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Advances in Clinical Toxicology
ISSN: 2577-4328

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

Deabes MMY[1]*, Amra HA[1], Damaty ELM[2] and Rowayshed GH[2]

[1]Food Toxicology and Contaminants Department, National Research Centre, Cairo, Egypt

[2]Food Science and Technology Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt

Research Article
Volume 3 Issue 3
Received Date: July 10, 2018
Published Date: July 26, 2018

***Corresponding author:** Mohamed M Deabes, Food Toxicology and Contaminants Department, National Research Centre, 33-El Bohouth street-Dokki, Cairo, 12311, Egypt, Email: mydeabes@yahoo.com

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 genra 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 (B_1, B_2 ,G_1 and G_2) 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 (B_1, B_2 ,G_1 and G_2) 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 (B_1, B_2, G_1 and G_2) 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 produc 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 B_1, B_2, G_1 and G_2 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 µ g/ ml.

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

Sixty samples were extracted in a warring blender with methanol/ dicholoromethan (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 Trifluroacetic 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 Myctoxins: The TLC procedure for detection and separation of mcotoxins 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-Ethylactate-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

aflatoxin B_1 and G_1 while that of B_2 and G_2 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				
				Aspergilluss	Fusarium	Penicillum	Alternaria	Rhizopus
Corn								
Cairo								
Winter	30	97	110	52	25	13	10	-
Summer	30	100	190	63	22	11	4	-
Giza								
Winter	30	100	130	53	15	27	5	-
Summer	30	98	163	57	17	20	6	-

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, Penicillium, 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, Pencillum, Fusarium, and Alternaria were 50, 35.5, 14.5, and 10 % on

corn samples in Cairo Governorate in winter season, and it was 63.3,24, 30.6 9.0 and 1.2 % during summer season of 2004.However, in the corn samples were collected from Giza governorate were found 53.8, 15.4, 353, and 5.5 %, in winter season, while in summer season were found 58.8, 23.8,33.8, and 6.3 %. The results also indicated that the dominant fungi genera were Aspergillus, Fusarium, Penicillium and Alternaria in all corn and peanut samples in both seasons Vaamonde, et al. [34] found that the incidence of Aflatoxigenic *A. flavus* strains was higher in peanuts (69%) than in wheat (13%) or soybeans (5%) while the ratio of CPA producers *A. flavus* isolated from all substrates was very high (94% in peanuts, 93% in wheat and 73% in soybeans). Isolates of *A. flavus* able to produce simultaneously Aflatoxins type B and CPA were detected in all substrates, suggesting the possibility of co-occurrence of these toxins.

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 cyclopiazonc 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 AflatoxinsB_1 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 [25,38]. Flavus, were found that 11 did not produce detectable Aflatoxins , 8 produced only Aflatoxins B1 and B2, and 13 produced all four Aflatoxins B_1, B_2 G_1 and G_2) 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 (1,200µg/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 o f A.flavus producing Aflatoxins (%)	Isolates o f A.flavus producing CPA (%)	Cyclopiazonic acid concentration (mg/L)		Total of Aflatoxins concentration (mg/L)	
						Range	Mean ± SE	Range	Mean ± SE
Corn	239	1520	98	64.5	64.5	0.4-32.50	12.56 ± 0.89	1.10 - 15.7	8.01± 0.48

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 (B_1, B_2 G_1 and G_2) 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 (B_1 and B_2),7/30 (23.3%) of both $AFG_1$and CPA, and 6/60 (20.0 %) of AFG_2 from corn grain samples collected from Cairo Governorate in winter season were contaminated with both of Aflatoxins (AFB_1, AFB_2 ,AFG_1 and AFG_2) and CPA . While in summer season, 11 samples out from 30 (36.3%) of AFB_1, 10/30 (20.0%) AFB_2 8/30 (26.6) of CPA and of both AFG_1 AFG_2 6 samples out from 30 were contaminated with both Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and CPA. The concentrations of both Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) 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.

	Aflatoxins and Cyclopiazonic Acid in Corn Samples											
Seasons	**Winter**						**Summer**					
Type of toxins	**AFB1**	**AFB2**	**AFG1**	**AFG2**	**Total**	**CPA**	**AFB1**	**AFB2**	**AFG1**	**AFG2**	**Total AFS**	**CPA**
Minimum concentration (µg/kg)	0.02	0.1	1.1	2.2	AFS	0.93	1.23	1.1	2.2	3	6.23	2
Maximum concentration (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 ± 42.47	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
No of positive samples	9	9	7	7		6	11	10	6	6		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.

	Aflatoxins and Cyclopiazonic Acid Concentrations (µg/kg)											
Seasons	**Winter**						**Summer**					
Type of toxins	**AFB1**	**AFB2**	**AFG1**	**AFG2**	**Total AFS**	**CPA**	**AFB1**	**AFB2**	**AFG1**	**AFG2**	**Total AFS**	**CPA**
Minimum	2.23	1.1	2.2	1	6.53	2	0.93	2.1	1.2	2	6.23	2.7
Maximum	30.5	17	14	13	60	51	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

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 (B_1, B_2) while, 6/30(20 %) of each AFG_1, AFG_2 and CPA were contaminated with Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) 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 (AFB_1 and B_2) and 7/30 (23.3 %) of both AFG_1 and AFG_2 and 10/30 (33.3%) of CPA were contaminated with aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2) and CPA in the Giza Governorate, in summer season respectively. This represent 23.3% and 33.3% aflatoxin (AFB_1) positive samples for corn grain samples collected from Giza Governorate in both season. The concentration of both aflatoxin (AFB_1, AFB_2 ,AFG_1 and AFG_2) 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 (AFB_1, AFB_2, AFG_1 and AFG_2) 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

µg/kg AFB_1 [39]. In Thailand 49% of 216 samples contained AFB1 at an average level of 424µg/kg [40]. In parts of India 100% of maize samples have been found contaminated with aflatoxin in the range of 6,250-15,600 ìg/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 30ºC, 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.

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

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.

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.

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.

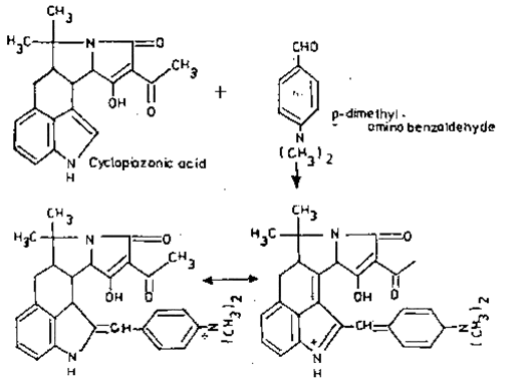

Figure 3: Color reaction of Cyclopiazonic Acid with ρ-di-methyl amino benzaldehyde [46].

The color reaction of Erlich's reagent (ρ-dimethylaminobenzaldehyde) with the indol group present in the CPA structure. The reagent ρ-dimethylaminobenzaldehyde reacts with α,β-unsaturated indoles, preferentially in the β-position, in presence of HCl to give colored cations, the condensation may be taking place at the α-position to give a purple cation, as illustrated in Figure 3.

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