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Detection the Presence of Ochratoxin A from *A.Niger* and *A.Ochraceus* Isolated From Wheat and Maize Grains by Using TLC and HPLC Techniques

Eman Salman Rahaif and IbtihalMuizAl-Hussaini

Department of Biology, Babylon University

emansalman90@yahoo.com

Abstract:

The main objective of this study was to detectionthe presence of OTA in wheat and maize grains in Babylon Governorate in Iraq.. Wheat (Triticum*aestivum*) and maize (*Zea may*) grains were used todetect OTA concentrations. The presence of OTA in grains was determined by TLC andHPLC. The results of TLC indicated that 5 of 6 strains of *A.niger* was produced of OTA and 4of 4 strains of*A.ochraceus* produced of OTA. The results of HPLC indicated that the concentration of OTA extract from *A.ochraceus* that isolated from maize grains recorded the highest concentration (98.3 , 124.9) ppb respectively, while the concentration of OTA extract from *A.niger* that isolated from wheat grains recorded the highestconcentration(8.14, 3.50) ppbrespectively, than that isolate from maize grains.

Keyword: Ochratoxin A, *A. niger*, *A. ochraceus*, TLC, HPLC, wheat, maize .

Introduction:

Mycotoxins are secondary metabolites formed by filamentous fungi such as *Aspergillus, Fusarium, Penicillium, Alternaria,* and *Claviceps* that develop on agricultural commodities under various climatic conditions. Mycotoxin can be found in cereals, cereal products, and meat, as well as feed, animal products, and soil. (Marin et al., 2013). Ochratoxin A, B, and C are the three forms of ochratoxin; OTA is the most common and dangerous mycotoxin found in foods. (A lhamoud*et al*.,2019). Ochratoxin A (OTA) is the most common mycotoxin in the ochratoxins community, and it's produced by several fungal species from the *Aspergillus and Penicillium* genera. (Cavin*et al.* 2007). *Penicillium (P.) verrucosum, Aspergillus (A.) ochraceus, and Aspergillu*s segment Nigri produce the most OTA (Magan and *Aldred 2005). P. verrucosum is the most common OTA-producing fungus in northern Europe, while A.* ochraceus is more important in warmer climate areas. (Cairns-Fuller *et al.* 2005). Cereals, seeds, groundnuts, raisins, coffee, beer, and wine, as well as some animal products, contain OTA. (Jorgensen,K.,2005). Cereals (wheat, barley, and oats) are the main source of human exposure to OTA (Duarte and Pena,2010). 5 ng/g OTA in raw cereal grains, 3.0 ng/g in cereal-processed products, 10 ng/g in coffee and dried fruits, and 2 _g/L in wine are the maximum limits. (Duarte *et al.*,2010).

OTA contamination is also prevalent in feedstuff, with cereals being the most commonly polluted feed product. (Streit*et al*.,2012). To determine the OTA in food, several methods have been developed, including cereals (barley, corn, wheat bran, and flour), coffee, wine, beer, and dried fruits (EFSA, 2006) The detection of OTA in wheat can be done using thin layer

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chromatography methods (Larsson and Moeller 1996).. Immune affinity (IMA) clean-up combined with high-performance liquid chromatography (HPLC) is used in the majority methods, accompanied by fluorescence detection (FD). HPLC coupled to mass spectrometry (MS) or tandem mass spectrometry (MS=MS) is quickly becoming a popular method for screening, identifying, and quantifying a wide range of mycotoxins, including OTA. (Visconti, Pascale, and Centoze 2001; Krska&Molinelli 2009;Lattanzio *et al*. 2007; Shephard et al., 2009). Reverse phase liquid chromatography is widely used to separate and detect ochratoxin A (OTA) in foods, feeds, and biological fluids (Scott, 2002). Alternative detection approaches include photodiode array detection (LC-DAD). the Food and Agriculture Organization (FAO) (IARC,1993).

Materials and Methods:

Sampling

The Grains (Wheat and Maize) were collected from the Silo of Babylon and AL Diwaniyah Governorate and Markets, at 3kg per sample. The samples were placed in sterile paper bags and labeled and brought to the laboratory.

Media and Growth Conditions.

The Fungi associated with Wheat and Maize grains used in this research were isolated by surface sterilization of the grain using a solution of sodium hypochlorate at a concentration of 1 % for a 2-5 minutes , followed by three washes with sterile distilled water. The grains was culturein Petri dishes contained PDA medium with 10 grains per dish and in three Repeated and dishes were placed in the incubator at 25˚temperture for a period of 7 days. Then the plates were examined for developing fungi .

Ochratoxin A Extraction

TheOchratoxin A was extracted from *A.nige*r and *A. ochraceus* according to Macdonald *et al*.,(1999), where three dishes were taken from the growth Fungi for a week at a temperature of 30˚C and cut the grown medium of Fungi in each plate and put these pieces in an electric blender and add 100 ml of a mixture consisting of water and chloroform in a ratio of 1:1, after which the mixture was filtered by a 4 layers of muslin cloth and then transfer the filtrate to a separating funnel and shake well. This extract was evaporated to dryness at 45˚C and the dried extract was stored at 4 ˚C.

Detection of Ochratoxin A in *A.niger* and *A. ochraceus*

1:Thin Layer Chromatography (TLC)

The TLC technique was used to detect the ability of *A.niger* and *A.ochraceus* isolated from wheat and maize grain . A(9:1) methanol : distilled water separation system used. A light straight line was made on the TLC plate 1.5 cm away from the base of the lower plate and then 1.5 µl of the standard ochratoxin A was applied by a capillary tube on the line. On the left side, the same amount of each sample was placed to the right of the standard Ochratoxin A After that, the spots were left to dry, then the TLC plates were placed in separation basin containing a mixture of methanol and distilled water 9:1 respectively and monitored until the mobile phase reached a distance of 1.5 cm from the upper end of the plate. The plate was left to dry and then examined under ultraviolet ray with a wavelength of 365nm. The revealed the presenced of the toxin by matching the location and color of the fluorescent of the extract with the location and color of Ochratoxin A (Sobolev and Dorner, 2002).

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2: High Performance Liquid Chromatography (HPLC)

Ochratoxin A was extracted from samples (5ml or 5gm) by homogenization with 20 mL acetonitrile : H2O (6:4, v=v) for 2 minutes. The extract was filtered and 4 mL of filtrate was diluted with 25mL phosphate buffer saline pH 7.4 (PBS). The samples were degassed in a sonic bath for 30 minutes, than the pH was adjusted to 7.2 using 2 M sodium hydroxide5 mL of acetonitrile is added to the sample and then stored until the analysis is performed.

HPLC Condition :

HPLC model SYKAMN (Germany) It was used forthiamethoxamanalyses and detection. The mobile phase was an isocratic acetonitrile : D.W : formic acid (50 : 47 : 3) at flow rate at 0.6 mL/min , column was C18 – ODS (25 cm * 4.6 mm) and the detector Florescent (Ex = 365 nm , Em = 445 nm).

Results and Discussion

The presence of OTA in wheat and maize grains was detected by using TLC and HPLC. Table 1 represent the RF values of OTA detection by TLC , 6 strains of *A.niger*, strains 2 and 4 have 2 spots, while strains 1, ,5 have only one spot and strain 3 have no spot. 4 stains of *A.ochraceus* strain 3 and 4 have 2 spots , and 1,2 have one spot.

Table 1: RF value of OTA detected by TLC for *A.niger*and*A.ochraceus*from wheat and maize grains

Fungi	Strain No.	Spot No.	RF %
A.niger	**1**	**1**	**85.88**
	2	**1**	**59.41**
		2	**85.29**
	3	**–**	**–**
	4	**1**	**84.70**
		2	**42.35**
	5	**1**	**63.52**
	6	**1**	**79.41**
		2	**91.76**
A.ochraceus	**1**	**1**	**58.23**
	2	**1**	**85.29**
	3	**1**	**81.76**
		2	**76.47**
	4	**1**	**84.70**
		2	**58.23**

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Table 2: Standard Ochratoxin A (0.25 ppb)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]
1	8.517	302.176	56.166	100.0	100.0	0.09
	Total	302.176	56.166	100.0	100.0	

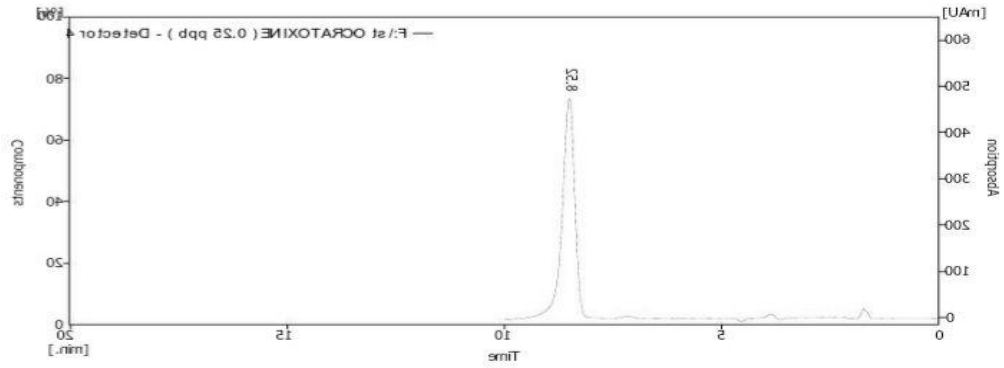

Figure 1:chromatogramstandard OCRATOXINE (0.25 ppb)

The chromatography conditions were adjusted starting from injection of the standard solution.. Table 2 shows the linearity from OTA analysis's by proposed method. Figure 1 presents the chromatograms obtained from OTA standard solution (0.25 ppb). The retention time 8.517 min and the area 302.176.

Table 3: HPLC Ochratoxin A concentration in *A.niger* and *A.ochraceus*

No	Con (ppb)	No	Con (ppb)
1. A.niger	**3.50**	***4. A.niger***	**2.25**
2. A.niger	**8.14**	***5. A.Ochraceus***	**98.3**
3. A.niger	**2.42**	***6. A.Ochraceus***	**124.9**

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Table 3 shows the concentration of OTA extracted from *A.niger* and *A.ochraceus* in wheat and maize grains obtained from HPLC .The OTA extracted from *A.ochraceus* in maize grains recorded the highest concentration than OTA extract from *A.niger* in wheat and maize grains and the concentration of OTA from *A.niger*in wheat grains was higher than maize grains .

Table 4: detection of Ochratoxin A in *A.niger*

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound name
1	1.367	124.571	16.488	15.6	15.5	0.13	***A.niger***
2	1.753	561.959	75.103	70.6	70.7	0.13	
3	3.900	71.043	8.352	8.9	7.9	0.16	
4	8.513	38.703	6.245	4.9	5.9	0.07	
	Total	796.276	106.187	100.0	100.0		

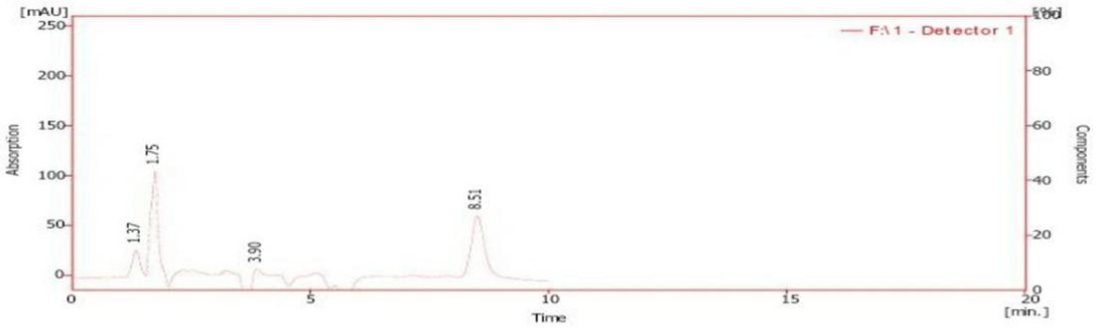

Figure 2: Chromatogram of Ochratoxin A in *A.niger*

Appropriate chromatographic conditions from the stationary phase and a step gradient polarity system in the mobile phase were used to detect the presence and concentration of OTA in *A.niger* from wheat grain. Table 4 and Figure 2 show that the OTA extract had a retention time of 8.513 minutes and area of 38.703 as opposed to the OTA standard retention time and area of 8.517 and.302.176. This result indicate the presence of OTA in *A.niger* in concentration 3.50 ppb.

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Table 5: detection of Ochratoxin A in *A.niger*

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound name
1	0.133	0.810	0.130	0.2	0.2	0.03	***A.niger***
2	1.367	122.391	15.716	34.8	26.7	0.13	
3	1.753	102.218	24.592	29.0	41.8	0.06	
4	3.900	25.605	3.929	7.3	6.7	0.10	
5	8.513	101.063	14.409	28.7	24.5	0.13	
	Total	352.088	58.776	100.0	100.0		

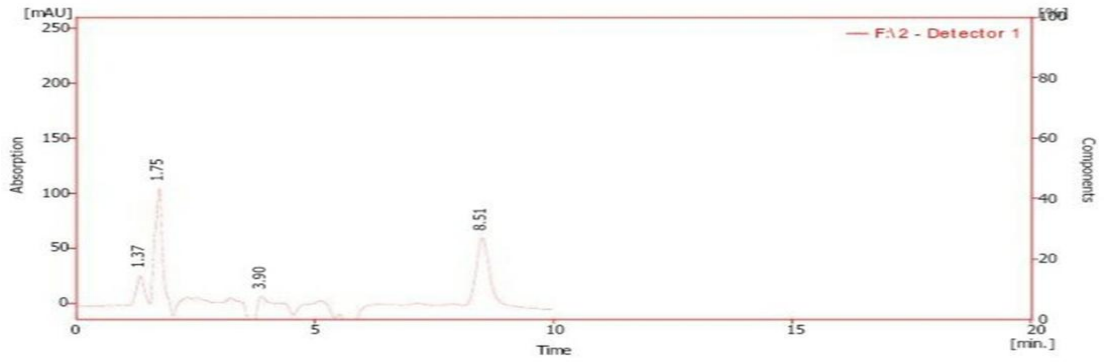

Figure 3: chromatogram of Ochratoxin A in *A.niger*

In wheat grains, the retention time of OTA extract A.niger was 8.513 min and the area was 101.063, as shown in table 5 and figure 3, compared to OTA standard retention time and area of 8.517 and 302.176, respectively. This indicates that OTA is present in A.niger at a concentration of 8.14 ppb.

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Table 6 detection of Ochratoxin A in *A.niger*

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound name
1	1.367	41.445	8.336	8.6	13.1	0.09	***A.niger***
2	1.753	135.110	26.375	28.0	41.4	0.06	
3	3.900	46.205	5.983	9.6	9.4	0.13	
4	5.127	51.326	2.524	10.6	4.0	0.13	
5	8.513	209.079	20.523	43.3	32.2	0.15	
	Total	483.164	63.742	100.0	100.0		

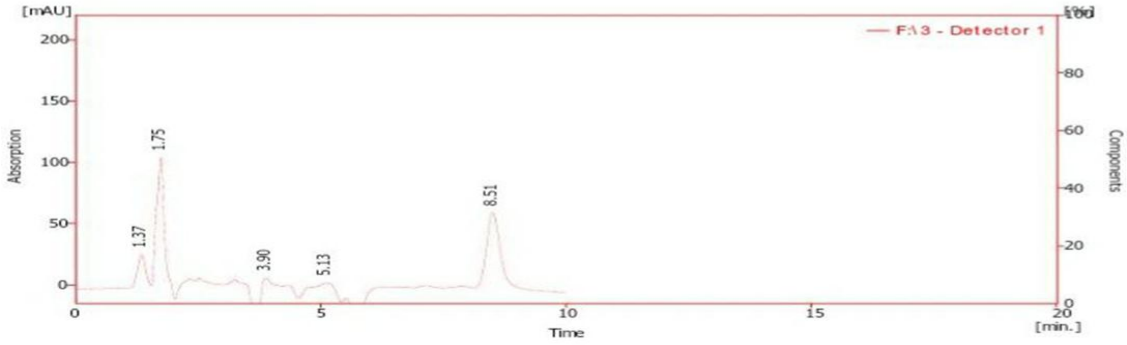

Figure 4: Chromatogram of Ochratoxin A in *A.niger*

In maize, the retention time of OTA extract from A.niger was 8.513 min and the area was 209.079, compared to OTA standard retention time and area of 8.517 and 302.176, respectively. This indicates that OTA is present in A.niger at a concentration of 2.42 ppb. table 6 and figure4

Table 7: detection of Ochratoxin A in *A.niger*

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No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound name
1	1.367	91.772	12.853	10.4	11.1	0.12	***A.niger***
2	1.753	440.768	66.578	49.8	57.4	0.12	
3	3.900	100.200	10.827	11.3	9.3	0.22	
4	8.513	252.696	25.808	28.5	22.2	0.17	
	Total	**885.435**	**116.066**	**100.0**	**100.0**		

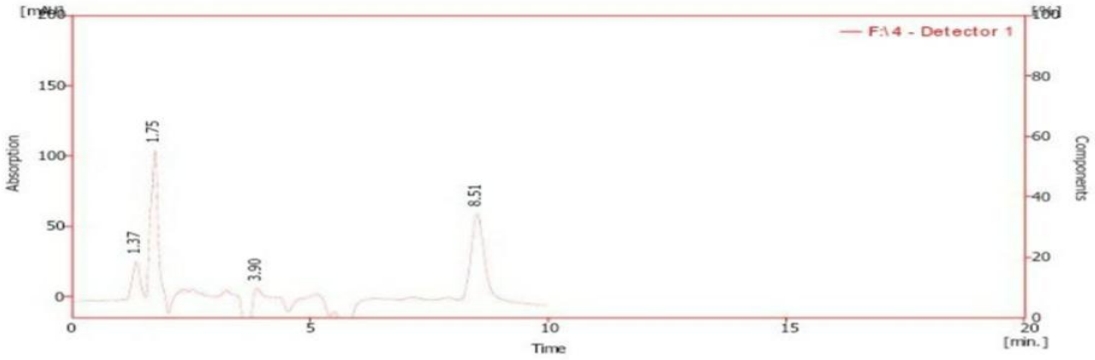

Figure 5: ChromatogramofOchratoxin A in *A.niger*

As shown in table 7 and figure5 , the retention time of OTA extract OTA in *A.niger* from maize grain was 8.513 min and area was 252.696 in compared with OTA standard retention time and area which was respectively 8.517 and 302.176. This result indicate the presence of OTA in *A.niger* in concentration 2.25 ppb.

Table 8: detection of Ochratoxin A in *A.ochraceus*

No	Reten. Time [min]	Area [mAU.s]	Heigh t [mAU]	Area [%]	Height [%]	W05 [min]	Compound name
1	1.367	176.530	20.199	11.4	12.8	0.15	***A.ochraceus***
2	1.753	724.367	84.010	46.8	53.0	0.15	
3	3.900	121.075	12.491	7.8	7.9	0.21	
4	5.127	120.245	6.963	7.8	4.4	0.36	
5	8.513	406.747	34.759	26.3	21.9	0.20	
	Total	**1548.964**	**158.422**	**100.0**	**100.0**		

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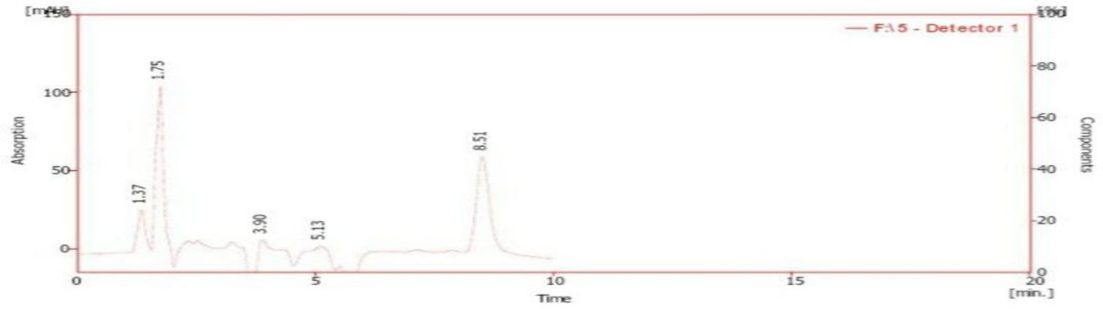

Figure 6: ChromatogramofOchratoxin A in *A.ochraceus*

The retention time of OTA in A.ochraceus from maize grain extract was 8.513 min and the area was 406.747, as shown in table 8 and figure 6, compared to the OTA normal retention time and area of 8.517 and 302.176, respectively. This indicates that OTA is present in A.ochraceus at a concentration of 98.3 ppb.

Table 9: detection of Ochratoxin A in *A.ochraceus*

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound name
1	1.367	179.218	20.192	11.3	12.6	0.15	***A.ochraceus***
2	1.753	553.891	75.216	34.9	47.0	0.13	
3	3.250	24.433	3.242	1.5	2.0	0.13	
4	3.900	100.200	10.827	6.3	6.8	0.22	
5	5.127	116.521	6.270	7.3	3.9	0.27	
6	8.513	614.021	44.331	38.7	27.7	0.24	
	Total	1588.285	160.078	100.0	100.0		

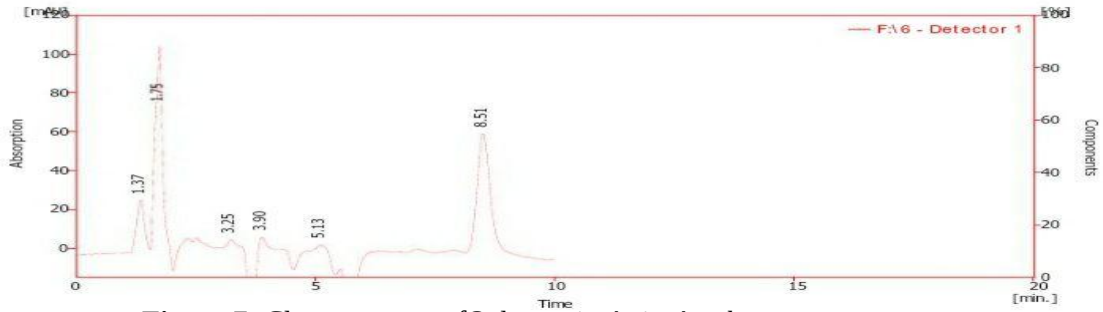

Figure 7: ChromatogramofOchratoxin A in *A.ochraceus*

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Table 9 and figure7,of OTA in *A.ochraceus* from maize grain where the retention time of OTA extract was 8.513 min and area was 406.747 in compared with OTA standard retention time and area which was respectively 8.513 and 614.021. This result indicate the presence of OTA in *A.ochraceus* in concentration 98.3 ppb. There are many studies which identify the presence of OTA in wheat and maize grains, Limay-Rios, Miller and Schaafsma, (2017) identify levels of OTA in wheat grains retailing and found to be lower than in stored wheat . 75% of *A. ochraceus*and *A.carbonarius*isolates produced OTA in a study of Brazilian maize grains, whereas only 3% of *A. niger*isolates were toxigenic (Taniwaki*et al*.,2003). Chandelier et al. (2004) discovered OTA contamination in cereal grain samples. Malir et al. (2006) looked for OTA in 114 cereal samples collected during harvest in the Czech Republic. According to Krogh et al.,(1973), detected the levels of OTA contamination in cereals range from 0.03 to 27.5 ppm.

Rizzo *et al*. (2002) The highest recorded levels of OTA in cereals in Europe were found in wheat (239 samples analysed) and rye (228 samples analysed) from Poland, with a maximum level of 2400 mgkg 1 for both cereals. Birzele et al. (2000) detected OTA in 43 freshly harvested wheat samples in Germany using the (ELISA) process. OTA was found in 60 percent of 125 wheat samples in France, the highest amount was found in a sample collected in a farm located in northern part of France and badly stored (Bedouret et al. 2001; Pfohl-Leszkowicz et al. 2007).

Conclusion

Theevaluation of the presence of OTA in wheat and maize grains was detected by using TLC and HPLC methods. The results of TLC indicated that 5 of 6 strains of *A.niger* was produced of OTA and 4of 4 strains of *A.ochraceus* produced of OTA. The HPLC results indicated that the concentration of OTA extract from *A.ochraceus* that isolated from maize grains recorded the highest concentration, while the concentration of OTA extract from *A.niger* that isolated from wheat grains recorded the highest concentration, than that isolate from maize grains.

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