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Evaluation of fungi and mycotoxins of smoked fish with special reference to some Aspergillus species.

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ABSTRACT

A total of 100 samples of smoked herring fish, 50 samples of whole fish, and 50 fillets were collected from different shops in Menofea Governorate. The samples were examined mycologically for counting, isolation, and identification of mould. The mean averages of total mould count were 3.5 $x10^5 \pm 1.14$ x 10^3cfu/g and 3.8 $x10^4 \pm 0.62$ $x10^2$ cfu/g for whole fish and fillets respectively. The predominant isolated strain is Aspergillus species particularly *A. flavus, A. niger, A. fumigatus* and *A. ochraceus* with a total percentage of 74%. The concentration values of Aflatoxins B1, B2, and Ochratoxin A ranged between 0.096 ppb -7.938 ppb, 0.075 ppb - 3.509 ppb, and 0.062 ppb – 1.219 ppb respectively. All samples are free from Aflatoxins G1 or G2. This study draws attention to the preparation and production of smoked herring fish to avoid possible health hazards from the Mycotoxins.

INTRODUCTION:

Fish is a major source of protein, particularly in Egypt. It is also an important source of vitamins, iodine and unsaturated fatty acids **(Abolagba and Melle 2008).** Fish is more susceptible to contamination, so it must go through some sort of processing or preservation. Otherwise, it will become unfit for ingestion by humans, and even after being treated, the fish may continue to be spoiled, especially if traditional procedures were applied **(Oparaku and Mgbenka 2012, Shewan 2000).** The technique of preserving food like fish involves several processes that prevent the growth of microorganisms such as the addition of growth-inhibiting substances or customized storage conditions by freezing or drying **(Akise et al. 2013).** For thousands of years, smoking has been applied to prepare and preserve food **(Krasemann, 2004).** Bad hygiene, insufficient cleaning, or preservation in open trays allow the fungal invasion, production of toxins, and spoilage of the product **(Hassan et al. 2009 and Fredrick et al. 2016).** During storage, the

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growth of fungi such as Aspergillus, Rhizopus and Penicillium species was enhanced **(Ayolabi and Fagade 2010).** According to **(Ayeloja et al. 2018),** *Aspergillus flavus, Fusarium oxysporum, Ceotrichium albidium*, Rhizopus species, Penicillium species, and Trichoderma species were isolated from Nigerian smoked fish. Also, **(Daramola et al. 2023)** examined smoked fish samples mycologically revealed seven types of fungi namely, *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus*, Rhizopus species, Alternaria, Candida species, Mucor species.

In seafood, some fungal species are capable of producing mycotoxins as aflatoxins (AFs), Fusarium mycotoxins, and ochratoxins (OTs) **(Nourbakhsh and Tajbakhsh 2021and Shamimuzzaman et al. 2022).** Mycotoxins are stable based on their chemical structures, and it is difficult to remove them from the food chain **(Huang et al. 2011).** The International Agency for Research on Cancer (IARC) has designated aflatoxins as a class 1 human carcinogen because they are the most potent metabolite and because they are also extremely hepatotoxic, mutagenic, and genotoxic **(IARC. 2012).** To ensure food safety, it is necessary to evaluate and quantify the metabolites in staple and vital food products given the harmful effects of aflatoxins on the human body, and also need to educate both the traders and the consumers on the risks involved in the consumption of such contaminated products.

MATERIAL AND METHODS:

1. Collection of samples:

Samples of smoked herring fish (n = 50), and smoked herring fillets (n = 50) were purchased from markets in the Menoufia governorate.

2. Preparation of the samples (AOAC, 2000):

The muscle of the smoked fish was thoroughly mixed and ground to obtain a uniform mass. The analysis was carried out as soon as possible or chilling the sample in the refrigerator to avoid decomposition.

3. Isolation of mould:

Ten grams (10g) of each sample was aseptically weighed into a sterile bottle containing 90 ml of sterile peptone water. The mixture was shaken vigorously using vortex mixer, and 5-fold serial dilutions were prepared (Samson et al. 2010). One milliliter of each dilution was dispensed in duplicate in sterile Petri dishes. Molten Saboraud dextrose agar to wincorporated and phenicol (0.5g/l) had been incorporated was added to the Petri dishes, which were gently rotated, the plates were allowed to solidify and were incubated at 25C° for 3-7 days.
The cultures were examined for growth at regular intervals and all observed colonies were subculture to obtain pure colonies, which were subsequently isolated and identified using morphological characteristics (**Alkenz et al. 2015),** macroscopy and microscopy **(Ellis et al. 2007and Samson et al. 2010).**

4. Aflatoxins and Ochratoxin A1 determination:

4.1. Apparatus and Equipment:

High-performance liquid chromatography (HPLC) used for aflatoxin determination was an Agilent 1100 HPLC system, Agilen Technologies, Waldbronn, Germany, equipped with quaternary pump model G 1311A, UV detector (Model G 1314A) set at 254nm wavelength. Also, auto sampler (model G1329A VP-ODS) and Shim pack (150× 4.6 mm) column (Shimadzu, Kyoto, Japan) were used. The Chemstation Software program was used to integrate and record the data. Liquid nitrogen and ultra-high purity (99%) argon gas were adopted. The present study used Easi-Extract Aflatoxin immunoaffinity columns.

4.2. Standard Aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2) and Ochratoxin A (OCA) solutions:

The stock standard solutions of AFB_1, AFB_2, AFG_1, AFG_2, and OCA were prepared by dissolving the solid standard in benzene: acetonitrile (98:2, v/v). According to AOAC (2000), the precise concentration was measured by using a Shimadzu UV-1601 PC spectrophotometer, Shimadzu Scientific Instruments, Japan. The stock solution was prepared by using an intermediate standard solution in benzene:

acetonitrile (98: 2, v/v) at a concentration of 9.855 ng ml. This solution was used to elaborate a calibration curve in the concentration range of 0.1 –9.8 ng/ml. All the solutions were stored at -18°C in amber vials.

4.3. Quantitative determination of aflatoxins (European Council 2006):

4.3.1. Sample extraction:

In a blender, 50 g of the prepared homogenized sample was mixed with 100 ml of acetone and 100 ml of water for 10 min, 10 g of diatomaceous earth was added and carefully mixed for 5 min then filtered by using Whatman No. 1 filter paper. To prepare the mixture, 0.01 ml of the filtrate was added to a 500 ml wide mouth glass stoppered Erlenmeyer volumetric flask. Then, 50 ml of 5% NaCl and 50 ml of hexane were added to the flask. The flask was gently shaken for five minutes at a speed of 2400 rpm on a mechanical shaker (IKA, GmbH, Germany). The hexane layer was discarded. After adding 50 ml of 5% NaCl and 150 ml of chloroform (3x50 ml) to the aqueous layer shake gently for 5 min each time. The chloroform layer was collected from the three extractions, dried over anhydrous sodium sulphate, and evaporated using a rotary evaporator. The residues were re-dissolved in 1 ml chloroform.

4.3.2. Clean-up procedure:

After adding 2 ml of 0.5% aqueous acetic acid to condition the column, the C18 column was loaded with 1 ml of the filtered extract and 4 ml of 0.5% acetic acid. Next, 0.5 ml of 20% Tetrahydrofuran (THF) in 0.5% aqueous acetic acid was used to wash the column. 2 ml of hexane was then added to the column tube, which was subsequently dried under nitrogen. After being cleaned with 3 ml of 25% THF in hexane, the column tube was dried for 1 minute in nitrogen. The retained aflatoxins were dried over a stream of nitrogen after being eluted with 2x2 mL from 1% THF in methylene chloride. The dried aflatoxins were reconstituted in 0.5 ml of toluene before the injection in HPLC.

4.3.3. HPLC determination:

Each aflatoxin was determined with HPLC at wave length 365 and 440 nm for excitation and emission, respectively. The mobile phase was composed of toluene, ethyl acetate, formic acid, and methanol (90:5:2.5:2.5, v/v) which was pumped with constant flow at 1.0 ml min. 20 ul of the reconstituted sample were injected in the HPLC at 24°C to get the optimum resolution of aflatoxins. Several blanks (methanol only) and aflatoxin standard solutions were injected. The assessment of the given samples was done in triplicates and the sample was regarded as positive for aflatoxin, if its retention time and peak corresponded to that of the standard. Calculations to get the level of each aflatoxin in the examined samples were carried out automatically by Agilent Chem Station Software System.

4.4. Quantitative determination of ochratoxin A (Toscani et al. 2007):

4.4.1. Sample extraction:

An aliquot of 10 g of the prepared sample and 100 ml of (chloroform: 85% orthophosphoric acid 100: 4, v/v) solution were mixed and homogenized in a blender for 2 min. After thoroughly filtering through Whatman No. 3 filter paper, sixty ml of the filtrate was transferred into a separating funnel and extracted twice with 5 ml of (buffer 0.2 M Tris-Hydrochloric acid: Acetonitrile 90:10, v/v). The upper aqueous layer was carefully gathered and well mixed.

4.4.2. Clean-up procedure:

Accurately, 50 ml of the aliquot was passed through the Agilent ZORBAX C18 (3 µm, 2.1x250 mm) column for cleanup. The column was washed by water and dried by air. Ochratoxin A was eluted with 2 ml methanol with a vacuum manifold. The methanol was dried under gentile nitrogen stream and the residue was re-dissolved in the mobile phase (water: acetonitrile: glacial acetic acid 49.5: 49.5: 1.0) before the injection in HPLC.

4.4.3. HPLC determination:

Ochratoxin A was assessed at wavelength 380 and 440 nm excitation and emission, respectively. The mobile phase was composed of water: acetonitrile: and glacial acetic acid

49.5: 49.5: 1.0, which was pumped with constant flow at 1 ml/ min. Typically, the same techniques and steps used for the determination of aflatoxins were applied to estimate ochratoxin A automatically by Agilent Chem Station Software System.

5. Statistical analysis

The analysis of data for mould count in smoked herring fish and smoked herring fillet using student's t- test are significantly different at a confidence interval of 95% ($p \leq 0.05$) and the result is expressed as mean ± SD.

RESULTS:

Table 1. Statistical analytical results of mould count (cfu/g) of examined whole (n=50) and fillet smoked herring (n=50)

Samples	No. of + ve samples	% of + ve samples	Min. cfu/g	Max. cfu/g	Mean± SD. cfu/g
Whole smoked herring	43	86 %	$3.25x10^2$	$8.9 x10^5$	$3.5 x10^{5a} \pm 1.14x 10^3$
Smoked herring fillets	33	66 %	$4.5x10^3$	$6.2x10^4$	$3.8 x10^{4b} \pm 0.86 x10^2$

Different letters are significantly different at $p \leq 0.05$.

Table 2. Incidence of isolated mould in examined samples

Samples	Whole smoked herring(n=50)		Smoked herring fillets(n=50)	
	No. of +ve samples	% of +ve samples	No. of +ve samples	% of +ve samples
Aspergillus species	37	74%	20	40%
A. flavus	13	35.1 %	10	50 %
A. niger	10	27 %	7	35%
A. fumigatus	7	18.9 %	5	25%
A. ochraceus	7	18.9 %	3	15%
Penicillium species	10	20%	8	16%
Mucor species	7	14%	5	10%

Table 3. Determination of Mycotoxins by HPLC (microgram/Kg "ppb")

	Whole smoked herring(n=50)				**Smoked herring fillets (n=50)**			
	Positive samples		**Concentration of mycotoxin**		**Positive samples**		**Concentration of mycotoxin**	
			Minimum	**Maximum**			**Minimum**	**Maximum**
Aflatoxin B_1	10	20%	0.096	5.247	14	28%	0.118	7.938
Aflatoxin B_2	7	14%	:0.075	2.561	10	20%	0.086	3.509
Aflatoxin G_1	0	0	0	0	0	0	0	0
Aflatoxin G_2	0	0	0	0	0	0	0	0
Ochratoxin A	7	14%	0.062	0.958	7	14%	0.105	1.219

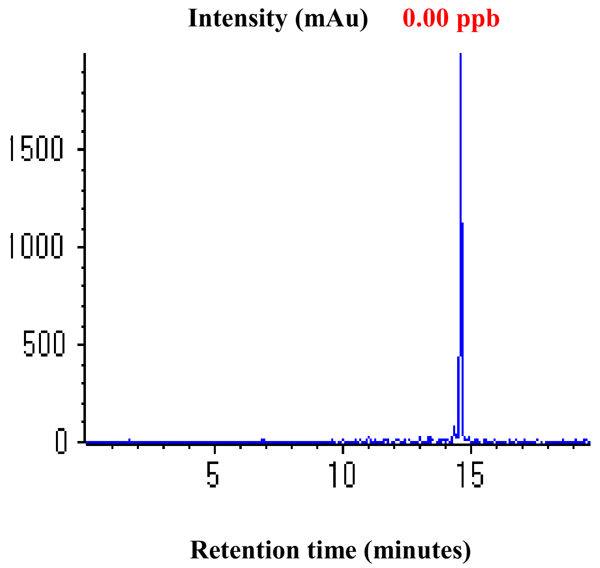

Figure (1): HPLC chromatogram of aflatoxin B1 A in the examined smoked herring sample (Negative)

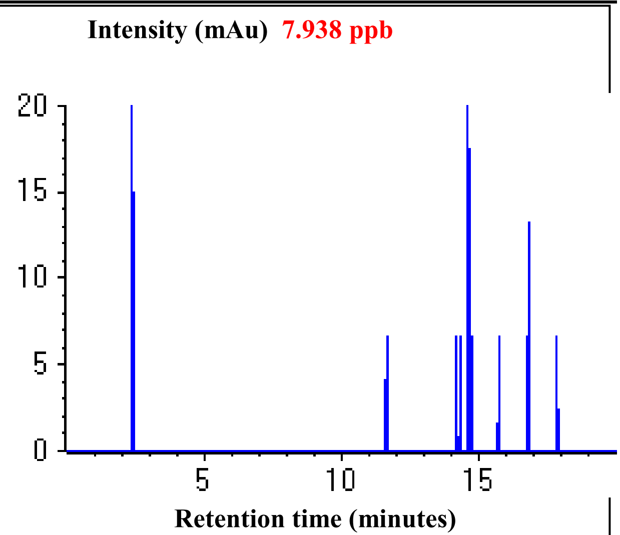

Figure (2): HPLC chromatogram of aflatoxin B1 A in the examined smoked herring sample (Positive)

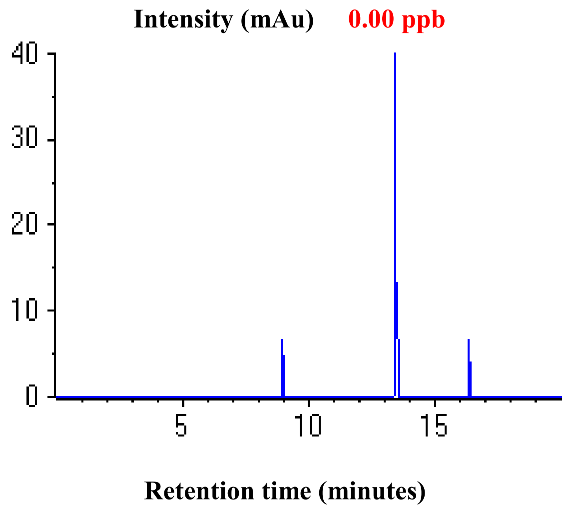

Figure (3): HPLC chromatogram of aflatoxin B2 A in the examined smoked herring sample (Negative)

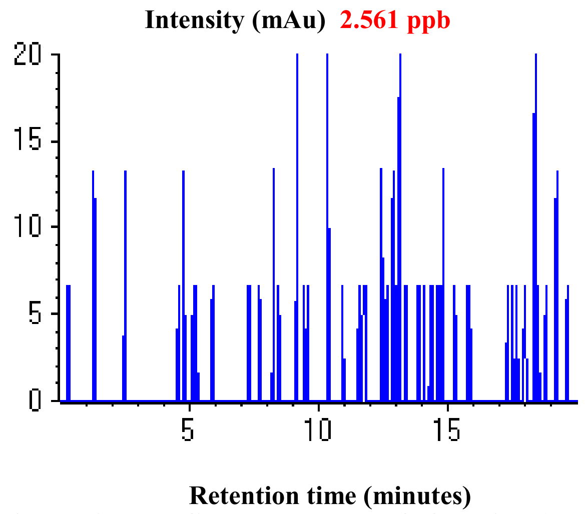

Figure (4): HPLC chromatogram of aflatoxin B2 A in the examined smoked herring sample (Positive)

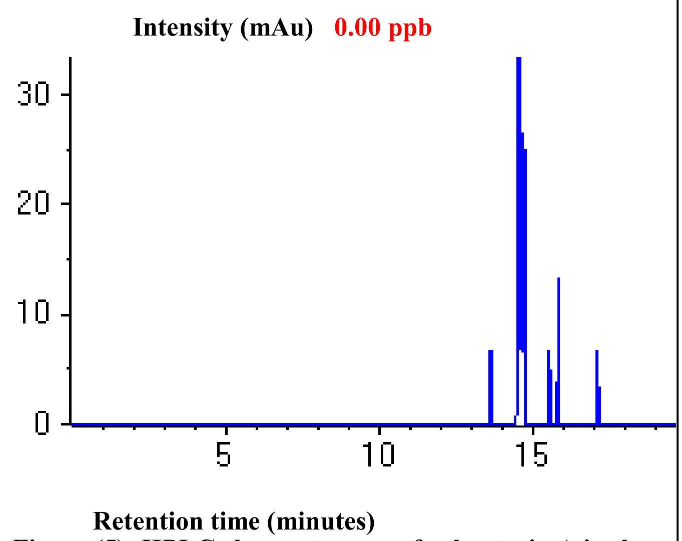

Figure (5): HPLC chromatogram of ochratoxin A in the examined smoked herring sample (Negative)

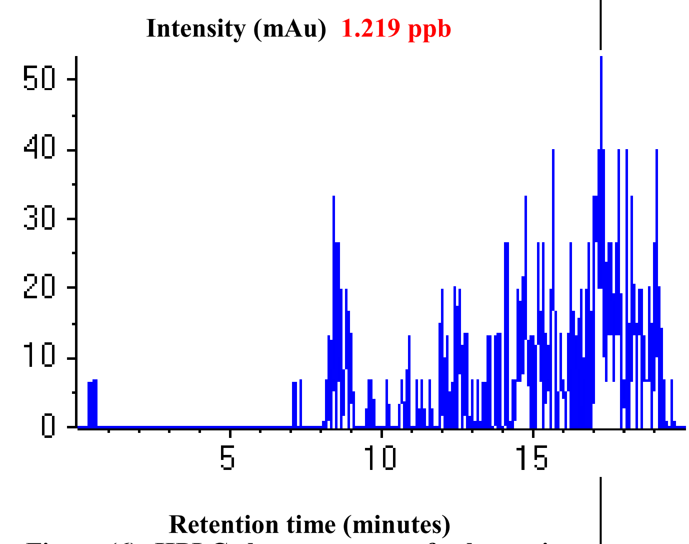

Figure (6): HPLC chromatogram of ochratoxin A in the examined smoked herring sample (Positive)

DISCUSSION

Inadequate sanitation before and/or during the handling, processing, shipping, and storage of fish and fish products may result in mold contamination of fish and fish products (**Hassan, 2003).** Dried or smoked may contain a variety of pathogenic microorganisms as well as fungi that can cause public health issues through their secondary metabolites (mycotoxins).

Mycotoxins pose a serious hazard to human health, especially when they co-occur naturally, where they may be more toxic and carcinogenic than when they are present alone. Acute toxicity from ingesting large concentrations of some mycotoxins, including aflatoxins, can result in serious sickness or even death. Long-term exposure to these metabolites at high concentrations can potentially result in more severe health issues such as cancer, weakened immune systems, and liver and kidney damage **(Al Jabir et al. 2019).**

Results shown in Table (1) revealed that the prevalence of mycotic contamination in smoked herring fish and smoked herring fillets is 86% and 66% respectively. These results are slightly similar to those obtained by **Ibrahim-Hemmat et al. (2017)** who revealed that the incidence of mould in the examined packed herring fish samples was 83.3%. Also, **Chinedu Adive et al. (2019)** revealed that the incidence of fungal contamination of smoked dried fish ranged from 67.6 % to 84.8%. The results also agree with **Aliyu et al. (2018)** found that 84% of smoked-dried fish samples were contaminated with fungi.

The higher level of fungal contamination (100%) in smoke-dried fish samples was reported by **Junaid et al. (2010),** however, **Job et al. (2016)** reported that the fungal contamination ranged from 15% - 85%. And also, **Abdel -Maksoud et al. (2010)** reported that the mycotic contamination of smoked herring is 40.91% and 31.82% for mould and yeast respectively.

The high percentage of mycotic contamination in this study may be due to uncontaminated salt, poor packaging, careless handling, and bad storage conditions.

The data presented in Table (1) showed a significant difference at $p \leq 0.05$ between smoked herring and fillet herring fish as the mean total mould count is $3.5 \times 10^5 \pm 1.14 \times 10^3$ cfu/g in smoked herring fish while the count is $3.8 \times 10^4 \pm 0.62 \times 10^2$ cfu/g in smoked herring fillets. These results are nearly similar to those reported by **Walter et al. (2020)** who found that smoked dried fish sold in Bida markets were contaminated with a varying load of fungi ranging from $5.44x10^5$ $\pm 1.21x10^6$ to $9.54x10^5$ ± 1.83 $x10^6$ cfu/g. The presented results agree with the results reported by **(Mounir et al. 2011, Olayemi et al. 2012 and Ibrahim-Hemmat et al. 2017).**

On the other hand, the mean values of total mould and yeast count of packed and unpacked smoked herring fish were 1.04x 10^2 $\pm$ 7.9 and 1.8x $10^2 \pm$ 2.3 CFU/g. respectively **(Khalifa and Mazyad 2009).**

The mycological examination of the presented samples table (2) revealed that the predominant mycotic species was Aspergillus species with a percentage of 74% and 40% in smoked herring fish and smoked herring fillets respectively, followed by Penicillium species (20% and 16%) then Mucor species (14% and 10%). The results are similar to those reported by **Foba et al. (2023**) who said that the predominant species were Aspergillus of the Glaucus group (39%), *Aspergillus niger* (36%), and Penicillium sp. (25%). **Ibrahim-Hemmat et al. (2017)** showed the same results that 80% of examined un packed smoked herring fish samples were contaminated with Aspergillus species followed by Pencillium species (50%). Also, **Abdoullahi et al. 2019, Fatima et al. 2021 and Daramola et al. 2023** reported the same results.

The data from Table (3) showed that the percentage of aflatoxins (B1 and B2) contamination of the examined whole smoked herring fish is 20% and 14% while 28% and 20% of smoked herring fillets are contaminated with aflatoxins (B1 and B2) respectively. The level of detectable aflatoxins (ranging between 0.075 - 7.938 ppb.) is below the permissible limits. These results are similar to the studies applied by **Fatima et al. (2021)** who reported that Aflatoxin B2, G1, and G2 were found at a range of 5.05 - 8.11 ppb in dried fish and meat from Ijebu-ode. Also, **Indra and Elasto (2020)** found that the concentration of aflatoxins ranged between 1.3 – 3.84 ppb in examined smoked dried fish in **Zambia. Besides, Akinyemi et al (2011)** reported that the concentration of aflatoxin in smoked dried fish samples ranged from 0.03 ppb to 1.15 ppb.

On the other hand, **Saad et al. (2020)** found that the examined fish products samples contained Aflatoxins B1, B2, G1, G2, and Ochratoxin A, with a high percentage of Aflatoxin B1(51.63 ±4.82µg/kg) in smoked herring. Also, a high concentration of AFB1 is detected in smoked fish **(Adebayo et al. 2008, Adesokan et al. 2016 and Fatima et al. 2021).**

All analyzed samples were free from aflatoxins G1 and G2. The same results are reported by another authers **(Abdoullahi et al. 2019).** On the other hand, **Saad et al. (2020)** detected aflatoxin G1/kg and G2/kg in smoked herring with an average of 25.06 ±3.18µg/kg and 16.22±1.39µg/kg respectively. Also, **Fagbohun and Lawal (2015)** detected AFG1 in 50 smoked dried fish samples in a concentration between 2.01–3.53 µg/kg.

Aflatoxin G1 and G2 were detected below detectable limits in the samples of smoked fish **(Fatima et al. 2021 and Saad et al. 2020)**

The presented results showed that the concentration of ochratoxin A in both whole smoked herring and smoked herring fillets is 14% from the examined samples with an average between 0.062 ppb and 1.219 ppb. These results agreed with the results of **Hassan et al. (2011).** While the mean values of Ochratoxin A/kg were 6.52±0.74µg/kg in the smoked herring fish **(Saad et al. 2020).**

Egyptian standard of smoked fish (228/2005) stipulated that smoked fish should be free from any visible mould growth and their toxins, so 14% and 34% of examined smoked herring fish and herring fillet respectively are accepted for consumption.

CONCLUSION

In this study, we concluded that the most contaminated samples with mould were whole smoked fish as the most predominant isolated strain was Aspergillus species, particularly *A. flavus, A. niger, A. fumigatus,* and *A. ochraceus*. Also, Aflatoxins B1, B2, and Ochratoxin A were detected while all samples were free from Aflatoxins G1 or G2. So, draws attention to the preparation and production of smoked herring fish to avoid possible health hazards from the mycotoxins**.**

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