, this study was carried out to isolate mycotoxic fungi namely A. flavus producing Aflatoxins, and Cyclopiazonic acid. Data in Table 2 indicate that, out of 239 Aspergillus spp. isolates which isolated from corn samples collected from Cairo and Giza Governorates, Concerning the frequency distribution of 98 isolates of A. flavus were tested for isolation of A. flavus 64.5 producing Aflatoxins isolated from corn. The results indicated that 64.5 of the isolates were A. flavus producing both of Aflatoxins and Cyclopiazonic acid form samples of corn. Regarding A. flavus, considerable variability in their mycotoxin-producing potential was found in concordance with others. The results showed that averages of 9.4 mg Aflatoxins /L of the YES medium were produced by A. flavus isolates from corn samples. On the other hand, the results showed that averages of 12.56 mg cyclopiazonic acid /L of the YES medium were produced by A. flavus isolates from corn. Resnik, et al. [35] found that 33 of 34 A. flavus isolates from corn produced CPA and only 5 of these strains produced Aflatoxins B1 simultaneously. Cyclopiazonic acid (CPA) has been found as a natural contaminant of corn and peanuts, often occurring together with the discovery of CPA production by A. flavus, 54 isolates of A. flavus were investigated for production of CPA and Aflatoxins [36]. Isolated 96 strains of Aspergillus Flavus from samples of stored grain [37]. They found that the Five strains produced Cyclopiazonic acid in the range of 0.5-30 mg/kg and 9 produced Aflatoxins B1 (0.1-14.8 mg/kg) but none of them produced both Cyclopiazonic acid and Aflatoxins. It was found that 28 of the 54 (52%) produced CPA whereas only 18 (33%) produced Aflatoxins. Isolates of A. flavus have been reported to co-produce Aflatoxins and CPA, as well as other toxins in different amounts. Mphande, et al. screened of 32 isolates for Mycotoxin production of A. flavus, were found that 11 did not produce detectable Aflatoxins, 8 produced only Aflatoxins B1 and B2, and 13 produced all four Aflatoxins B1, B2, G1 and G2 in varying amounts. Only 6 of the A. flavus isolates produced Cyclopiazonic acid at concentrations ranging from 1 to 55 μg/kg. The one A. parasiticus isolate screened also produced all the four Aflatoxins (1,200 μg/kg) but did not produce Cyclopiazonic acid. When the raw peanut samples (n = 120) were analyzed for total Aflatoxins, 78% contained Aflatoxins at concentrations ranging from 12 to 329 μg/kg.

| Source | Isolates of Aspergillus tested (No.) | Isolates of Aspergillus flavus (No.) | Isolates of A. flavus producing Aflatoxins (No.) | Isolates of A. flavus producing Aflatoxins (%) | Isolates of A. flavus producing CPA (%) | Cyclopiazonic acid concentration (mg/L) | Total of Aflatoxins concentration (mg/L) |
|--------|-------------------------------------|-------------------------------------|-----------------------------------------------|-----------------------------------------------|----------------------------------------|--------------------------------------|-----------------------------------------|
| Corn   | 239                                 | 1520                                | 98                                            | 64.5                                          | 64.5                                   | 0.4-32.50                            | 12.56 ± 0.89                            |

Table 2: Natural occurrence of Aspergillus flavus isolated from raw corn t samples collected from Cairo and Giza Governorates and its ability for Aflatoxins and cyclopiazonic acid (CPA) production in YES medium.

Natural Co-occurrence of Aflatoxins and Cyclopiazonic Acid (CPA) in Corn

It is worthy to mention that the current investigation to detect the Aflatoxins (B1, B2, G1 and G2) and cyclopiazonic acid (CPA) levels and /or frequencies in the Egyptian corn. Hundred and twenty samples of corn (60 from Cairo and 60 from Giza Governorates) were collected from retail markets in Cairo and Giza Governorates during winter and summer seasons. The data in Tables 3 & 4 showed the natural occurrence of Aflatoxins and cyclopiazonic acid in corn grain samples in the winter and summer season during 2004/2005...
collected from Cairo and Giza Governorates. The results in Table 3 indicated that 9 samples out from 30 (30.0%) of both Aflatoxins (B₁ and B₂), 7/30 (23.3%) of both AFG, and CPA, and 6/60 (20.0%) of AFG₂ from corn grain samples collected from Cairo Governorate in winter season were contaminated with both of Aflatoxins (AFB₁, AFB₂, AFG₁, and AFG₂) and CPA. While in summer season, 11 samples out from 30 (36.3%) of AFB₁, 10/30 (20.0%) of AFB₂/30 (26.6%) of CPA and of both AFG₁, and AFG₂ 6 samples out from 30 were contaminated with both Aflatoxins (AFB₁, AFB₂, AFG₁, and AFG₂) and CPA. The concentrations of both Aflatoxins (AFB₁, AFB₂, AFG₁, and AFG₂) and CPA were ranged from (0.02-22.0, 0.10-12.0, 1.10-12.50 and 2.20-13.0) and 0.93-35.0 µg/kg in the corn samples were collected from Cairo Governorate in the winter season, while in the summer season were found ranged of concentrations (1.23-45.5, 1.10-19.0, 2.20-23.0, 3.0-10.0 and 2.0-56.0), for Aflatoxins and CPA, respectively.

| Type of toxins | AFB1 | AFB2 | AFG1 | AFG2 | Total | CPA | AFB1 | AFB2 | AFG1 | AFG2 | Total AFS | CPA |
|---------------|------|------|------|------|-------|-----|------|------|------|------|----------|-----|
| Minimum (µg/kg) | 0.02 | 0.1  | 1.1  | 2.2  | AFS   | 0.93| 1.23 | 1.1  | 2.2  | 3.0  | 6.23     | 2.0 |
| Maximum (µg/kg) | 22   | 12   | 12.5 | 13   | 3.42  | 35  | 45.5 | 19   | 23   | 10   | 78.3     | 56  |
| Mean ± SE     | 18.7±2.35 | 8.6±1.2 | 9.7±1.56 | 9.81±1.56 | 57.24±4.27 | 21.70±4.08 | 20.52±3.79 | 10.56±1.8 | 11.36±2.93 | 6.75±1.04 | 39.96±7.28 | 24.06±6.5 |
| Total No. of sample | 30 | 30 | 30 | 30 | 5.65 | 30 | 30 | 30 | 30 | 30 | 30 | 8 |
| % of positive samples | 30 | 30 | 23.3 | 23.3 | 20 | 36.6 | 33.3 | 20 | 20 | 26.6 |

Table 3: Natural occurrence of Aflatoxins and cyclopiazonic acid in corn samples collected from Cairo Governorate during winter and Summer Seasons.

| Type of toxins | AFB1 | AFB2 | AFG1 | AFG2 | Total AFS | CPA | AFB1 | AFB2 | AFG1 | AFG2 | Total AFS | CPA |
|---------------|------|------|------|------|----------|-----|------|------|------|------|----------|-----|
| Minimum       | 2.23 | 1.1  | 2.2  | 6.53 | 2        | 0.93| 2.1  | 1.2  | 2    | 6.23 | 2.7      |     |
| Maximum       | 30.5 | 17   | 14   | 60   | 61       | 35.5| 17   | 20   | 18   | 75   | 46       |     |
| Mean ± SE     | 19.67±3.21 | 10.37±2.04 | 9.53±1.67 | 8.87±1.97 | 54.47±7.12 | 25.3±6.7 | 20.19±2.96 | 11.23±1.93 | 11.9±2.67 | 9.64±2.25 | 46.49±7.47 | 23.17±5.15 |
| Total No. of sample | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 |     |

Table 4: Natural occurrence of Aflatoxins and cyclopiazonic acid in corn sample collected from Giza Governorate during winter and summer seasons.

The results in Table 4 show the natural occurrence of Aflatoxins and cyclopiazonic acid in corn grain samples in the winter and summer seasons collected from Giza Governorate. The results indicated that 7 samples out from 30 (23.3%) of each aflatoxin (B₁, B₂) while, 6/30 (20%) of each AFG₁, AFG₂ and CPA were contaminated with Aflatoxins (AFB₁, AFB₂, AFG₁, and AFG₂) and CPA respectively in winter season from corn samples collected from Giza Governorate. On the other hand, 10 samples out from 30 (33.3%) of each aflatoxin (AFB₁ and B₂) and 7/30 (23.3%) of both AFG₁ and AFG₂ and CPA were contaminated with aflatoxins (AFB₁, AFB₂, AFG₁, and AFG₂) and CPA in the Giza Governorate, in summer season respectively. This represent 23.3% and 33.3% aflatoxin (AFB₂) positive samples for corn grain samples collected from Giza Governorate in both season. The concentration of both aflatoxin (AFB₁, AFB₂, AFG₁, and AFG₂) and CPA were ranged from (2.23-30.5, 1.10-17.0, 2.20-14.0, and 1.0-13) and 2.0-51.0 µg/kg in the corn samples, in the winter season, whereas in the summer season the concentrations of both Aflatoxins (AFB₁, AFB₂, AFG₁, and AFG₂) and CPA were ranged from (0.93-35.5, 2.10-17.0, 1.20-20.0 and 2.0-18.0) and 2.7-46.0 µg/kg for Aflatoxins and CPA, respectively. In a survey of peanut products in North America, 19% of 1416 samples examined were contaminated with an average level of 1

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µg/kg AFB1 [39]. In Thailand 49% of 216 samples contained AFB1 at an average level of 424 µg/kg [40]. In parts of India 100% of maize samples have been found contaminated with aflatoxin in the range of 6,250-15,600 µg/kg [41]. Aflatoxins are present in food chain Consumption of aflatoxin in many parts of the world varies from 0 to 30,000 ng/kg/day [42]. The factors such as high temperature and low moisture can result in cracks in the seed and subsequent invasion by the fungus. Temperature and moisture are the dominant factors that affect aflatoxin contamination of corn. Environmental conditions most favorable for maximum growth and aflatoxin production by A. flavus are temperatures greater than 30°C, maximum relative humidity of greater than 85%, and water activity of 0.98 to 0.99 [43]. Thus, A. flavus can infect with proper moisture/temperature conditions during storage almost any stored product [44]. Regional differences in aflatoxin contamination of crops may be attributable to climatic conditions and to agricultural practices that increase susceptibility of plants to invasion by A. flavus [45]. Aflatoxin formation in groundnut is favored by prolonged end of season drought and associated elevated temperature [46].

In general, the TLC technique is simple and does not require special equipment, but most of the published methods suffer from the excessive time needed for analysis and/or inaccuracy of the obtained results. It had been shown previously that spray reagents may lead to variable results because the intensity of fluorescent color is dependent on many difficult-to-control factors such as time, amount of reagent applied to the sample spot, and development of background color.

It had been shown that the CPA detection by the direct spraying of the apparel (Figure 1) Ehrlich’s reagent on the separated CPA spots using the TLC technique resulted in a weak-unstable developed color intensity giving unobvious detection and inaccurate quantitative determination by densitometric procedure.

Thereupon, the current study try to resolve this problem by modification a novel incubating the CPA spots detection with indirect apparel color reagent which giving stable spots color an obvious detection and an accurate quantitative determination by using the densitometric procedure, as shown in the Figure 2.

Figure 1: Thin Layer Chromatography of Cyclopiazonic Acid Standard separation by used direct spraying of the apparel Ehrlich’s reagent.

In this technique, covered surface of TLC plate free of CPA was sprayed with an apparel Ehrlich’s reagent and dried. Then, it adhered together to the other covered surface of the other TLC plate on which the spotting of CPA in the tested sample extract or the CPA standard solution was developed in toluene-Ethylactate-90% formic acid (6 : 3 :1, v/v) and incubated 2 hours at 35°C to give a purple spots in Figure 2.

1,2,3,4 = Random samples of corn ,and spot of 5; 6 standard of CPA.
Figure 2: Thin Layer Chromatography of Cyclopiazonic Acid separation of samples and standard by the new method of indirect apparel Ehrlich’s reagent.
The color reaction of Erlich's reagent ($\rho$-dimethylaminobenzaldehyde) with the indol group present in the CPA structure. The reagent $\rho$-dimethylaminobenzaldehyde reacts with $\alpha,\beta$-unsaturated indoles, preferentially in the $\beta$-position, in presence of HCl to give colored cations, the condensation may be taking place at the $\alpha$-position to give a purple cation, as illustrated in Figure 3.

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