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Isolation of Asperigllus flavus and detection of total aflatoxins residues in feed by HPLC

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A B S T R A C T

Mycotoxins are natural secondary toxic anti nutritive metabolite products of fungi, that contaminate the food stuff , high performance liquid chromatography (HPLC) is one of chromatographic techniques that mainly used in quantitative and qualitative of most mycotoxin residues . A total of 100 random samples of yellow corn and silage feed samples were collected from farms in Ismailia and Suez governorates to detect total aflatoxins residues. Aspergillus flavus were isolated from 86 % , 80 % of yellow corn and silage feed samples. Total aflatoxins residues were detected by HPLC with the means average of Aflatoxin B1, G1, B2, G2 and total aflatoxins residues respectively, in examined 50 yellow corn feed samples were 74.66±12.53 µg/kg, 30.14±6.65 µg/kg,16.13± 2.15 µg/kg ,7.42± 3.28 µg/kg and 85.89 ± 34.94µg/kg. While in examined silage feed samples the mean average were 85.18 ± 17.48 µg/kg,24.02 ± 4.81 µg/kg, 11.79± 2.25 µg/kg, 5.20±1.65 µg/kg and 82.52 ± 18.77 µg/kg. HPLC is suitable technique for the quantification of aflatoxins because of its accuracy and high sensitivity

Key words: *Total aflatoxins residues – yellow corn – silage feed - HPLC*

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1. INTRODUCTION

Mycotoxins are abiotic hazards that represent a worldwide threat to human health and welfare owing to their toxicity and occurrence in food and feed (Sun et al., 2017).Among the reported mycotoxins (>400species), only a small number of mycotoxins are considered of economic importance due to their high prevalence and high contamination levels in food and feed products. These mycotoxins include aflatoxins especially aflatoxins B1 (AFB1), ochratoxin
A (OTA), zearalenone (ZEA), trichothecenes (deoxynivalenol[DON], T-2), fumonisins like fumonisin B1 (FB1), beauvericin(BEA), citrinin (CTN), alternaria toxins (alternariol [AOH], alternariolmonomethyl ether [AME], tentoxin [TEN], tenuazonicacid [TeA], altenuene [ALT], altertoxins [ATXs], Alternariaalternate f. sp. lycopersici toxins [AAL-toxins], and iso-tenuazonic acid[iso-TeA}).(EFSA , 2011) , (Ferre , 2016) , (Marin et al., 2013) , (Shi et al., 2018).Aflatoxins (AFs) are secondary metabolites of fungi (e.g.,Aspergillus flavusand A. parasiticus). Aflatoxins occur naturally and can be found in common food and feedstuffs such as rice, peanuts, yellow corn, silage and soybean meal (Anjum et al., 2012) , (Oplatowska et al., 2016). The four major analogues—aflatoxins B1 (AFB1), B2 (AFB2), G1(AFG1), and G2 (AFG2)—are the most important members because they all pose a potential risk to human and animal health if food and

feedstuffs have been contaminated. The B and G classes depicting the blue and green fluorescence emitted by their metabolites under ultraviolet (UV) light, and the sub-type 1 and 2 refers to the major and minor compounds respectively (Tav et al., 2007). In particular, the toxicity of AFB1 can range from levels that may cause immune system suppression to the induction of teratogenic, mutagenic, and carcinogenic activities (Khayoon et al., 2012), (Manzanares et al., 2010), which is collectively classified as a carcinogen (Group 1) by the International Agency for Research on Cancer (IARC) (IRAC , 2002).The Food and Drug Administration (FDA) has established an “action level” of 20 ppb for aflatoxins in yellow corn in interstate commerce. Fungus and mycotoxin residues are one of the cause of mycotoxicosis which characterized by immunosuppression , hepatotoxicity , nephrotoxicity , carcinogenicity, mutagenicity , loss of egg production , law growth rate and teratogenicity. FAO reported that 25% of world agriculture commodities are polluted with mycotoxins(Li et al., 2012) ,(Shephard, 2008) and (Simons, 2009) .

Aflatoxin determination in food and feed is currently performed by high-performance liquid chromatography (HPLC) . (CAST, 2003) , (Kolosova et al., 2006) and (Kos et al., 2016) . High-performance liquid chromatography with fluorescence detection (HPLC-FLD) is considered as the most reliable instrument for the quantification of AFs due to its accuracy and high sensitivity (Sirhan et al., 2014).

2.MATERIAL AND METHODS

Feed samples100 samples of feedstuffs were collected from farms in Ismailia and Suez governorates for investigation of fungal contamination and detection of total aflatoxins residues types aflatoxin "B1, G1, B2 and G2 ". These samples divided as 50 of each as yellow corn, silage after isolation of fungi we extracted from 30 samples only of each category total aflatoxins residues by HPLC. These samples were introduced in formation of formula of feed manufacture which Introduced as poultry and large animals meals. The samples were collected during summer and winter seasons of years 2015 & 2016 and brought to residues analysis unit Reference laboratory for Veterinary Quality Control on Poultry production- Animal Health Research Institute , Dokki, Giza .

2.1.Media:

(Sabourauds dextrose agar medium (SDA) according to (Cruickshank et al., 1975).

Dichloranrose Bengal chlormphenicol agar (DRBC) according to (King et al., 1979).

,Dichloran rose Bengal yeast extract sucrose agar (DRYS) according to (King et al., 1979).

Czapek –Dox agar Medium according to (Al Doory , 1980).

2.2.Standards of aflatoxins :

Total aflatoxins standards were obtained from Sigma (St. Louis, MO, USA) of 99% purity. Preparing of working solution was in acetonitrile and stored at -20 ºC in amber glass vials over a period of 12 months. Preparing of aflatoxins Standard stock solutions were in acetonitrile according to the (AOAC, 1995) and (IRAC, 2002).

2.3.chemicals and Reagents:- Liquid Chromatography mobile phase:(M. Ph.): according to : (Khayoon et al., 2010):

2.4. Stain:-

Lactophenol cotton blue stain according to (Leanor, 1978).

2.5.Apparatuses:-

Liquid chromatography (HPLC)Agilent Series 1200 quaternary gradient pump, Series 1200 auto sampler, Series 1200 UV and fluorescence detector, and HPLC 2D Chemstation software (Agilent - Germany).FLD signals for compound, which was excitation at 360nm and emission at 440nm. Analytical column the chromatographic (Extend-C18, Zorbax

column, 4.6 mm, 250 mm, 5 µm, Agilent Co.).

2. 6.isolation of molds according to (Refai, 1979).

2. 7.preparation of sample homogenate: Of the aseptically mixed samples, 25gm were put into a stomacher jar containing 225ml of BPW .

2. 8.Dilution: The homogenate samples were mixed by shaking and 1 ml was transferred into a tube containing 9ml of BPW then mixed and vortex.

2.9.Mold identification: The incubated plates were examined macroscopically and microscopically. The selection of individual colonies according to their morphological characters and microscopic examination. Aspergillus were classified according to the key of (Al-Doory, 1980), (Raper, 1965) and (Refai, 1988). But the identification of other molds were carried out according to (Conner et al., 1992).Stock cultures were made from isolates then examined on Czapek- Dox malt extract and potato dextrose agar slopes for identification. Identification of the colonies were carried out by observation of macroscopic and microscopic characteristics of mold colony.

2.10.Macroscopic examination:

This according to observation of the rate of growth of colonies , texture and pigmentation on the surface and reverses sides over a period of week.

2.11.Microscopic Examination:

Direct microscopic examination: From 4-6 days old colony a piece was transferred to clean glass slide then one drop of lactophenol cotton blue was added . The slide was covered with a glass cover then examined microscopically.

2.12.Slide culture techniques according to (Ajello et al., 1963).

Detection of total aflatoxins in yellow corn and silage feed samples by High Performance Liquid Chromatograph "HPLC" according to (Khayoon et al., 2010):

HPLC conditions operations:_according to Khayoon et al., (2010).

Sample Extraction: according to Akiyama, et al., (1996)

Statistical analysis:-The obtained data were computerized and analyzed for significance, calculation of standard error and variance according to (Feldman et al., 2003).

3. RESULTS

The results showed in Table (1) illustrated the prevalence of main fungal species in examined feed samples. Aspergillus species were isolated from 84% and 80 % of examined yellow corn , silage samples ,respectively. Mucor was isolated from 12% and 4% of examined yellow corn, silage samples, respectively. Rhizopus was isolated from 12 % and 8% of examined yellow corn and silage, respectively. Penicillum was isolated from 6% and 8% of examined yellow corn , and silage samples, respectively. Fusirium was isolated from 10% and 4% of examined yellow corn and silage samples, respectively. Teluromyces were isolated from 2% of examined yellow corn samples.

Regarding the conventional identification of different Aspergillus species, in table (2) the most predominant isolate was A. flavuswhich was recovered from yellow corn at the rate of (66%) followed by A. parasiticus(16%) and A. niger (2%). While in examined silage samples, A. flavus was recovered from(60 %), A. parasiticus(10%) , A. niger(2%) and A. ochraceus from (2%) .It was clear from the result that there are differences in the level of contamination which may be due to the exposure of the examined samples to different climatic condition during preparation , transportation and storage.

This current study in table (3) illustrated that analytical results of aflatoxin residues (µg/kg) in examined yellow corn and silage feed samples by HPLC. The result showed that 12 out of 30 examined yellow corn samples contain AFB1 by 40 % with minimum 106.54

µg/kg, maximum 166.24 µg/kg by a mean 74.66+/- 12.53 µg/kg. AFG1 were found in 9 out of 30 examined yellow corn samples by 30% with minimum 0.572 µg/kg, maximum 74.5 µg/kg by a mean 30.14+/- 6.65 µg/kg. AFB2 were found in 9 out of 30 examined yellow corn samples by 30% with minimum 1 µg/kg, maximum 22.52 µg/kg by a mean 16.13+/- 2.15 µg/kg. AFG2 were found in 9 out of 30 examined yellow corn samples by 30% with minimum 0.21 µg/kg, maximum 26.34 µg/kg by a mean 7.42+/- 3.28 µg/kg. Total aflatoxins were found in 16 out of 30 examined yellow corn samples by 53.3% with minimum 0.792 µg/kg , maximum 193.14 µg/kg by a mean 85.89+/- 34.94 µg/kg. For silage samples , the result showed that 14 out of 30 examined silage samples contain AFB1 by 47 % with minimum 0.15 µg/kg , maximum 201.33 µg/kg by a mean 85.18+/- 17.48 µg/kg . AFG1 were found in 10 out of 30 examined silage samples by 33% with minimum 0.6 µg/kg, maximum 50 µg/kg by a mean 24.02+/- 4.81 µg/kg. AFB2 were found in 9 out of 30 examined silage samples by 30% with minimum 0.1 , maximum 20 µg/kg by a mean 11.79+/- 2.25 µg/kg . AFG2 were found in 10 out of 30 examined silage samples by 33% with minimum 0.5 µg/kg, maximum 16 µg/kg by a mean 5.20+/- 1.65 µg/kg . Total aflatoxins were found in 20 out of 30 examined silage samples by 67% with minimum 0.11 µg/kg, maximum 201.33 µg/kg by a mean 82.52+/- 18.77 µg/kg .

Table (1) prevalence of main fungal species in examined feed samples.

Types of sample	Asperigllus species		Mucor		Rhizopus		Penecillum		Fusirium		Teluromyces	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Yellow corn n= 50	42	84	6	12	6	12	3	6	5	10	1	2
Silage n=50	40	80	2	4	4	8	4	8	2	4	-	-

% types of samples according to samples of each one n=50.

Table (2): Prevalence of Aspergillus species in examined feed samples:

Feed type	Aspergillus flavus		Aspergillus parasiticus		Aspergillus niger		Aspergillus ochraceus	
	No.	%	No.	%	No.	%	No.	%
Yellow corn n=50	33	66	8	16	1	2	-	-
Silage n=50	30	60	5	10	4	8	1	2

% percent of presence of Aspergillus species in yellow yellow corn and silage feed samples.

Table (3): Statistical analytical results of aflatoxin residues (µg/kg) in examined yellow corn and silage feed samples by using HPLC (n= 30).

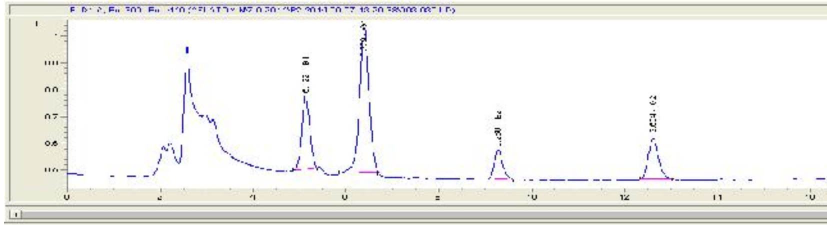
Toxin type	Yellow corn					Silage				
	No	%	Min.	Max.	Mean +/- SE	No.	%	Min.	Max.	Mean +/- SE
AFB1	12	40	16.54	166.24	74.66+/-12.53	14	47	0.15	201.33	85.18+/- 17.48
FG1	9	30	0.572	74.5	30.14+/-6.65	10	33	0.6	50	24.02+/-4.81
AFB2	9	30	1	22.52	16.13+/-2.15	9	30	0.1	20	11.79+/-2.25
AFG2	9	30	0.21	26.34	7.42+/-3.28	10	33	0.5	16	5.20+/-1.65
TAF	16	53.3	0.792	193.14	85.89+/-34.94	20	67	0.11	201.33	82.52+/-18.77

1. Macroscopical & Microscopical characterization of molds

Fig.1.Macroscopical characterization of Asperigallus flavus

Fig. 2.Microscopical characterization of Asperigallus flavus

Fig. (3) Chromatogram of standard of total aflatoxins 40µg/kg.



This chromatogram Showed no excipient compound interference between peaks of the pure standard and peaks of spiked feed samples. Retention times of the analytes and for aflatoxin B1 Rt =5.01 min, for aflatoxin G1 Rt =6.379 min , for aflatoxin B2 Rt = 9.269 min and for aflatoxin G2 Rt =13.04 min

.Fig .(4) Calibration curve of total Aflatoxins residues (10-360) µg/kg by using HPLC.

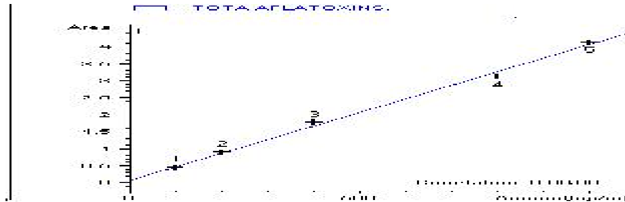


Fig.(5)Chromatograms showing C 19 yellow corn feed sample contain total afltoxins residues .

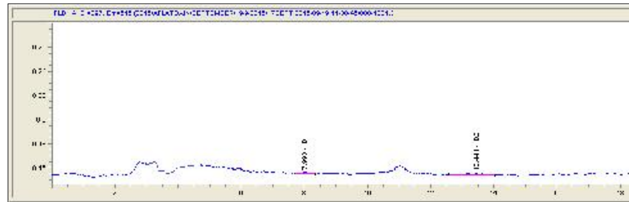


Fig.(6) Chromatograms showing S 7 silage feed sample contain total afltoxins residues .

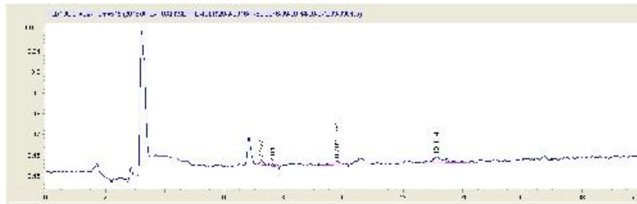


Fig.(7) Chromatograms showing blank sample.

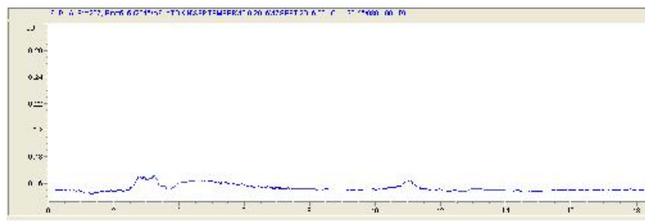


Fig.(8)Chromatograms showing reagent blank.

4. DISCUSSION

The highest incidence Aspergillus species were in yellow corn by (86 %) followed by silage (80%).These findings were in agreement with the results found by and (Hassan et al., 2008).

These results were lower than those found by. (Hassan et al., 2012) who reported that the most common isolated moulds from feed were Aspergillus spp. (100%), Fusariumspp. (24%), Mucor spp. (28%), Pencillium spp.(52%), Cladosporium spp.(8%) and Alternaria spp.(28%) due to the highest humidity levels ,very bad storage conditions of feed. In addition, 25%
of the world's crop production is contaminated with mycotoxins.

These findings were in agreement with the results of (EL- Hamaky et al., 2001) and (Hassan et al., 2016), who recovered most of these fungi from the examined samples. On the other hand, Aspergillus flavus and A. ochraceus were recorded to a risk to public health hazard due to production of aflatoxins that cause some degree of acute toxicity when consumed in high amounts which are potential carcinogen. In developing countries, it appears that there is a direct correlation between dietary aflatoxins intake and the incidence of liver cancer (Bhatnagar and Ehrlich 2002) and (FDA, 2000).

AFB1 is the extremely potent carcinogenic type of aflatoxins that responsible for carcinogenicity that grouped a human carcinogen by world health organization reported by (Anklam et al., 2002). Aflatoxins (AFs) are usually found in various agricultural commodities (Meissonnier et al., 2008), which are known to be very dangerous mycotoxins. The exposure to aflatoxin B_1 can result in suppressed immune response, malnutrition, proliferation of the bile duct, centrilobular necrosis, fatty infiltration of the liver, hepatic lesions and even hematoma's. It is one of the most commonly found metabolites and has a highest toxigenic effects (Richard, 2007).The maximum residual levels for aflatoxin B1 set by (FAO, 2003) in food were ranged from (1-20 µg/kg). These results were higher than those obtained by (Abd- EL Kader et al., 2015) who found that AFB1 was 40% B1 was observed to (6 out of 15 samples) of all analyzed samples with ELISA methods. The average of minimum and maximum 7.14 to 11.45 µg/kg in ELISA compared to HPLC minimum and the maximum 4. 22 and 7.05 ng/g by HPLC. This refers to bad Storage condition , high temperature and law humidity were the key factors in yellow corn contamination with AFB1.

These results were higher than those by. (Hassan et al., 2010) who detected aflatoxins in 30% of feed samples with the mean value of 3.4 ± 0.1 ppb. This increasing levels of results were due to configuring the media for proliferation of fungi and producing of mycotoxin.

This results were in competence with the results obtained by (Traistaru and Moldovan 2012) who found that HPLC is suitable for the quantification of the lower content of aflatoxins as HPLC is highly performance ,pressure , precise, sensitive and accurate chromatographic technique important in detection and determination of qualitative and quantitative analysis based up on the adsorption as well as partition, due to the nature of stationary phase if solid or liquid respectively which can separate mixture of compound in chemistry and biochemistry with the purpose of identifying , quantifying , purifying the individual components of the mixture.

4. CONCLUSION

HPLC is accurate precise routine analysis for detection and determination of total aflatoxins residues in feed of qualitative and quantitative analysis due to its highly sensitive and accurate technique .

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