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Original Research

Detection of Polycyclic Aromatic Hydrocarbons Concentrations in Egyptian Raw and Sterile Milk

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Abstract

This study was conducted to determine the polycyclic aromatic hydrocarbons (PAHs) levels and health risk of farm raw milk and ultra-heat treated (UHT) sterile market milk collected from different sources at Mansoura Province in Egypt using gas chromatography- mass spectrometry (GC- MS) during different seasons from August 2021 to December 2021. The results showed that the total amount of Σ18 PAHs levels was within the range of 11.778 – 26.331 µg/kg in farm milk samples and 1.151 – 2.946 µg/g in market UHT sterile milk. The results proved that the highest mean level of Σ PAHs in farm milk samples was 17.931 µg/kg followed by that of market sterile milk samples 2.123 µg/kg. European Commission (EC) has established safe level in milk for regulations require the concentrations of Benzo(a) pyrene (BaP) and the total Σ PAH4 to be less than 1.0 µg/kg. Mean concentration of BaP residues that was detected in farm milk samples was 0.251µg/kg with a range of 0.000 – 1.124 µg/kg and was not-detected in all market milk samples. Mean concentrations of Σ PAH4 levels were 0.561 µg/kg within the range of 0.046 – 2.433 µg/kg in farm milk and 0.047 µg/kg within the range of 0.012 – 0.110 µg/kg in market milk samples. These results were slightly higher than the critical limit set by the European Food Safety Authority (EFSA). The assessed dietary exposure was established by comparing the Estimated Daily Intake (EDI) with Acceptable Daily Intake (ADI). By comparing the obtained results, we found that for the BaP, the EDI for farm raw milk can be exceeded the maximum levels set in Regulation 1881/2006 (EFSA) for PAHs in milk, but UHT market sterile milk not exceeded the maximum levels. Therefore, there should be concerns regarding the effects of the consumption of different kinds of raw milk on the local population.

KEYWORDS
Benzo[a]pyrene, EDI, Farm raw milk, GC-MS, Market UHT sterile milk. Polycyclic aromatic hydrocarbons (Σ PAHs), Σ PAH4, Σ PAH8.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are chemical carcinogenic mixtures consisted of hydrogen besides carbon particles in a cyclic arrangement and have > 2 fused benzene rings Sampaio *et al.* (2021). They are generated from organic substances that incompletely burning of in anthropogenic processes and natural Amirdivani *et al.* (2019). Because of this great variance in these compounds, PAHs generally recognized nervous toxicity, carcinogenic and endocrine Seralini *et al.* (2022). PAHs enter the human body through inhalation, ingestion, or dermal contact, with main exposure (88–98%) from contaminated food consumption Alomirah *et al.* (2011).

The absorption of PAHs is enhanced via their high solubility in lipids, being lipophilic, and this permits their binding with the cell membrane Duan *et al.* (2016). Cows can be exposed to PAHs through soil, water, and air, thus milk and animals' feed could be contaminated by such substances Sun *et al.* (2020). PAHs could be excreted in milk because of their capability of crossing the blood mammary barrier Grova *et al.* (2002).

Raw milk, which contains more triglycerides, contains greater levels of PAHs. Based on their physical and chemical properties, PAHs are highly soluble in fats and are retained by food rich in fats Kishikawa *et al.* (2003). The lipophilic nature of such compounds enhances their accumulation in the fatty tissue and then is excreted in milk Dobrinas *et al.* (2016). Furthermore, the manufacturing and heat treatment of milk may result in production of PAHs, and thus could be detected in pasteurized and ultra-high-temperature processing (UHT) milk samples Ciecierska and Obiedzinski (2010). Benzo[a]pyrene (BaP) is the main PAH which has carcinogenic effect Zhang *et al.* (2021). Since PAHs have variable forms, the European Commission (EC, 2011) has identified safe levels of BaP, benzo[a]anthracene (BaA), chrysene (CHR), and benzo[b]fluoranthene (BbF) as four major PAHs (PAH4). These regulations require levels of BaP and total PAH4 to be < 1.0 µg. kg^{-1} Chenggang *et al.* (2020). BaP was found to have the most remarkable carcinogenic effects Rajendran *et al.* (2014). An intake of B(a)P (> 10 µg/kg/d) can harm the health (as reported by Joint FAO/WHO Expert Committee on Food Additive (JECFA, 2015). Gas chromatography–mass spectrometry (GC-MS) is the method with high specificity and sensitivity, which is extensively used in food safety assessment and is applied for detection of

PAHs Xu *et al*. (2021). Therefore, this study aimed at estimate the concentrations of PAHs in different Egyptian farm milk and UHT sterile market milk samples from different sources in Mansoura Province in Egypt utilizing GC-MS; and at evaluating the health risks related to the consumption of such products.

MATERIALS AND METHODS

Milk Samples

A total of fifty samples of different types of milk were used in this study including 20 market UHT sterile milk samples (samples of commercially available milk brands, with different packing dates and batch numbers, were obtained from multiple places in various regions at Mansoura city, Egypt) and 30 farm raw milk samples (which were packed from different farms at different dates from multiple places in various locations at Mansoura city, Egypt) were obtained during the period from August 2021 to December 2021.

Raw farm milk samples of 500 ml were aseptically collected from farms in dark sanitary capped glass bottles stored at -20°C. Each sample was transferred in a separate labeled aluminum foil to avoid oxidation and photo-irradiation process Marquès *et al*. (2016). Samples were collected in glass containers with plastic caps and were delivered to the Environmental and Food Pollutants laboratory for immediate analysis within 4 hours.

Milk samples were collected and prepared for detection of residues of 18 PAHs compounds (naphthalene, Naphthalene, 2-methyl-, Naphthalene, 1-methyl-,acenaphthylene, acenaphthene, Fluorene, phenanthrene, anthracene, Fluoranthene, Pyrene, BaA, CHR, BbF, Benzo[k]fluoranthene, BaP, Indeno(1.2.3-cd) pyrene, Dibenz[a,h]anthracene and benzo(ghi)perylene).

PAHs reference standards

A mixture (18 compounds) of PAHs standards including Acenaphthene 1000 µg/mL, Acenaphthylene 2000 µg/mL, Anthracene 100 µg/mL, BaA 100 µg/mL, BbF 100 µg/mL, Benzo[k] fluoranthene 100 µg/mL, Benzo[ghi]perylene 200 µg/mL, BaP 100 µg/mL, CHR 100 µg/mL, Dibenz[a,h]anthracene 200 µg/mL, Fluoranthene 100 µg/mL, Fluorene 200 µg/mL, Indeno[1,2,3-cd] pyrene 100 µg/mL, 1-Methylnaphthalene 1000 µg/mL, 2-Methylnaphthalene 1000 µg/mL, Naphthalene 1000 µg/mL, Phenanthrene 100 µg/mL, Pyrene 100 µg/mL was obtained from Sigma-Aldrich company (Darmstadt, Germany) (PAH Mix 3, ampule of 1 mL concentration in methylene chloride: methyl alcohol (1:1). The standard solutions were kept in the dark at 4°C when not in use.

Extraction of milk samples

Extraction and preparation of milk samples were applied according to Kishikawa *et al*. (2003). In brief, thawing of milk samples was performed in water bath at 37 °C for 5 minutes before being analysed. Two grams of milk sample underwent saponification with 4.0 ml of 0.4 M NaOH in EtOH:H2O (9:1, v/v) at 60 °C for half an hour. The resultant solution underwent extraction twice using 2.0 ml of n-hexane. The latter underwent evaporation to dryness, the residue dissolved in 100 µl of acetonitrile and was filtered through a filter paper (0.45 µm) and the aliquot was analyzed. An aliquot of 1 µL of such solution was injected into the GC/MS (Agilent Technologies 7820A/5975 MSD GC-MS) for analysis.

Clean-up of samples

Clean up of extracted milk samples were applied according to Villeneuve *et al*. (1999). Briefly, clean-up process was achieved with a silica/alumina column. Aromatic hydrocarbons underwent elution with 30 ml of hexane and dichloromethane (9:1)(v\v). The eluent volume was then decreased to 1 ml and analyzed in the GC/MS.

GC/MS analysis

Samples were injected into GC/MS that present in (Environmental and Food Pollutants laboratory-Faculty of Agriculture-Fayoum University-Fayoum-Egypt).

Analysis was performed according to Kumari *et al*. (2012) by the Agilent GC/MS system, Model: 7890B, Agilent Technologies Company-USA. Helium (99.99%) was served as a carrier gas at a flow rate of 1.8 mL/minute. Initially, the temperature was set at 70°C then increased to 250°C (rate = 15°C/minute), and lastly to 315°C (rate = 5°C/minute) with a holding time of five minutes. The temperatures used were 270°C for the ion source, 150°C for the quadrupole and 315°C for the transfer line. The MS was operated in full scan mode with a range of 50 - 320 m/z and ionization energy of 70 eV.

Statistical analysis

Quantitative data were described using range minimum, maximum, mean, and standard error of means. Mann Whitney test (U) is used to link PAHs occurrence and its concentration in raw milk and market sterile milk. A result was considered significant if P-value was less than 0.05.

RESULTS

Occurrence and concentration of (Σ 18PAHs) residues in Egyptian milk

PAHs were reported in 83.9% in raw milk samples within the range of 11.778 – 26.331 µg/kg and mean concentration of the 18 PAHs compounds (Σ18 PAHs) was 17.931±0.576 µg/kg. While from UHT market sterile milk, PAHs were detected in 77.8% of examined samples within a range of 1.151–2.946 µg/kg and the mean concentration was 2.123±0.083 µg/kg as shown in Table 1. In the current study, the occurrence and mean concentration of PAHs in raw milk is higher compared with sterile market milk.

Occurrence and concentrations (µg/kg) of different PAHs residues (Σ4 PAHs levels, Σ8 PAHs levels) in raw farm milk and market sterile milk

Raw farm milk

In the current study, total PAH4 incidence was detected about in 76.7% of samples with a level less than 1.0 µg/kg. In addition, 23.3% of samples recorded levels with more than 1.0 µg/kg. Total concentration PAH4 ranged between 0.046 and 2.433 µg/kg and mean level was 0.561±0.133 µg/kg. While total mean PAH8 all samples were detected in a range of 0.068–5.449 µg/kg and mean level of 0.840±0.240µg/kg as shown in Table 1.

UHT market sterile milk

In sterile market milk samples, total PAH4 occurrence was detected in all samples in a level less than 1.0 µg/kg. Total concentration of PAH4 ranged between 0.047 and 0.008 µg/kg while the mean concentration was 0.012 – 0.110 µg/kg. While total mean PAH8 was detected in all samples within a range of 0.012 – 0.120 µg/kg and mean level was 0.053±0.008µg/kg as shown in Table 1. In both total concentrations PAH4 and PAH8 between raw farm milk and market sterile milk there were significant difference at $P<0.05$.

Comparison between the concentrations (µg/kg) of different PAHs residues especially (BaP) detected in market and farm milk

BaP was found in 86.7% of total raw milk samples with a mean concentrations less than 1 µg/kg (0.251±0.072 µg/kg) within a range of 0.000 – 1.124 µg/kg and in 13.3% of samples exceeded slightly above 1 µg/kg without detection of BaP, Indeno(1.2.3-cd)pyrene, Dibenz[a,h]anthracene and Benzo[ghi] perylene in all examined sterile market milk samples. In sterile market milk, BaP was found in 100% of all samples at levels less than 1 µg.kg^{-1}. Comparison between the concentrations (µg/kg) of different PAHs residues detected in market and farm milk shown in Table 2 and Figure 2, revealed that there were significant difference between raw farm milk and market sterile milk in all PAHs residues except in Indeno(1.2.3-cd)pyrene and Benzo(ghi) perylene, the difference was non-significant. Chromatogram of extracted milk sample as shown in Figure 1 revealed that Phenan-

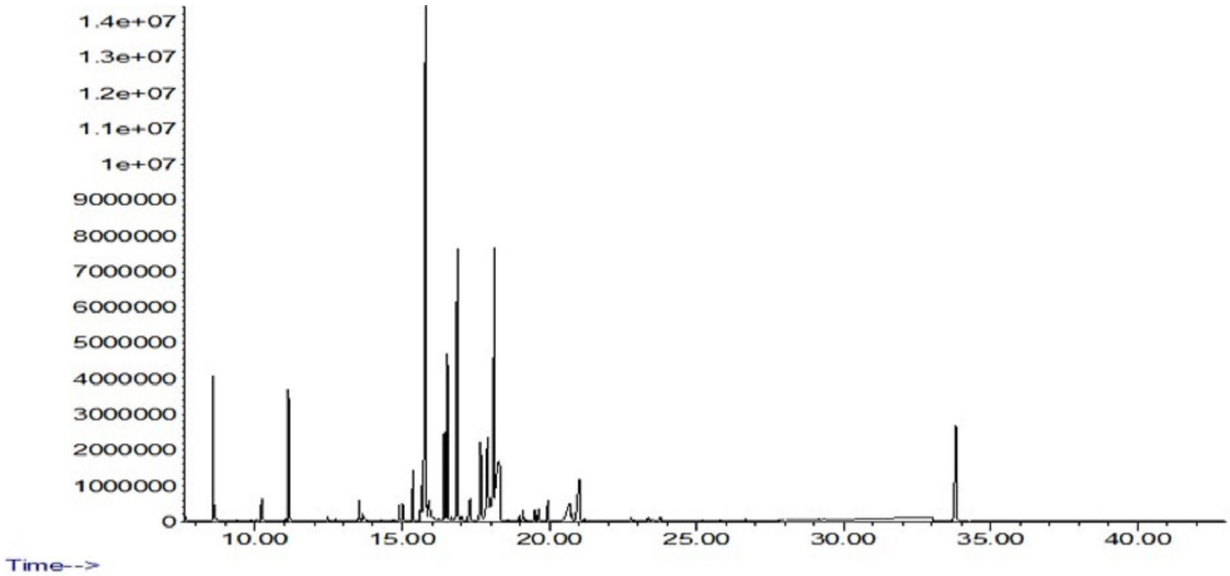

Figure 1. Examples of extracted farm milk sample by GC-MS total ion chromatogram of eighteen PAHs (Chromatogram of extracted raw milk samples).

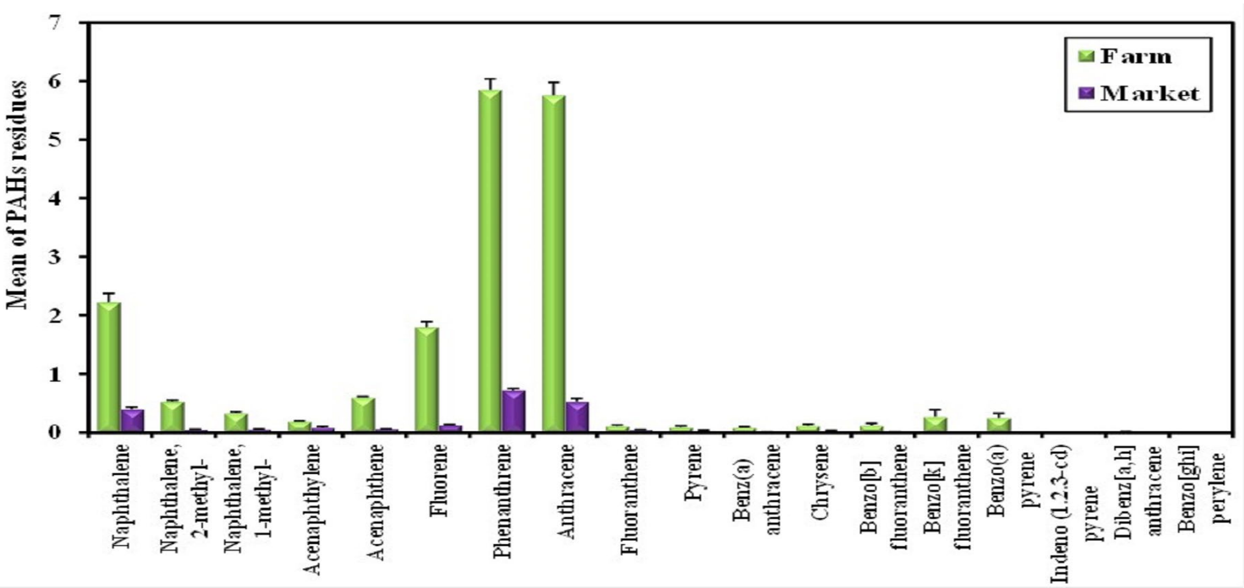

Figure 2. Comparison between farm and market according to different PAHs residue.

Table 1. Concentration of Σ18, Σ4 PAHs and Σ8 PAHs in farm and market milk samples.

	Σ18 PAHs			Σ 4 PAHs					Σ 8 PAHs		
	Min	Max	Mean±SE.	Min	Max	Mean±SE.	No. of possible samples exceed acceptable level according to (EC) NO.	%	Min	Max	Mean±SE.
Farm milk	11.778	26.331	17.931±0.576	0.046	2.433	0.561±0.133	7	23.30%	0.068	5.449	0.840±0.240
Market milk	1.151	2.946	2.123±0.083	0.012	0.11	0.047±0.008	ND.	0%	0.012	0.12	0.053±0.008

SE: Standard error; ND: Not Detected
*: refer to statistically significant difference was detected between Concentration of Σ18, Σ4 PAHs and Σ8 PAHs in farm and market at $p \leq 0.05$.

threne was the compound of the highest residue in both farm milk and sterile market milk samples with a mean concentration of 5.850±0.190 and 0.722±0.028 µg/kg, respectively, while in all sterile milk samples, BaP, Indeno(1.2.3-cd)pyrene, Dibenz[a,h] anthracene and Benzo[ghi]perylene were non-detectable. UHT samples showed low levels of PAH (1.3 µg/g [Σ[PAHs]) compared to raw milk (2.123µg/g Σ[PAHs]). Phenanthrene, naphthalene, anthracene and fluorine, respectively were found in the highest concentration of PAHs in farm milk. The lowest PAHs level was 0.198 µg/kg in pasteurized milk.

Public health significance and daily dietary intakes of PAHs

The daily dietary intake was estimated by the comparison of the Estimated Daily Intake (EDI) versus the Acceptable Daily Intake (ADI). This was presented as a percent (ADI%). The values of the maximum and mean EDI for BaP (mg/d) in milk are shown in Table 3. In farm raw milk, Max. EDI for B(a)P was 0.225 µg/kg with mean concentration 0.050±0.014 µg/kg, while the max. EDI for Σ4 PAHs levels 0.253 µg/kg with mean concentration 0.054±0.015µg/kg and the max. EDI for Σ8 PAHs levels was 0.328 with mean concentration of 0.062±0.018µg/kg. On the other hand in UHT sterile market milk Max. EDI for B(a)P was not-detected, while the max. EDI for Σ4 PAHs levels 0.001µg/kg with mean concentration 0.0004±0.00004µg/kg and the max. EDI for Σ8 PAHs levels was 0.001 with mean concentration 0.001±0.0001µg/kg. By comparing the obtained results presented with EFSA and EPA, both Σ4 PAHs and Σ8 PAHs samples were not exceeded the levels. UHT milk demonstrated a lower level of Max. EDI PAHs contamination in comparison with raw milk, which suggests that the importance of the heat treatment during milk production.

DISCUSSION

Consumption of PAHs-contaminated feed and grass may be associated with elevated concentrations of such contaminants in milk and its products Amirdivani *et al.* (2019). The concentrations of total PAHs are significantly variable between different types of samples depending upon the fatty content, nature, as well as the production method Iwegbue and Bassey (2013). Our results agree with Abou-Arab *et al.* (2014) and Raza and Kim (2018) who found that the highest mean PAH concentrations were reported in raw farm milk followed by commercial raw milk (1.011 µg/kg versus 0.370 µg/kg). Shariatifar *et al.* (2020) found that pasteurized milk had the lowest PAHs concentrations (0.87±0.18 µg/kg) as PAHs contamination of milk is also influenced by various sterilization techniques (ultrahigh-temperature processing [UHT] and pasteurization) and skimming. On the other hand, in another study (Simoneit, 2002; Naccari *et al*., 2011; Zelinkova and Wenzl, 2015) revealed that pasteurized UHT milk showed greater PAH concentrations in comparison with raw milk, suggesting that these high PAH concentrations could be because of heat treatment of milk during production.

PAHs residues detected in raw farm milk directly indicate the quality of milk and dairy products while indirectly indicate environmental pollution where the milk is produced Naccari *et al*. (2006). In 2011, EC according to European Food Safety Authority (EFSA, 2008) report, stated in the Regulation (EU) No. 835/2011 that BaP alone not sufficiently indicates the presence of PAH in foods, and that the sum of 4 PAHs (CHR, BbF, BaA, and BaP) is the most appropriate measure to recognize PAHs behaviour in foods. Besides, the current maximum limits were set in this Regulation. According to the European Commission (EC), the regulations require levels of BaP and of total PAH4 to be < 1.0 µg/kg. EFSA (2008) established that the following 8 PAHs (BaA, CHR, BbF, BaP, IndP, BkF, Bghi and DahA) for evaluation

Table 2. Concentration of different PAHs residues in farm and market milk samples.

	PAHs residues	Naphthalene	Naphthalene, 2-methyl-	Naphthalene, 1-methyl-	Acenaphthylene	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benz(a) anthracene	Chrysene	Benzo[b] fluoranthene	Benzo[k] fluoranthene	Benzo(a) pyrene	Indeno (1.2.3-cd) pyrene	Dibenz[a,h] anthracene	Benzo[ghi] perylene
Farm	Min.	1.126	0.257	0.179	0.128	0.3	1.081	2.169	3.585	0.044	0.012	0.011	0.014	0	0	0	0	0	0
(n = 30)	Max.	4.685	0.877	0.791	0.457	0.752	3.682	8.169	9.585	0.667	0.724	0.302	0.73	1.032	3.015	1.124	0.017	0.071	0.036
	Mean	2.226	0.528	0.329	0.185	0.597	1.801	5.85	5.759	0.107	0.087	0.082	0.112	0.116	0.266	0.251	0.001	0.009	0.003
	SE.	0.826	0.124	0.116	0.072	0.086	0.523	1.04	1.221	0.118	0.135	0.072	0.152	0.226	0.662	0.396	0.003	0.018	0.009
Market	Min.	0.126	0	0.017	0.002	0.016	0.001	0.597	0.072	0.006	0.004	0.001	0.002	0.002	0	0	0	0	0
(n = 20)	Max.	0.866	0.129	0.124	0.365	0.09	0.2	0.901	0.976	0.084	0.125	0.021	0.09	0.021	0.016	0	0	0	0
	Mean	0.394	0.045	0.051	0.079	0.059	0.122	0.722	0.526	0.045	0.029	0.01	0.027	0.01	0.005	0	0	0	0
	SE.	0.163	0.038	0.03	0.093	0.015	0.069	0.124	0.243	0.025	0.039	0.005	0.031	0.006	0.005	0	0	0	0
U		0.0*	0.0*	0.0*	84.0*	0.0*	0.0*	0.0*	0.0*	103.0*	107.0*	19.0*	115.0*	105.0*	94.0*	40.0*	260	230.0*	260

SE: Standard error; U: Mann Whitney test
p: p value for comparing between the two studied groups
*: refer to statistically significant difference was detected between Concentration of different PAHs residues in farm and market milk samples at $p \leq 0.05$.

Table 3. Estimated Daily Intake (EDI) in terms of BaP (mg/day).

	Farm (n = 30) Max. Estimated Daily Intake (EDI)	Farm (n = 30) Mean±SE. Estimated Daily Intake (EDI)	Market (n = 20) Max. Estimated Daily Intake (EDI)	Market (n = 20) Mean±SE. Estimated Daily Intake (EDI)	EFSA Recommendation	EPA Recommendation
B(a)P	0.225	0.050±0.04	ND	ND	0.001	0.001
Σ4 PAHs levels	0.253	0.054±0.015	0.001	0.0004±0.00004	0.002	0.002
Σ8 PAHs levels	0.328	0.062±0.018	0.001	0.001±0.0001	0.002	0.002

EPA: Environmental Protection Agency; EFSA: European Food Safety Authority.

of oral carcinogenicity in foods caused by contamination with PAHs and advocated that these 8 PAHs (PAHs8) or the PAHs4: BaA, CHR, BbF and BaP or the subgroup of PAHs 2: CHR and BaP are the most appropriate to indicate the existence of PAH in food. From an analysis of PAH2, PAH4 and PAH8 in respect to BaP, it was found that PAH2 (CHR + BaP) couldn't be used as a valuable marker to BaP in raw milk; PAH4 was demonstrated to be the main contributor for PAH contamination in all evaluated milk samples; and PAH8 did not provide much more importance in comparison with PAH4 (FSAI, 2015; Naccari *et al*., 2006). There were significant differences between total concentrations of PAH4 and PAH8 in raw farm milk and market sterile milk at $P<0.05$. In another study, Santonicola *et al*. (2017) found that 77.7% of samples had PAH4's concentrations higher than limits set by the European Food Safety Authority (EFSA, 2008). Such results signify a concerning health risk related to consuming milk-based baby foods. Likewise, Badibostan *et al*. (2019) only one sample was detected to exceed the limit of 1 μg/kg set by the EC (2011) for PAH4 (1.43 μg/kg). Kacmaz (2016) found that the highest mean value of 4PAHs in all UHT milk samples was 0.84±0.57 μg kg-1 whereas the lowest in raw milk samples was 0.10±0.06 μg kg-1. The samples were below the limit of 1 μg/kg set by the EU (not-detected to 0.14 μg/kg w/w). Iwegbue and Bassey (2013) reported that out of 20 milk brands evaluated, 14 samples had intake values for BaP, PAH 2 and PAH 4, whereas the remaining 8 brands had intake value of zero for PAH 8.

Generally, PAHs containing four fused rings, such as BaA and CHR, have weak carcinogenic effects as compared with compounds containing ≥ 5 rings, such BaP, which have the potential of genotoxicity and carcinogenicity and therefore are considered among organic pollutants of public health issues Nisbet and LaGoy (1992). A daily intake of BaP > 10 ng/kg can result in harmful effects in human (JECFA, 2015).In a previous study, Rawash *et al*. (2018) detected BaP in 45% of analyzed raw milk samples, the levels ranged between 0.01 and 0.41 μg/g with a median concentration of 0.12 μg/g and only two of the analyzed samples contained 7 of carcinogenic PAHs with a mean level (0.46 μg/g). Santonicola *et al*. (2017) found that 18.2% of samples had levels of BaP, which exceeded the acceptable limit set by EU European Food Safety Authority (EFSA, 2008). Such results signify that there may be public health issue linked to milk consumption.

International Agency for Research on Cancer (IARC, 2010) categorized BaP as group 1 carcinogen (i.e. carcinogenic to humans), BaP was shown to be a marker of the carcinogenetic concentrations in foods. It can enhance the reactive oxygen species' formation, causing DNA damage. Toxicological studies showed that certain PAHs can also produce mutagenic/genotoxic effects Huang and Penning (2014). Many PAHs possess toxic, mutagenic, and carcinogenic effects. BaP is particularly regarded as carcinogenic Zhang *et al*. (2021). Naphthalene can cause hemolysis after its inhalation or ingestion in large volumes (Chen and Liao, 2006).

CONCLUSION

Collectively, both farm raw and UHT sterile market milk contain PAHs in various concentrations that might in some extent exceed the recommended critical limits especially in raw farm milk. Milk and its products are daily used, thus the exposure to PAHs is unavoidable, highlighting the carcinogenic potential of such compounds on long term exposure particularly for young children. It is therefore necessary to apply strict regulations on the production of milk and dairy products to reduce the concentrations of PAHs and minimize health problems.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related.

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