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Rate of contamination of some beef and pork meat products with some mould and its mycotoxins

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Abstract

The present work was designed to study occurrence of moulds and their aflatoxin residues in some beef and pork meat products and their hazards on public health. Forty samples of different meat products including minced beef, sausage beef, minced pork and sausage pork (ten each) were collected randomly from different supermarkets and shops in Cairo and Giza governorates and examined for detection of moulds and aflatoxins .The obtained results indicated that the mean counts of moulds were 2.98 ± 0.304, 3.08±0.237, 2.55± 0.178 and 2.631 ± 0.19 (log cfu /g) in minced beef, sausage beef, minced pork and sausage pork, respectively. The isolated species were belong to 3 species (*Aspergillus*, *Cladosporium* and *Penicillium* spp.). Beef and pork sausage samples were heavily contaminated with moulds more than other samples examined (80% and 60%). *Aspergillus spp*. were the most prevalent species recovered from the examined meat product samples. *Penicillium* could not be isolated from the examined beef products samples while it could be isolated from the minced and sausage pork products by (33.3%). *A. flavus* and *A. niger* were the most prevalent *Aspergillus* spp., recovered from the examined meat product samples. Aflatoxins (B_1, G_1 and G_2) could be detected in the all types of analyzed meat products samples with different concentrations, while B_2 was detected in beef sausage only. Also AFB_1 was the most prevalent toxin in the contaminated samples. The public health significance of isolated mould species and aflatoxin as well as recommended hygienic measures to keep meat products safe were discussed.

Keywords*: Minced beef, minced pork, sausage beef, sausage pork, moulds, aflatoxins.*

Introduction

Food of animal origin has an important role in the balanced diet and in recent years there is a rapid expansion of meat products processing plants to face the continuous increasing demand of meat product with low cost, high nutritive value and safe for human consumption. But the microbial contamination (including fungal contamination) is main problem in the process of food production. As meat with high nutritional value foodstuff, it's considered an excellent medium for growth and multiplication of different moulds genera as well as aflatoxin production **(Hanssen, 1995).**

Moulds are filamentous fungi widely distributed in nature and may contaminate our food supply and feedstuffs including roughages and concentrates affecting their quality leading to severe economic losses and a public health problem **(Marwa *et al*., 2013 and Elsayed *et., al* 2018).**

Health problems associated with high levels of airborn mould spores were recorded including allergic reactions, asthma episodes, irritations of the eye, nose and throat, sinus congestion, and other respiratory problems **(Fisk *et al*., 2010)**. In addition to the ability of some mould species produce secondary metabolite myco-

toxins causing public health hazard **(Pal, 2002; Bennett, and Klich, 2003 and Richard, 2007).**
Aflatoxins occupy the most important group among mycotoxins produced by certain kind of moulds that are found naturally all over the world. Two closely related species, Aspergillus flavus and Aspergillus parasiticus, under favourable conditions including high temperatures and humidity are mainly responsible for production of aflatoxins of public health significance. Contamination of food and feed commodities resulting in serious health threat to humans and livestock and a significant economic burden as an estimated 25% or more of the world's food crops could be destroyed annually (**WHO, 2018).**
Chronic exposure for aflatoxin has several health consequences including liver cancer, teratogenic effects and immunosuppression. Consumption of food containing aflatoxin concentrations of 1 mg/kg or higher causing aflatoxicosis **(WHO, 2018).**
Therefore, the current study was planned out to evaluate the prevalence of moulds species that may be present and determine the quantities of aflatoxins residues in some beef and pork meat products sold in the Egyptian markets.

Materials and Methods

1- Collection of samples:

Forty random samples of minced beef, sausage beef, minced pork and sausage pork (10 of each) were collected from different shops at Giza city. Samples were transferred under hygienic conditions in sterile polyethylene bags without delay and transported to the laboratory for mycological examination and aflatoxins detection.

2- Preparation of samples: (ISO 6882-2/2003)

Twenty five grams of each sample were transferred aseptically finely grounded and mixed with 225 ml of sterile 1% peptone water and serial decimal dilutions for microbiological examination were prepared.

3- Isolation and Identification of moulds: (APHA, 2001)

It was applied using pour plate technique of already prepared samples on Dichloran rose Bengal Chloramphenicol agar. The inoculated plates were incubated at 25°C±1°C for 7 days and the total mold count/gm was calculated. Counted isolated mould colonies were selected, purified and identified individually by macroscopic and microscopic characteristics under oil immersion lens according to **Pitt and Hocking (1999).**

4- Quantitative analysis of total aflatoxins residues (In the Reference Lab of Animal Health Research Institute) according to (Anklam *et al.,* 2002 and Abd El Monem *et al.,* 2015).

Total aflatoxins standards were obtained from Sigma (St. Louis, MO, USA) of 99% purity. Working solution was prepared in acetonitrile and stored at -20 ºC in amber glass vials over a period of 12 months.

4.1. Apparatus and Equipment

Liquid chromatography: (HPLC) Agilent Series 1200 using fluorescence (FLD) detector with which was excitation at 360nm and emission at 440 nm. The chromatographic separation was performed with a reversed-phase column (Extend-C18, Zorbax column, 4.6 mi.d., 250 mm, 5 µm, Agilent Co.). Solid-phas extraction (SPE) columns: -Bond Elute C18.HLB Oasis cartridges (6 ml), Electro non digital balance, Mincer, Shaker, nitrogen evaporator, vacuum manifold and acrodiscs (0.45µm)

4.2. Chemical reagents:

Deionized water (HPLC-grade). Acetonitrile, Methanol "HPL grade" were obtained from Sigma Aldrich and Acetic acid diluted in water. 20% citric acid from Merck (Darmstadt, Germany), dichloromethane, Hexane, petroleum ether, acetone and trifluoro acetic acid were obtained from Fischer-scientific (Leicestershire, UK).

4.3. Preparation of samples for aflatoxin extraction.

Meat products samples were homogenized and frozen at -20ºC in the dark till the time of analysis

4.4. Extraction of aflatoxins.

Extraction of total aflatoxins residues from meat products tissues was done according to.

SPE (Solid Phase Extraction) and derivatization step was done according to, twenty µl were injected into HPLC.

4.5. Liquid chromatographic conditions.

To achieve the optimum resolution of the aflatoxins injection volum 20 µl , flow rate of 1.0 ml/min. The column temperature was at 30 and flurescence (FLD) detection was carried out at excitation at 360nm and emission at 440nm. Liquid Chromatographic mobile phase: Isotonitrile mode using 60:20:20 water / methanol acetonitrile mixture as the mobile phase according to **Abd El- Moneme *et al*., (2015).**

5. Statistical Analysis

The obtained results were statistically evaluated by using Statistical Package for Social Sciences (SPSS) according to **Sabine and Brian (2004).**

Results and Discussion

Meat is preferred by millions of people all over the world as a major source of animal protein, but it is considered a favorable media for growth of various pathogens including mould, so it act as hygienic hazard problem on the consumer **(Elsayed *et al*., 2018).**

Table (1). Mean values of total mould count (log cfu/g ±SE) in the examined meat product samples (10 each).

Types of examined samples	Positive samples		Mould count		
	No.	%	Minimum	Maximum	Mean ±SE
Minced beef	5	50	2.3	3.90	2.98± 0.304
Sausage beef	8	80	2.3	3.95	3.08±0.237
Minced pork	3	30	2.3	2.9	2.55± 0.178
Sausage pork	6	60	2.69	3.90	2.631 ± 0.19

S.E.: Stander Error
Sausage beef should be free from mould (Egyptian standard 1972/2005)

Table (2). Prevalence of identified mould genera in the contaminated meat products samples.

Types of examined samples	Positive samples	Aspergillus flavus		Aspergillus niger		Cladosporium cladosporidiae		Penicillium solitum	
		No.	%	No.	%	NO.	%	NO.	%
Minced beef	5	3	60	1	20	2	40	ND	ND
Sausage beef	8	5	62.5	2	25	4	50	ND	ND
Minced pork	3	1	33.3	2	66.6	1	33.3	1	33.3
Sausage pork	6	4	66.6	1	16.6	4	66.6	2	33.3

ND: Not Detected

Results in **table (1)** revealed that moulds were isolated from all types of examined meat products with different rates and the sausage products had higher incidences of contamination (80% and 60% of beef and pork samples, respectively) than that for both minced beef and pork samples (50 and 30%, respectively).

The mean total count of mould in minced beef was 2.98 ± 0.304 (log cfu/g) and the result is nearly similar to (2.44±1.6 log cfu /g) that recorded by **Elsayed *et al*. (2018)** and higher than the result (1.57 ± 0.11 log cfu/g) recorded by **Sachindra *et al*. (2005)**. While higher results were recorded by **Zakarya *et al* (2014)** who reported the total mould count in examined minced meat samples was 3.0×10^3 cfu /g.

However, the mean count of mould in the examined sausage beef samples was 3.08±0.23 (log cfu/g) which agree with results (3.76 ± 2.96 and 3.32 ±3.09) that obtained by **Brr *et al*. (2004)** and **Howayda *et al*., (2015)** . While lower results obtained by **Fatma (2016) and**

Elsayed, *et al.* (2018) as they recorded (2.93 ±2.64 and 2.83 ±2.07cfu/g), respectively and higher result (5.63 log cfu/ g) was recorded by **Coolborn and Adenike (2016).** Also **(*Hassan et al., 2014)*** concluded that the highest mean count of mould recorded in sausage samples among different examined meat products was (4.20 ± 4.09 log cfu/ g).

In the same table the results showed that low mean count of mould in the examined minced pork samples (2.55± 0.178 log/cfu/g) if compared with the results obtained by **Omorodion and Odu (2014)** who recorded that the mean count of mould in pork meat ranged from 4.78 to 5.27 logcfu/g.

Moulds could be isolated from 60% of the examined sausage pork with a mean count 2.631 ± 0.19 (log cfu/g)**.** On the contrary, **(Hakim and Randa, 2015)** could not isolate moulds from the examined minced or sausage pork samples.

According to the **Egyptian standard schedules (1972/ 2005)** sausage beef should be free from mould, while for minced meat there is no standard limit for mould was mentioned as well as there is no standard schedules for pork meat products.

Results in **table (2)** revealed that the identified moulds were belong to 4 genera; *Aspergillus flavus, A. niger*, *Cladosporium cladosporidiae and Penicillium solatium* and among them ; A. flavus was the predominant mould isolated from all examined samples .

In minced beef, the prevalence of *A. flavus* and *A. niger* were 60% and 20% respectively. *Cladosporium cladosporidiae* prevalence was 40% while *Penicillium* species could not be isolated from this product. Lower results obtained by **Elsayed *et al.* (2018)** who found 34.28% and 14.28% of *A. flavus and A. niger,* respectively in the same product. While **(Hassan *et al.*, 2014)** could isolate *Asperagillus* spp. from all examined minced meat samples where *A. flavus* was the most predominant isolated spp.

In sausage beef**,** *A. flavus was* isolated from 62.5% of isolated spp. followed by genus *Cladosporium cladosporidiae* in 50%. while *A. niger* could be isolated in a lower percentage (25%) while *Penicillium* species could not be isolated from this product**.** Lower results obtained by **Brr *et al.* (2004)** who isolated both of *A. flavus* and *A. niger* from 23.1% of the examined sausage samples. While (**Elsayed *et al.*, 2018)** recorded that 30% and 36.6% of the identified spp. in sausage samples were *A. flavus* and *A. niger***,** respectively.

Results of examined pork meat products revealed that**;** the prevalence of *A. flavus and A. niger* in minced meat were 33.3% and 66.6 %, respectively, while both of *Cladosporium cladosporidiae and Penicillium solitum were* isolated with the same ratio (33.3%) out of the examined samples and in sausage samples, the prevalence of *A.flavus* and *A. niger* were 66.6% and 16.6% ,respectively and 66.6% and 33.3% for *Cladosporium cladosporidiae* and *Penicillium solitum ,* respectively. (**Omorodion and Odu, 2014)** could isolate *Asperagillus* and *Penicillium* spp, from (46.15%) and (20.08%) of examined pork meat samples, respectively.

As moulds are microorganisms widely distributed in nature, the conditions under which meat and meat products are sold is very important, so meat products may be contaminated in markets and butcher shops through air dust during their improper viewing for sale in the open air as well as via contaminated tools, cross contamination from workers and insufficient cleaning and disinfection of the working areas may lead to musty or moldy aroma during preparation **(Ostrý, 2001;Ina, 2005 and WHO, 2018).**

In addition, storage temperature is one of the major controlling factors of food quality and safety because of its influence on microbial growth rates as low temperature can reduce the growth rate of many microorganisms. **(WHO, 2007).**

Aspergillus and Penicillium spp. have been reported in association with infections and disease either superficial (skin and nail infections) or invasive infections as chronic granulomatous sinusitis, osteomyelitis, otomycosis, pneumonia, endocarditis and urinary tract infections **(Howard, 2002, 2003; Walsh *et al.*, 2004; Zhang *et al.*, 2005; Hedayati *et al.*, 2007; Person *et al.*, 2010; Khan and Karuppayil, 2012).**

Mycotoxins are produced by certain moulds. Several types of aflatoxin occur in nature, but four aflatoxins B_1, B_2, G_1 and G_2 are particular-

ly dangerous to human and animal AFB1 is the most toxic and is classified as a human carcinogen **(IARC, 2002).** Also they are very heat resistant to most forms of food and feed processing and are difficult to be eliminated from foods **(Mehrdad, 2007 and Patriarca and Pinto, 2017).**

Table (3). Statistical analysis of aflatoxin residues (ppb) in the analyzed meat product.

Types of Toxin		Analyzed meat products			
		Minced beef N=10	**Sausage beef N=10**	**Minced pork N=10**	**Sausage pork N=10**
TAF	t / N (%)	7/10 (70%)	9/10 (90%)	8/10 (80%)	10/10 (100%)
	Min.	0.152	0.112	0.47	0.799
	Max	1.663	3.759	1.918	5.108
	Mean	1.041 ±0.295	1.91±0.675	1.130±0.208	2.525±0.527
AFB_1	n / t (%)	3/7(42.8%)	9/9 (100%)	6/ 8(75%)	10/10(100%)
	Min.	0.128	0.112	0.026	0.397
	Max.	0.674	3.759	1.799	4.144
	Mean	0.371±0.16	1.99±0.501	0.783±0.416	2.270±0.65
AFB_2	n / t (%)	ND	3/9(33.3%)	ND	ND
	Min.	ND	0.757	ND	ND
	Max.	ND	2.095	ND	ND
	Mean	ND	1.464±0.387	ND	ND
AFG_1	n / t (%)	3/7(42.8%)	1/9(11.1%)	5/8(62.5%)	8/10(80%)
	Min.	0.152	0.042	0.119	0.067
	Max.	0.195	0.042	0.57	0.964
	Mean	0.169±0.013	0.042	0.439±0.083	0.396±0.141
AFG_2	n / t (%)	3/7(42.8%)	ND	4/8(50%)	1/10(10%)
	Min.	0.973	ND	0.458	0.287
	Max.	0.988	ND	0.812	0.287
	Mean	0.980±0.004	ND	0.635±0.102	0.287

ND: Not Detected TAF: Total aflatoxin SE: Stander Error /: per
N: number of analyzed samples n: number of samples contaminated with B_1,B_2,G_1 or G_2
t :number of samples contaminated with TAF
The acceptable level of aflatoxins is 20 ppb (EC, 2006 and FDA, 2011).

Fig. (1): Calibration curves of aflatoxin B1,B2,G1 and G2 by using HPLC

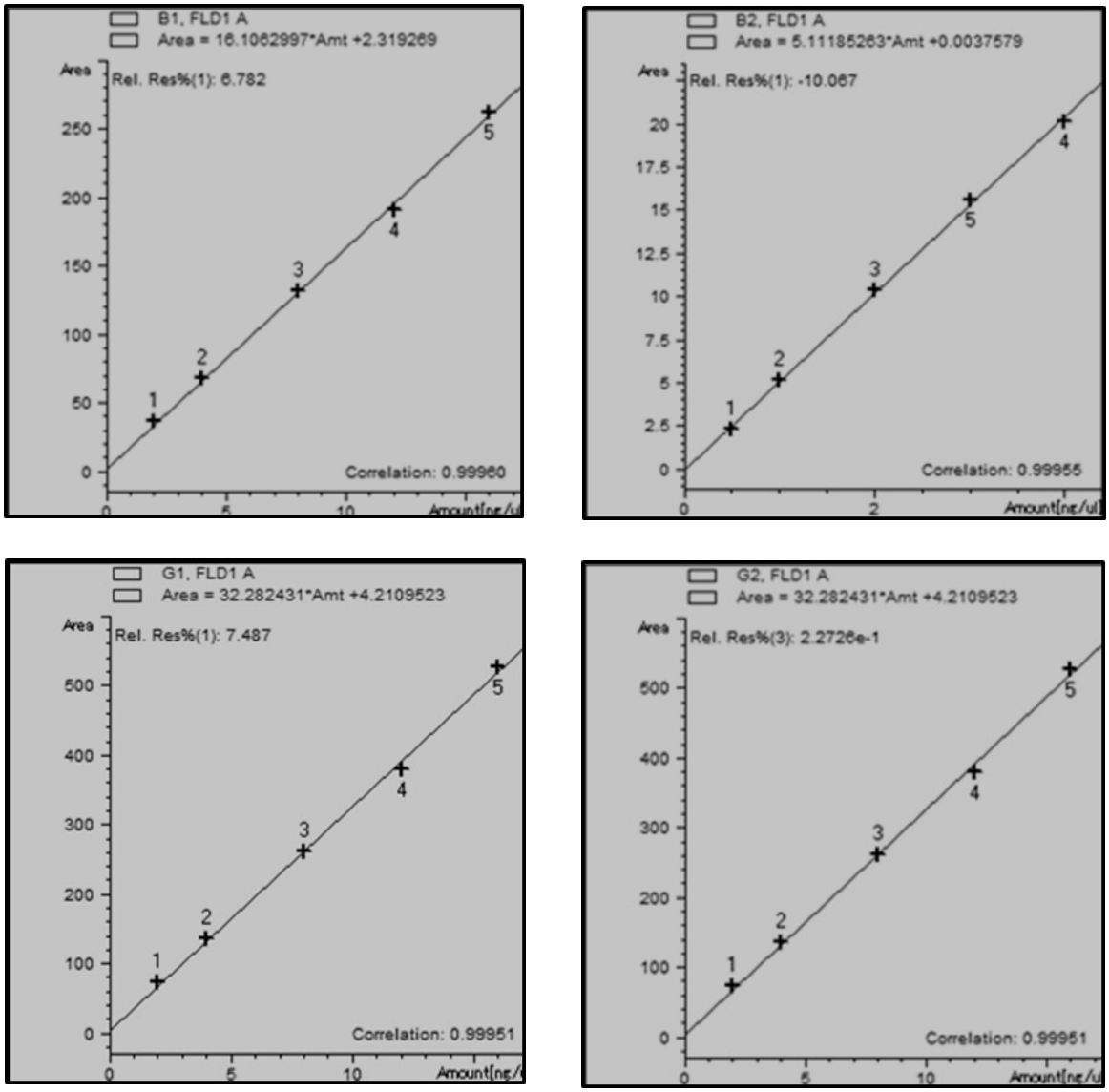


Table (4). Regression equation parameters

Values of toxins	Parameters				
	Linearity range	**Correlation coeffi-cient**	**Sloper**	**Intercept**	**Regression equation**
B1	275–1375 µg/kg	0.99950	16.1052997	2.319269	Area=16.1052997 *amt +2.319269
B2	275–1375 µg/kg	0.99955	5.11185203	0.0037679	Area=5.11185203 *amt +0.0037679
G1	275-1375µg/kg	0.9951	32.282431	4.219523	Area=32.282431 *amt +4.219523
G2	275-1375µg/kg	0.9951	32.282431	4.219523	Area=32.282431 *amt +4.219523

Table (5). Cocentration of aflatoxins (B_1, B_2, G_1,G_2 /ppb) spiking in blank muscle and their responding peak response automatically using HPLC

#	RT	Signal	Compound	Lvl	Amt[ng/ul]	Area	Rsp.Factor	Ref	ISTD	#
1	13.519	FLD1 A	B1	1	2.000	36.874	5.4239e-2	No	No	
				2	4.000	67.969	5.8850e-2			
				3	8.000	132.150	6.0538e-2			
				4	12.000	191.030	6.2819e-2			
				5	16.000	262.360	6.0986e-2			
2	18.719	FLD1 A	G1	1	2.000	73.925	2.7055e-2	No	No	
				2	4.000	135.980	2.9416e-2			
				3	8.000	263.070	3.0411e-2			
				4	12.000	381.310	3.1471e-2			
				5	16.000	526.850	3.0369e-2			
3	23.454	FLD1 A	G2	1	0.500	2.297	2.1765e-1	No	No	
				2	1.000	4.100	2.4390e-1			
				3	2.000	16.389	1.2203e-1			
				4	3.000	22.252	1.3482e-1			
				5	4.000	29.626	1.3501e-1			
4	29.200	FLD1 A	B2	1	0.500	2.302	2.1720e-1	No	No	
				2	1.000	5.201	1.9227e-1			
				3	2.000	10.415	1.9203e-1			
				5	3.000	15.625	1.9200e-1			
				4	4.000	20.154	1.9847e-1			

Aflatoxins standard concentrations as showen in **table (5)** were prepared and treated according to described extraction procedure and their corresponding peaks response (area under peak) were illustrated in **table (5)** and **fig. (1).** The calibration curve was calculated by linear regression equation method as for B_1: y=16.1052997*amt +2.319269, B_2: y=5.11185203*amt +0.0037679, for both G_1 and G_2: y= 32.282431*amt +4.219523 where y symbol indicated area under peak and amt symbol indicated concentration of aflatoxins as shown in **table (4).** Linearity existed within range 275 - 1375 µg/kg with a correlation coefficient 0.9995. Retention times of the analytes are shown on the recorded **fig. (2, 4 and 5)** and **table (5)** were for aflatoxin B_1 Rt. =13.521min., for G_1 Rt. =18.734 min. and for G_2 Rt. =23.345 min. But retention times of the analytes are shown on the recorded **fig. (3)** were for aflatoxin B_1 Rt. =10.451min., B_2 Rt. = 29.692 min. and for G_1 Rt. =27.203 min.

In **table (3)** the analysis of meat products samples revealed that the total aflatoxins (TAF) as well as B_1, B_2, G_1 and G_2 aflatoxins could be detected and estimated in the all types of analyzed meat products samples with different incidences and concentrations.

The results revealed that for minced beef samples, from 10 samples only 7 samples (70%) were contaminated with aflatoxins with mean value 1.041±0.295 ppb, and each of AFB_1, G_1 and G_2 was detected in 3 different samples only (42.8%) of contaminated samples and G_{2s} recorded the highest mean value among them (0.98 ±0.004 ppb) as B_1 and G_1 recorded mean values (0.371± 0.16 and 0.169± 0.013 ppb, respectively) while B_2 not detected in any sample. **Hassan *et al* . (2014)** could detect aflatoxin residues B_1, B_2, G_1 and G_2 in minced meat (3.62 ± 0.88, 3.40 ± 0.82, 4.24 ± 0.85, 2.83 ± 0.60 ppb), respectively.

However, 90% of the sausage beef samples were contaminated with aflatoxins residues with mean concentration 1.91±0.675ppb where B_1 was the predominant toxin as it was detected in the all contaminated samples (100%) followed by B_2 that detected in only 3 (33.3%) samples with mean value (1.99±0.501and 1.464±0.387 ppb), respectively, on the other hand G_1 was detected in one sample only with a concentration 0.042 ppb while G_2 could not be detected in any sample. On contrary **(Howayda, 2008)** detected the aflatoxin resi-

dues in (46.66%) of sausage samples and (**Zohri *et al*., 2014)** could extract aflatoxin B_1 from only 10 % of the same product, also **(Shaltout *et al*., 2014)** could extract B_1, B_2, G_1 and G_2 aflatoxins in sausage samples by higher concentrations 9.03 ± 1.17, 5.57 ± 0.72, 3.35 ± 0.49 and 2.33 ± 0.29 ppb, respectively, while lower results were obtained by **Morshdy *et al*. (2015)** who detected the aflatoxins B_1, B_2, G_1 and G_2 with mean values of 0.69 ± 0.22, 0.31 ± 0.15, 0.62 ± 0.28 and 0.16 ± 0.05 ppb, respectively.

Fig. (2): Chromatograms of Total Aflatoxins residues in minced beef sample

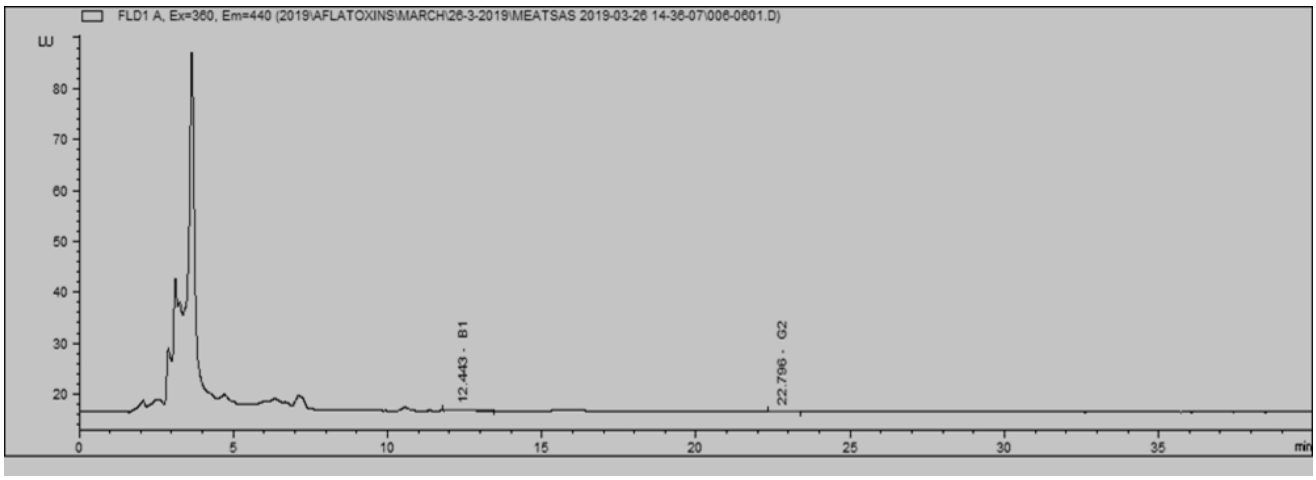
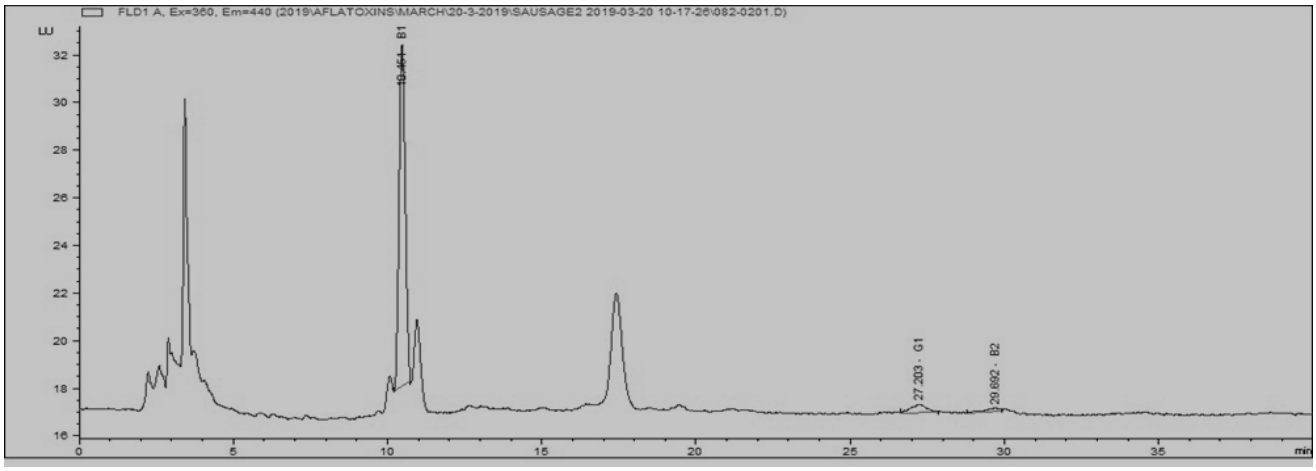


Fig. (3): Chromatograms of Total Aflatoxins residues in sausage beef sample



For pork products, data in the same table revealed that 80% and 100% of examined minced and sausage pork samples were contaminated with aflatoxins and the mean concentration of TAF was higher in sausage samples (2.525 ± 0.527 ppb) than that detected in minced meat (1.130 ± 0.208 ppb).

Otherwise, B_1 and G_1 represented the main aflatoxins detected in the two products samples as they were detected in (75% & 100%) and (62.5% &80%) of contaminated minced meat and sausage samples with mean concentrations (0.783 ± 0.416 & 2.270 ± 0.65 ppb) and (0.439 ± 0.083 & 0.396 ± 0.141 ppb), respectively. While G_2 could be detected in both minced and sausage pork samples in 4 (50%) and 1 (10%) samples only with concentrations (0.635 ± 0.102 & 0.287 ppb), respectively while both of pork products samples were free from AFB_2.

Fig. (4): Chromatograms of Total Aflatoxins residues in minced pork sample

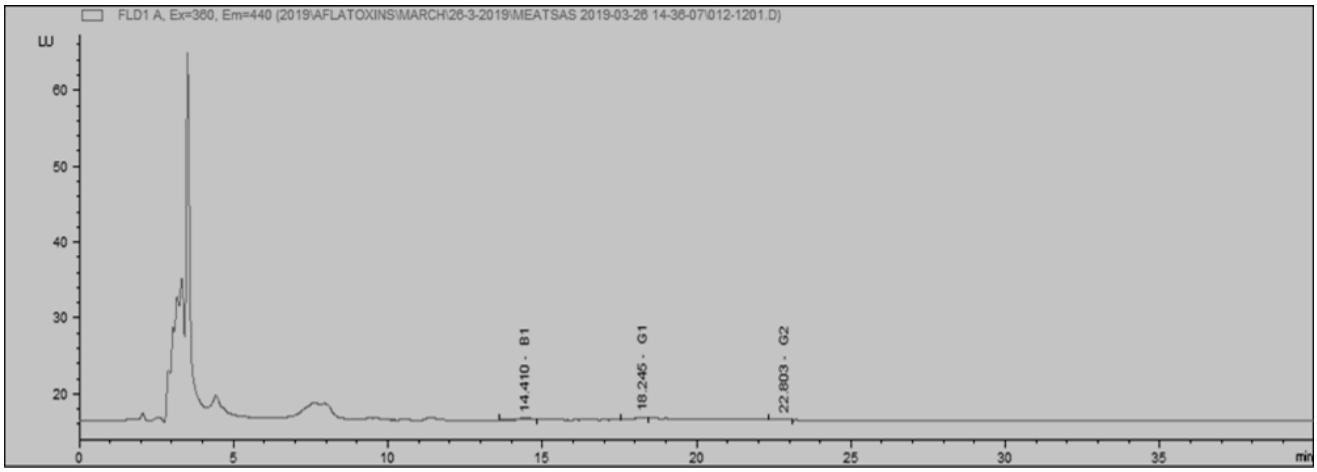
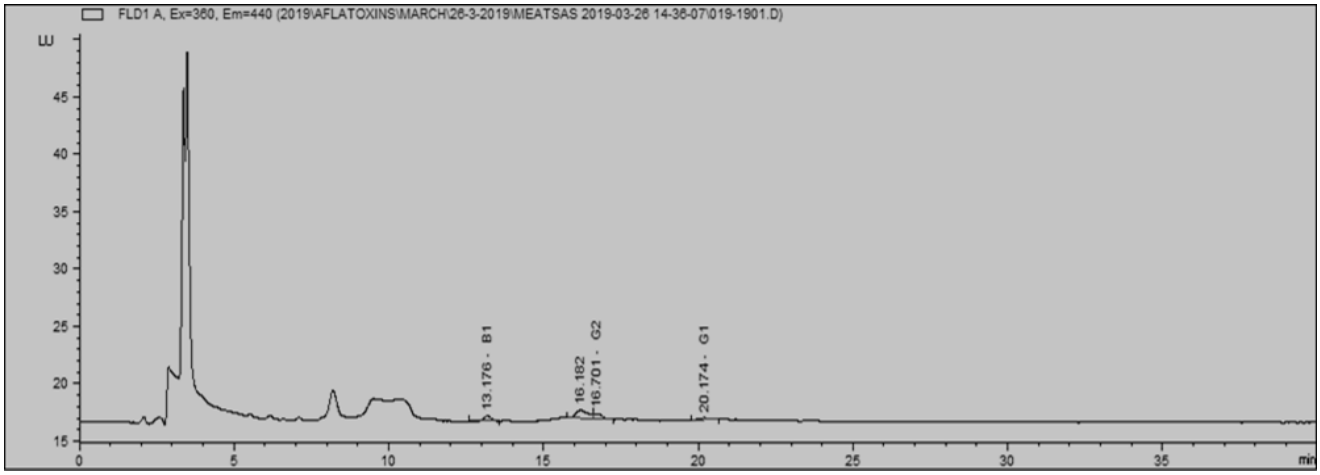


Fig. (5): Chromatograms of Total Aflatoxins residues in sausage pork sample



Aflatoxins contamination of the examined meat products samples could be originated either from the animal tissues previously fed on aflatoxin contaminated feed**. (Badr *et al*., 2017; Osman *et al*., 2017 and Choochuay *et al*., 2018).**

Moreover, food additives as spices including red chilli, black pepper, dry ginger and flavourings that added in food processing as sausage represent a source of secondary mycotoxin contamination of the final products as they are susceptible substrates for mycotic growth and mycotoxin production, they remain throughout processing and storage **(Maria *et al*., 2001, Miakova *et al*., 2002 and Jeswsal & Kumar, 2015).** And their improper storage conditions (warm and humid storage environments) leads to levels of contamination much higher than those found in the field **(WHO, 2018).**

The obtained results revealed that aflatoxins residues were detected in all types of analysed meat products in levels below the international permissible limits. Food and Drug Administration **(FDA, 2011)** established regulatory working guidelines on the acceptable levels of aflatoxins in human foods set at 20 ppb for total aflatoxins and the maximum permissible limit recommended by **European community (EC) No 1881/2006** in food for human consumption of 10 µg/kg (10ppb) for total aflatoxins B_1, B_2, G_1 and G_2,.

Based on past outbreaks it has been estimated that consumption of an AFB1 dose of 20–120 µg/kg /day over a period of 1–3 weeks is acutely toxic and potentially lethal therefore; the international risk managers as the Codex Alimentarius Commission, established very low maximum levels for mycotoxins in food due to their severe toxicity and their cumulative effect as the maximum levels of B_1 and total aflatox-

ins set by the Codex in cereals are (2 and 4 µg/kg)and (5 and10 µg/kg for spices), respectively.
Exposure of human to mycotoxins either directly or indirectly through the ingestion of meat products from animal fed on contaminated feedstuffs resulting in death in acute case due to intoxications or to hepatocellular carcinoma as a chronic case due to their accumulation effect **(Miliţă *et al.*, 2010)**

Conclusion and Recommendations
Meat products in this study were subjected to various degree of mould and aflatoxins contamination through meat processing so, strict hygienic measures including all steps of manufacturing, handling during the production stages of meat products must be applied :-

1-Proper investigation and hygienic storage of animal feed and food additives are highly recommended to avoid fungal and subsequently mycotoxins production.
2- Slaughtering and preparation of carcasses should follow the good hygienic measures.
3- Application of strict hygienic measures including all steps of manufacturind and handling during the production stages of the products.
4- Meat products that for sale must be kept refrigerated and covered to prevent exposure to mold spores in the air as using plastic wrap.
5- Educational programs and training courses must be applied for meat handlers and workers.
6- Strengthen the control of the state on markets to ensure the application of these hygienic measures.

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