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Original Article

Detection of polycyclic aromatic hydrocarbons (PAHs) in cold and hot smoked Mullet fish (*Mugil cephalus*) products by using GC-MS.

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ABSTRACT: The aim of this study is to determine the levels of polycyclic aromatic hydrocarbons in cold and hot smoked mullet fish obtained from two fish farms (A and B) localized at El-Fayoum Governorate, , Egypt during November 2020. It found The 5 compounds of PAHs; acenaphthylene, acenaphthene, fluorene, fluoranthene and pyrene were detected in both cold and hot smoked fish samples from the two farms (A) and (B), while phenanthrene and anthracene were detected only in cold smoked samples from farm (A) and (B). The total PAHs in the cold and hot smoked Mullet fish samples obtained from farm (A) were 42.9 and 12.1 µg/kg, respectively, while in cold and hot smoked samples obtained from farm (B) were 32.1 and 11.2 µg/kg, respectively. Benzo (a) pyrene (BaP) compound that considered as indicator for carcinogenic PAHs was not detected in both cold and hot smoked Mullet fish. Also, PAH4; benzo (a) pyrene, benzo (a) anthracene, benzo (b)fluoranthene and chrysene did not detected in both the cold and hot smoked Mullet fish. Categories of PAHs concentration are considered a minimally contaminated (10 to 99 µg/kg) compared with the maximum recommended levels. Based on our results, it could be concluded that Benzo (a) pyrene compound was not detectable in all smoked samples which are considered as a safe product for consumption.

Key words: Fish, Smoking, PAHs, GC-MS
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1. INTRODUCTION

Smoking is a traditional preservation technology that combines the effect of salting, deposition of smoke components and drying. It produces the characteristic taste, color and flavor that is much appreciated by consumers and extends its shelf-life via the effects of dehydration, anti-microbial and anti-oxidant of the smoke compounds (Pagu *et al.* 2013). Smoke contains many different components, such as aldehydes, ketones, alcohols, acids, hydrocarbons, esters, phenols, ethers, etc (Doe, 1998).

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Polycyclic aromatic hydrocarbons (PAHs) are generated by the incomplete combustion of wood during smoking process. Food can become chemically contaminated by polycyclic aromatic hydrocarbons (PAHs) due to thermal treatments during preparation and manufacturing such as smoking, roasting, baking, and frying (Ishizaki et al. 2010). Gómez-Estaca *et al.* (2011) noticed that the traditional smoking techniques involve treating of pre salted whole or filleted fish with wood smoke from incomplete burning of wood that comes into direct contact with the product can leads to its contamination with PAHs if the process is not adequately controlled or if very intense smoking procedures are employed. Therefore, it is probably that smoked fish contains PAHs, some of which might be carcinogenic. This has increased the risk of PAHs contamination through consumption of smoked fish. From the public health point of view, food safety organizations are of growing concern globally regarding PAHs residues if it is present in foods above the recommended levels that could pose serious public health concerns. This study was designed to determine the concentrations of polycyclic aromatic hydrocarbons in cold and hot smoked mullet fish products obtained from two fish farms localized in Fayoum governorate, Egypt during November, 2020

2. MATERIAL AND METHODS

Mullet fish (*Mugil cephalus*) samples were obtained from two different fish farms in Fayoum Governorate, Egypt during November, 2020. The two farms (A) and (B) are irrigated by draining waters from El-Batts and El-Wadi drain waters, respectively. The averages of weight and length of fish samples were 305±40g and 33±2cm for raw samples obtained from farm (A) and 255±50g and 30.5±1.5cm for raw samples obtained from farm (B). The fish samples were immediately transported in ice boxes from the two farms to the laboratory of Fish Processing Technology, Shakshouk Station for Fish Research, National Institute of Oceanography and Fisheries (NIOF), Egypt. Fine refined table salt of sodium chloride (BONO) produced by Egyptian Salts and Minerals Company (EMISAL) was used. It composed of 98.5% sodium chloride, 30-70 ppm of potassium iodate and 0.3% of humidity. Sawdustas the source of smoke was purchased from carpentry workshop at Fayoum city.

2.1.Smoking process

The traditional methods of cold and hot smoking were carried out according to the method described by Abd El-Mageed (1994) with some modifications using smokehouse at Shakshouk, Fish Research Station, (NIOF). The smokehouse had inside the dimensions of 2.20 × 1.0 × 3.5 m with perforated metal sheets placed at 75 cm above the smoke source. Mullet fish samples were immersed for 2 hours in brine solution containing 10% NaCl at a ratio of 1:1 (w/v). The samples were rinsed with tap water for 1 min to remove the excess of salt then drained and semi-dehydrated at 25-28°C for 2 hours. The smoking process was carried in the smoke house using sawdust as smoke source. In the cold smoking; the samples were hooked in the smokehouse above the smoke source by about 2.5 m for 8-10 hours at the temperature of 35 - 40°C, while in hot smoking the samples were hooked in the smokehouse about 1.5 m above the smoke source for 5 - 6 hours at 50 - 90°C. After smoking the fish samples were cooled under ambient temperature.

2.2.Polycyclic Aromatic Hydrocarbons (PAHs) determination:

(PAHs) were determined in Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Food (QCAP), Agricultural Research Centre, Giza Governorate, Egypt.

2.3.Chemicals and Reagents

Acetone (Riedel-dehaen, purity 99.8%), acetonitrile (Sigma-Aldrich, purity>99.9%), toluene (Merck),

dichloromethane chromatography grade, and n-hexane (purity >99.0%) were the solvents used. Agilent QuEChERs salts and buffers were pre-packaged in anhydrous packages for EN 15662 containing 4g of magnesium sulfate (MgSO4), 1g of sodium chloride (NaCl), 1g of sodium citrate, and 0.5g of disodium citrate sesquihydrate. Silica gel (60–120 mesh, Fluka) was activated at 150◦C for 12 hours prior to use. A 1000μg/ml stock solution of 14 PAHs includes naphthalene, fluorene, fluoranthene, benz(a)anthracene, chrysene, pyrene, benzo(b) fluoranthene, benzo(k) fluoranthene, Benzo(a)pyrene, acenaphthene, phenanthrene, anthracene, acenaphthylene, and pyrene-d10 (surrogate standard) and reference standards obtained from Sigma-Aldrich with purity > 95% were prepared, while benzo (g,h,i) perylene and dibenz (a,h) anthracene were obtained as readymade of 100μg/ml in methylene chloride and indeno [1,2,3-cd] pyrene 200μg/ml in methanol. A 1μg/ml working solution of all 16 PAHs was prepared in toluene. Calibration mixtures with concentration 2, 10, 50,100 and 500 ng/ml were prepared from serial dilution of the working solution in toluene where pyrene-d10 maintained at level 50 ng/ml in all calibration levels and all stored in refrigerator at 4◦C. Polyethylene 50 ml tubes with screw cap and 15ml tubes contain1g magnesium sulfate were obtained for sample extraction. Centrifuge up to 4000 rpm (HeraeusLabofuge 400), Vortex, Automatic Pipettes (HirschmannLaborgerate) suitable for handling volumes of 10μl to 100μl and100μl to1000μl, 10 ml solvent dispenser (HirschmannLaborgerate) for Acetonitrile. The glassware were washed with detergent and water then rinsed with acetone and dried at 90 ◦C before use.

2.4. Sample Extraction

The validation procedure needs to be considered, the context of fitness for purpose and cost benefit criteria (Khorshid *et al.* 2015). About 10g of fish sample was weighted in 50 ml Teflon centrifuge tube, 50μl of10μg/ml pyrened10 was added which acts as surrogate standard of 50μg /Kg, and each set of 6 replicates was spiked with 20, 100, and 500μl of 1μg/ml spiking mixture to get 2, 10, and 50μg/kg, respectively. 10 ml of acetonitrile was used for extraction, shaken for 2 minutes, mixed with Agilent QuEChERs, shaken for 1 minute, and centrifuged at 4000 rpm for 5 minutes. Aliquots of the resulting supernatant were transferred to Teflon tube containing MgSo4, vortexed for 30 seconds, and centrifuged at 4000 rpm for 2 minutes; 4 ml of the acetonitrile layer was transferred into 50 ml flask and then evaporated near to dryness.

2.5. Clean up of PAHs

Samples Packed by Solid Phase Extraction (SPE) Steps. All fish extracts were subjected to packed solid phase clean up cartridge which was prepared in-house as follows. Plug a glass wool on 10 ml length syringe; 1g 20% deactivated silica gel and 0.2 MgSo4 were weighted and conditioned with 5ml of n-hexane/dichloromethane (3:2), the sample extract loaded to the cartridge using 10 ml of elute (n hexane/dichloromethane). Collect fractions in a 50 ml flask, evaporate on rotary evaporator at 40◦C near to dryness and dissolve in 2 ml toluene and then apply to GCMS for analysis.

2.6.GC-MSD conditions

Agilent 6890N series gas chromatography instrument equipped with 5975 series mass selective detector and Agilent GC Column of model J&W HP-5ms Ultra Inert with the specifications (30m length, 0.25mm internal diameter, 0.25μm film thickness) were used for both qualitative and quantitative determination of PAHs. Helium gas was used as the carrier gas; the column was maintained at a constant flow rate of 1.3 ml/min. The back injector line was maintained at 260◦C. Injection volumes were 1.0μl in the splitless mode. The column temperature was initially held at 90◦C for 2 min, ramping to180◦C at a

rate of 15°C/min, held at 180°C for 15 min, ramping to 250°C at a rate of10°C/min, held for 2 min, ramping to 290°C at a rate of 10°C/min, and held for 10 min. The mass spectrometer was operated in the ionization mode and spectra were acquired using a mass range of 45-450 m/z. Quality control and assurance of each patch were passed by monitoring the performance of the GCMS and the mass selective detector daily by tuning the mass detector and monitoring the sensitivity and linearity of the calibration curve, respectively, and also analyzing blank sample to confirm that there in contamination effect on the results during analysis.

3. RESULTS AND DISCUSSION

3.1.Concentrations of PAHs in smoked Mullet fish products;

The results in Table (1) show the concentrations (µg /kg) of polycyclic aromatic hydrocarbons (PAHs) that detected in cold and hot smoked Mullet fish obtained from farms (A) and (B).The results indicated that 5 compounds of PAHs; acenaphthylene, acenaphthene, fluorene, fluoranthene and pyrene were detected in both cold and hot smoked fish samples from the two farms (A) and (B), while phenanthrene and anthracene were detected only in cold smoked samples from the two farms (A and B). Benzo (a) pyrene (BaP) compound that is considered as indicator for carcinogenic PAHs was not detected in both cold and hot smoked Mullet fish. Also, PAH4; benzo (a) pyrene, benzo (a) anthracene, benzo (b)fluoranthene and chrysene did not detected in both cold and hot smoked Mullet fish. Also, it could be noticed the higher levels of PAHs compounds were found in the cold smoked samples from the two farms; (A) and (B) than in the hot smoked samples.

Table (1): Concentrations of polycyclic aromatic hydrocarbons (PAHs) detected in smoked Mullet fish obtained from farms (A) and (B)

No.	PAHs (µg/kg)*	Mw**	Farm (A)		Farm (B)	
			Smoked fish		Smoked fish	
			Cold	Hot	Cold	Hot
1	Naphthalene	128	ND	ND	ND	ND
2	Acenaphthylene	152	4.3	2.2	2.5	1.7
3	Acenaphthene	153	3.8	1.5	3.5	1.4
4	Fluorine	166	7.6	2.7	4.2	2.0
5	Anthracene	173	3.1	ND	1.9	ND
6	Phenanthrene	178	9.2	ND	7.0	ND
7	Fluoranthene	202	2.5	1.9	3.5	1.4
8	Pyrene	202	12.4	3.8	9.5	4.7
9	Benzo(a)anthracene	228	ND	ND	ND	ND
10	Chrysene	228	ND	ND	ND	ND
11	Benzo(b)fluoranthene	252	ND	ND	ND	ND
12	Benzo(k)fluoranthene	252	ND	ND	ND	ND
13	Benzo(a)pyrene	252	ND	ND	ND	ND
14	Dibenzo(a,h)anthracene	275	ND	ND	ND	ND
15	Benzo(g,h,i)perylene	276	ND	ND	ND	ND
16	Indeno(1,2,3, cd)pyrene	276	ND	ND	ND	ND
	Total PAHs		42.9	12.1	32.1	11.2

*On wet weight basis,**Mw: Molecular weight, Farm (A): Irrigated from El-Batts drain, Farm (B): Irrigated from El-Wadi drain, ND: Not detected.

The results also indicated that the smoked samples obtained from farm (A) contained higher levels of PAHs compounds than that in smoked samples obtained from farm (B). The total PAHs in the cold and hot smoked Mullet fish samples obtained from farm (A) were 42.9 and 12.1µg/kg, respectively, while in cold and hot smoked samples obtained from farm (B) we 32.1 and 11.2 μg/kg, respectively. The values of

total PAHs concentrations in the investigated cold and hot smoked samples were found to be very low than the levels recorded by Silva *et al.* (2011) who stated that the concentrations of total PAHs in smoked Catfish *(Arius heude loti*), sole (*Cynoglossussenegalensis*) and *hake* by using sawdust as a source of fuel were 2058.1, 1395.2 and 856.2 µg/kg, respectively. Zelinkova and Wenzl (2015) found that the hot smoking resulted a higher PAH levels than in cold smoking. Moreover, Abo-Zeid (2020) indicated that the concentration of total PAHs in cold smoked Cat fish was 369.5 *µ*g/Kg. Also, Mohamed *et al*. (2020) stated that the total PAHs contents of cold smoked Mullet fish from two different farms; (A) and (B), were 23.6 and 11.9 µg/kg, respectively and indicated that benzo (a) pyrene, PAH4 and PAH8 did not detected in all the smoked samples.

From the results outlined in Table (10), it could be decided that the low concentrations of PAHs as well as the non-detected benzo (a) pyrene revealed that Mullet fish samples smoked by the two different methods posed no health risks. The higher concentrations of the PAHs compounds in the cold smoked samples could be attributed to the longer time of the exposure to smoke in cold smoking than in hot smoking. The variations of PAHs levels between the cold smoked and hot smoked samples might be due to the procedures used for smoking process; surface of fish exposed to the smoke, combustion temperature, smoking time, oxygen accessibility and density of smoke (Basak *et al.* 2010). Similar results were found by El-Lahamy *et al*. (2016) who reported that hot smoking method could be safer and deemed fit for human consumption than cold smoking although, the results reveal that the fish samples smoked by the two methods do not constitute a health risk, as the benzo (a) pyrenewas not detected.

3.2.Toxic Equivalent Factors (TEFs) and B (a) P Equivalent of PAHs found in smoked Mullet fish samples

Toxic equivalent factor (TEF) is an estimate of the relative toxicity of the individual (PAH) fractions compared to benzo (a) pyrene. Even if this presentation of PAHs content is empirical because the effects of PAHs in a mixture are insufficiently understood, with this approach it is possible to express PAH contamination of food by a single value as reported by Isioma *et al.* (2017), Vincent *et al*. (2007) and AFSSA (2003). Benzo[a] Pyrene (BaP) has been well characterized as the most potent carcinogenic PAH after dibenz [a,h] anthracene. Therefore, the total PAH concentration is expressed as Benzo[a] Pyrene Equivalent (BaP_{eq}) to illustrate the toxic potency (Perugini *et al.* 2007).

The BaP_{eqi} was calculated as the sum of BaP_{eqi} value for individual PAHs determined in the smoked mullet fish. The BaP_{eqi} value was calculated for each PAH from its concentration in the sample (C_{PAHi}) multiplied by its toxic equivalency factor (TEF_{PAHi}) as reported by Nisbet and LaGoy, (1992) as shown in the following equation:

$$BaPeq = \Sigma (BaP_{eqi}) = \Sigma (C_{PAHi} \times TEF_{PAHi})$$

The toxic equivalent factors (TEFs) and B [a] P Equivalent of PAHs in cold and hot smoked mullet fish obtained from the two fish farms (A and B) are presented in Table (2). After cold and hot smoking of farm (A), the B[a]P Equivalent of Acenaphthylene, Acenaphthene, Fluorene, Anthracene, Phenanthrene, fluoranthene and Pyrene were 0.0043, 0.0038, 0.0067, 0.031, 0.0092, 0.0025 and 0.0124; respectively, and the total B [a] P Equivalent was 0.069 in cold smoked sample. In the case of hot smoked samples obtained from farm (A), the values of Acenaphthylene,Acenaphthene, Fluorene, fluoranthene and Pyrene were 0.0022, 0.0015, 0.0027, 0.0019, 0.0038,

respectively and the total B [a] P Equivalent was 0.0121.

On the other side, B[a]P Equivalent of Acenaphthylene,Acenaphthene, Fluorene, Anthracene, Phenanthrene, fluoranthene and Pyrene for smoked products of farm (B) were 0.0025, 0.0035, 0.0042, 0.019, 0.007, 0.0035 and 0.0095, respectively and the total B[a]P Equivalent was 0.0492 in cold smoked samples. Also, the values of Acenaphthylene, Acenaphthene, Fluorene, fluoranthene and Pyrene were 0.0017, 0.0014, 0.002, 0.0014 and 0.0047, respectively and the total B [a] P Equivalent was 0.0112 for hot smoked samples obtained from farm (B).

Form the above discussed data it could be concluded that the $\sum$ (BaP_{eqi}) values for cold smoked samples obtained from both farms (A) and (B) were higher than in hot smoked sample from the two farms, which may be attributed to the longer period of cold smoking than in hot smoking that consequently increased the chance to the PAH compounds to penetrate the fish body. Also, $\sum$ (BaP_{eqi}) of cold smoked products from farm (A) was higher than farm (B); which may be due to the less weight of fish obtained from farm A compared to farm B and the exposure to smoke components is higher than big fish weights.

Table (2): Toxic Equivalent Factors (TEFs) and B[a]P Equivalent of PAHs found in cold and hot smoked Mullet fish

Compound*	TEF**	Farm (A)				Farm (B)			
		Smoked fish				Smoked fish			
		Cold		Hot		Cold		Hot	
		Conc (µg/kg)	BaP eqi	Conc (µg/kg)	BaP eqi	Conc (µg/kg)	BaP eqi	Conc. (µg/kg)	BaP eqi
Naphthalene	0.001	ND	-	ND	-	ND	-	ND	-
Acenaphthylene	0.001	4.3	0.0043	2.2	0.0022	2.5	0.0025	1.7	0.0017
Acenaphthene	0.001	3.8	0.0038	1.5	0.0015	3.5	0.0035	1.4	0.0014
Fluorene	0.001	7.6	0.0067	2.7	0.0027	4.2	0.0042	2.0	0.002
Anthracene	0.01	3.1	0.031	ND	-	1.9	0.019	ND	-
Phenanthrene	0.001	9.2	0.0092	ND	-	7.0	0.007	ND	-
Fluoranthene	0.001	2.5	0.0025	1.9	0.0019	3.5	0.0035	1.4	0.0014
Pyrene	0.001	12.4	0.0124	3.8	0.0038	9.5	0.0095	4.7	0.0047
Benzo(a)anthracene	0.1	ND	-	ND	-	ND	-	ND	-
Chrysene	0.01	ND	-	ND	-	ND	-	ND	-
Benzo(b)fluoranthene	0.1	ND	-	ND	-	ND	-	ND	-
Benzo(k)fluoranthene	0.1	ND	-	ND	-	ND	-	ND	-
Benzo(a)pyrene	1	ND	-	ND	-	ND	-	ND	-
Dibenzo(a,h)anthracene	1	ND	-	ND	-	ND	-	ND	-
Benzo(g,h,i)perylene	0.01	ND	-	ND	-	ND	-	ND	-
Indeno(1,2,3,c)pyrene	0.1	ND	-	ND	-	ND	-	ND	-
$\sum$ (BaP_{eqi})			0.069		0.0121		0.0492		0.0112

*On wet weight basis,Farm (A): Irrigated from El-Batts drain, Farm (B): Irrigated from El-Wadi drain, **TEF: Toxic equivalent factor, BaPeqi[a]: P equivalent.

3.3.Molecular weight of PAHs in smoked Mullet fish

The temperature range of 500–900°C is known to favor the production of high molecular weight PAHs compounds from thermal breakdown of lignin in lignocelluloses during wood combustion and also from pyrolysis of fats in fish. The increase in the concentration of low molecular weight hydrocarbons over the smoking can be suggested to have been influenced by low fat and pyrolysis resulted from melted dropping onto the heat source. This is due to the average temperature of the smoking processes does not favor the production of high molecular weight compounds of PAHs. (Maga, 1988; Bartle, 1991, Nakamura *et al.* 2008; Essumang *et al*. 2013 and Chukwujindu *et al*. (2016).Table (3) shows the molecular weights (MW) of PAHs in cold and hot smoked mullet fish obtained from the two

fish farms. The total concentration of the low molecular weights (LWM) of PAHs was higher than the medium molecular weights (MMW) in both smoked fish farms samples.

Table (3): Total mean concentration (µg / kg) of PAHs in cold and hot smoked mullet fish according to their molecular weights

Molecular weight*	Farm (A)		Farm (B)	
	Concentrations of PAHs (µg /kg)		Concentrationsof PAHs (µg /kg)	
	Cold smoked	Hot smoked	Cold smoked	Hot smoked
LMW	28	12.8	19.1	5.1
MMW	14.9	5.7	13	6.1
HMW	ND	ND	ND	ND

On wet weight basis, Farm (A): Irrigated from El-Batts drain, Farm (B): Irrigated from El-Wadi drain, * **HMW**: high molecular weight, **MMW**: medium molecular weight, **LMW**: low molecular weight.

LMW levels in cold smoked samples obtained from farm (A) were the highest value in all samples, recorded by 28 µg / kg followed by cold smoked samples from farm (B) recorded by 19.1 µg / kg, while hot smoked samples recorded 12.8 and 5.1 µg / kg for farm (A) and (B) respectively. Generally cold smoked samples in both farms contained higher levels of LMW and MMW compounds. Also, the levels of LMW compounds were higher than MMW in most of the samples. The HMW compounds were not detected in all samples. This may be due to the lipophilic nature of the PAHs and it may be that the skin of fish protected them from the high molecular weight PAHs than low molecular weight as reported by Mohammadi *et al.* (2013). Most of the carcinogenic PAHs fall within the group of the HMW (EFSA, 2002).

Categories of PAH concentration in cold and hot smoked Mullet fish

Seyedeh et *al.* (2013) reported that the categories of PAHs concentration as not contaminated (<10 µg/kg); minimally contaminated (10-99 µg/kg); moderately contaminated (100-1000 µg/kg) and highly contaminated (> 1000 µg/kg).

Category of PAH concentration (µg/kg) in the cold and hot smoked samples is illustrated in Table (4). Concentrations of PAHs were 42.9 and 12.1 µg/kg in cold and hot smoked fish from farm (A), respectively after smoking and 32.1 and11.2µg/ kg in cold and hot samples from farm (B), respectively. Based on these results, categories of concentration of PAH are considered a minimally contaminated (10-99 µg/kg) in all smoked samples for both treatments compared with the recommended levels as set by Seyedeh et *al.* (2013).

Table (4): Categories of PAH concentration (µg/kg) in the studied cold and hot smoked samples

Farm (A)				**Farm (B)**			
Cold smoked		**Hot smoked**		**Cold smoked**		**Hot smoked**	
ΣPAHs	**Category**	**ΣPAHs**	**Category**	**ΣPAHs**	**Category**	**ΣPAHs**	**Category**
42.9	Minimally contaminate	12.1	Minimally contaminate	**32.1**	Minimally contaminate	11.2	Minimally contaminated

4.CONCLUSION

In conclusion, Benzo (a) pyrene is one the most carcinogenic PAHs, and used as indicator for safety of smoked fish, European Commission limited the maximum acceptable concentrations of benzo (a) pyrene at 2 ppb for smoked fish. Benzo (a) pyrene not found in smoked products and the categories of concentration of PAH are considered a minimally contaminated compared within ternational recommended levels.

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