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Technical note

Polycyclic aromatic hydrocarbons (PAHs) in four milk brands sold in Mexico City: evaluating three fat extraction methods

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Abstract:

Polycyclic aromatic hydrocarbons (PAHs) are recognized as emerging pollutants in milk due to their risk to human health. Identification and quantification of PAHs requires analytical methods that allow more accurate and complete estimates. An analysis was done of the sixteen PAHs considered priority by the U.S. Environmental Protection Agency in whole milk from Mexico City, and this used to compare three milk fat extraction procedures. Of the four milk brands analyzed, three were ultrapasteurized (UHT) and one was pasteurized (HTST). The milk was acquired from March-June 2016. Three extraction methods were

tested: saponification (method A); detergent solution extraction (method B); and liquid-liquid extraction (method C). The PAH profiles from each method were generated by gas chromatography with a flame ionization detector. Three of the four milk brands (75 %) were positive for at least one of the sixteen analyzed PAHs. Profiles differed by extraction method with only low molecular weight compounds in method A, both low and high molecular weight compounds in method B, and higher recovery rates of low and high molecular weight compounds in method C. This method produced better recovery rates for low (58.7-12.3) and high molecular weight PAHs (81.8-8.0) than in method B (low molecular weight = 15.0-8.0, high molecular weight = 58.0-21.0).

Key words: Polycyclic aromatic hydrocarbons, Extraction methods, Milk, Gas chromatography.

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Polycyclic aromatic hydrocarbons (PAHs) include over one hundred different chemicals formed during incomplete combustion of organic matter and released into the environment in large quantities[1,2]. Due to their persistence and toxicity, the U.S. Environmental Protection Agency (EPA) has included sixteen PAHs (Table 1) in its list of persistent organic pollutants[3].

Table 1. The sixteen polycyclic aromatic hydrocarbons (PAHs) listed as pollutants by the US EPA, by molecular weight

PAHs	Abbreviation	Molecular weight (g/mol)
Low molecular weight (LMW):		
Naphthalene	NAP	128
Acenaphthene	ANA	154
Acenaftilene	ANY	152
Fluorene	FLU	166
Phenanthrene	PHE	178
Anthracene	ANT	178
High molecular weight (HMW):		
Fluoranthene	FLT	202
Pyrene	PYR	202
Benzo(a)anthracene	BaA	228
Chrysene	CHR	228
Benzo(b)fluoranethane	BbF	252
Benzo(k)fluoranethane	BkF	252
Benzo(a)pyrene	BaP	252
Benzo(g,h,i)perylene	BPE	276
Indeno (1,2,3-cd)pyrene	IPY	276
Dibenzo(a,h)-anthracene	DBA	278

EPA, 1998[3].

These compounds occur worldwide as particulate matter in the air[4], and can accumulate in soils and grasses[5,6]. If lactating cows eat fodder containing PAHs, these can then be detected in milk and derived dairy products[7-11]. Contamination of milk with PAHs depends on environmental factors such as exposure source, cow lactation stage, animal health status and breeding system[12,13].

Consumption of milk containing PAHs poses a risk to human health. The European Union (EU) has consequently established maximum residue levels of 1 to 35 µg kg fat in different foods for benzo(a)pyrene (BaP) and the combination of BaP, benzo(a)anthracene (BaA) , benzo(b)fluoranthene (BbF) and chrysene (CHR)[14].

No official method exists for quantification of PAHs in milk, but two methodologies are currently in use: 1) gas chromatography with an ionizing flame detector and mass spectrometry[15,16]; and 2) high-resolution liquid chromatography with a fluorescence detector[7,8,17]. Various procedures have been used for sample preparation, including saponification, liquid-liquid extraction (LLE), and cleaning by column chromatography, or

more recently, solid phase extraction (SPE)[18,19,20]. However, their results can differ. For example, direct identification and quantification of PAHs in milk by saponification with subsequent extraction, or by fat extraction followed by purification, produce different PAH profiles, and tend to identify phenanthrene (PHE), anthracene (ANT), fluorene (FLU), pyrene (PYR), BaA and CHR. The present study objective was to evaluate the efficacy of three fat extraction methods in the identification and quantification of the presence of PAHs in four brands of milk.

Four brands of whole milk (three ultrapasteurized [UHT] and 1 pasteurized [HTST]) were randomly selected. Three samples were collected for each brand (n= 12) during March-June 2016 in supermarkets in the Coyoacán delegation of Mexico City, Mexico. All samples were stored for no more than 5 d after purchase in the Instrument Analysis Laboratory of the Metropolitan Autonomous University-Xochimilco (Universidad Autónoma Metropolitana). The UHT samples were stored in a cool, dry place, and the pasteurized sample under refrigeration (5 °C). Before beginning the extraction process samples were homogenized in a water bath (40 °C) for 30 min, manually stirring every 5 min. The samples were processed with one of three extraction methods:

Method A: Saponification. This was done following an established method[17], with modifications. Briefly, 8 ml 0.4 M sodium hydroxide solution in ethanol was added to 4 ml (4 g) milk. The mixture was homogenized for one minute in a vortex and placed in a thermal bath at 40 °C until almost dry (1 ml). It was completely dried under a nitrogen flow, reconstituted in 1,000 µl isooctane and stored at -20 °C until analysis.

Method B: Detergent solution extraction. Sample (250 ml) and 250 ml detergent solution (50 g sodium hexametaphosphate in 24 ml Triton X -100 dissolved in 1 l water) were added to a 500 ml flask. The flask was vigorously stirred by being placing in a water bath at 90 °C, and inverting every 15 min until fatty matter had separated out in the neck of the flask. The fat was removed from the flask, filtered at 50 °C through No. 4 Whatman filter paper in the presence of anhydrous sodium sulfate and stored in glass tubes at -20 °C until analysis[21].

Method C: Liquid-liquid extraction (AOAC 989.05). Sample (150 ml) and 0.5 g ethylenediaminetetraacetic acid (EDTA) were added to a separation funnel, stirred for one minute and allowed to sit for 2 min. Methanol (50 ml) was added to the funnel and the solution stirred again for 1 min. This operation was repeated, adding 50 ml diethyl ether and 50 ml petroleum ether. It was set aside to allow separation of the organic phase (supernatant). The lower layer was drained off and the supernatant passed through No. 1 Whatman filter paper, adding 5 g anhydrous sodium sulfate. The organic phase was rotory evaporated at 40 °C, transferred to a 5 ml bottle and stored at -20 °C until analysis.

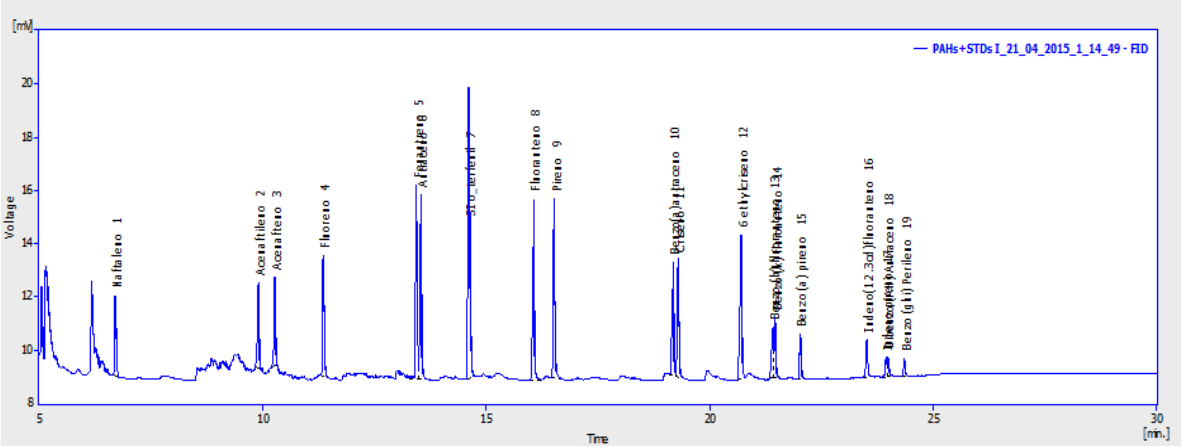
The saponified fat sample was slowly deposited in a column containing 6 g silica gel in its inferior portion and 1 g anhydrous sodium sulfate. Hexane (20 ml) was added and the organic

phase marked as F1. Using a different flask, 30 ml 9:1 hexane-dichloromethane (v/v) were added and allowed to flow in slowly. When it arrived at the level of sodium sulfate, 20 ml 1:1 hexane-dichloromethane (v/v) were added. The entire organic phase was collected in a single flask and marked as F2 (recovered PAHs). This phase was rotary evaporated at 40 °C until almost dry (1 ml), transferred to an amber vial and completely dried under a nitrogen flow. It was reconstituted in 250 µl isooctane and stored at -20 °C until analysis[19].

A high-resolution digital gas chromatographer with self-sampler (Shimadzu GC 2010) was used with a PTV injector at 250 °C in Splitless mode with a 1 min sampling time, 5.0 ml min^{-1} purge flow, and 5 ml min^{-1} septum purge. Nitrogen was the vehicle gas and was used at a 9.8 ml min^{-1} flow rate. The column was an HP5-MS (30 m length x 0.025 mm ID x 0.25 mm thickness). The temperature sequence was as follows: initial temperature 40 °C for 3 min; increased to 50 °C at 2 °C/min; increased to 160 °C at 3 °C/min; increased to 210 °C at 5 °C/min; increased to 255 °C at 7 °C/min; increased to 265 °C at 4 °C/min; increased to 300 °C at 5 °C/min; 300 °C for 5 min. Chromatographic analysis was done with the GG solution software.

Sample extract (1 µl) was injected into the column of a chromatographer (Agilent GC 5890). A capillary column (30 m length x 0.25 mm ID x 0.25 mm thickness) (Rtx-5Sil MS, Restek Bellafonte, PA, USA) was used along with a precolumn (2 m length x 0.53 mm ID) (Siltek, Restek). The vehicle gas was helium at a constant flow rate of 1 ml/min. Injector temperature was set at 3 °C above device temperature at all times. The run temperature sequence was as follows: 1 min at 100 °C; increased to 300 °C at 5 °C/min; 15 min at 300 °C. Analyte detection was done with a mass spectrometer (Agilent MS 5972) in electron impact mode at 70 eV ionization energy, and using single ion monitoring[22] (Figure 1).

Figure 1: Mass spectrometry chromatogram identifying sample peak to determine correspondence to native compound



Internal standard: orthoterphenyl (peak 7); 6 ethyl chrysene (peak 12); indeno[1,2,3-cd]fluoranthene (peak 16).

Chemicals were reagent quality and solvents were HPLC quality; all were acquired from J.T. Baker Chemical, USA. Analyte identification and quantification were done with a mixture of sixteen PAH compounds recommended in the method EPA 610 (Chemicalservice, USA): naphthalene (NAP); acenaphthalene (ALC); acenaphthylene (ACY); fluorene (FLU); phenanthrene (PHE); anthracene (ANT); fluoroanthracene (PMA); pyrene (PYR); benzo(a)anthracene (BaA); chrysene (CHR); benzo(b)fluoranthene (BbF); benzo(k)fluoranthene (BkF); benzo(a)pyrene (BaP); dibenzo(ab)anthracene (DBA); benzo(ghi)perylene (BGP) and indeno(cd)pyrene (IcdPy) (Table 1).

Extraction by saponification (Method A)[17] identified only LMW PAHs. Extraction with the detergent solution (Method B)[19] identified both LMW and HMW PAHs; 66.66 % were LMW and 33.33 % were HMW (Table 2).

Table 2: Polycyclic aromatic hydrocarbons (PAHs) concentration (µg g^{-1}) in milk determined with methods A and B

PAHs	Method A	Method B
NAP	0.066	Nd
ALC	0.200	0.372
ACY	0.066	Nd
FLU	Nd	0.915
PHE	Nd	7.153
ANT	5.385	14.924
FLT	Nd	Nd
PYR	Nd	3.773
BaA	Nd	0-.056
CHR	Nd	0.044
BbF	Nd	1.264
BkF	Nd	0.750
BaP	Nd	0-.114
DBA	Nd	4.061
BGP	Nd	Nd
IcdPy	Nd	1.641
Sum of 16 PAHs	5.717	35.067
Sum of 4 PAHs	0.0	1.478
Sum of LMW PAHs	5.717 (100 %)	23.365 (66.6 %)
Sum of HMW PAHs	0.00	11.702 (33.4 %)

Extraction methods= A: saponification and direct extraction; B: detergent solution extraction.
Nd= not determined; µg g^{-1}: microgram PAH per gram milk fat.

Extraction of PAHs from milk by saponification (Procedure A) produced a PAH profile different from previous studies which report a predominance of HMW PAHs with higher concentrations of PHE and ANT, as well as a LMW PAHs proportion of 50 to 68% of total PAHs[17,23]. Absence of HMW compounds when using Method A may be due to low sample concentrations, as observed elsewhere[24]. However, milk sample size (4 ml) was not enough to exceed PAH detection limits under the present conditions (flame ionization detector).

Low HMW PAH concentrations have been reported in infant dairy formulas[18,25], and whole and UHT milk[17]. Low molecular weight (LMW) PAHs (2 and 3 rings), particularly NAP, ACE and ACY, have not been reported in various studies[9,17,23], or were recovered at percentages less than 50%, possibly due to their high volatility[17]. Saponification time and temperature play an important role in recovery rates. For example, in the present study

detection temperature was 40 °C, similar to the 60 °C saponification temperature[24], and various LMW compounds were detected. In a previous study saponification was done at 80 °C and only PHE and ANT were detected[23]. This suggests that saponification temperature is a critical factor when extracting PAHs[9].

In Method B the milk fat was not saponified and was run through a purification column, allowing identification of 66.6 % LMW PAHs and 33.4 % HMW PAHs. This profile is similar to the 75.5 % LMW and 24.5 % HMW proportions reported for 31 milk samples from Brazil and Argentina[7]. The LMW PAH proportion is within the 40 to 69 % range reported for fresh milk from farms near an industrial area[19]. Differences between these studies may be due to milk fat extraction method since one study used organic solvents[7] and another a detergent solution[19].

Most studies using direct saponification of samples have employed mass-coupled or fluorescent detectors, which allow quantification of low PAH concentrations[17,18,24]. However, when using gas chromatography with flame ionization detection, a larger amount of milk fat is needed to achieve adequate sensitivity. Extraction with a detergent solution produces sufficient amounts of fat although PAHs may be lost due to the temperature (90 °C) to which samples are subjected.

Recovery rates in methods B and C, as confirmed by GC-MS, were highly variable, with higher rates of HMW PAHs recovered (Table 3). This variability among LMW and HMW PAHs was probably due to fat extraction method and rotary evaporation temperature. Under the evaluated conditions the most appropriate method was C since it attained recovery rates ranging from 45.3 to 95.1 %. These are similar to those reported in another study using organic solvents for fat extraction in which recovery rates ranged from 40 to 125 %, although individual PAH compounds were not identified[26]. Rates in a study of human milk varied from 42 to 101 %, using the boiling point, with an R^2 of 0.779[27]. Particularly high recovery rates (95 to 98 %) have been reported for powdered milk when using an ultrasound bath and subsequent column purification[15], and when using a solid phase microextraction system (87.6 to 112 %)[28].

Table 3: Recovery rates of polycyclic aromatic hydrocarbons (PAHs) in milk using two extraction methods (mean ± standard error)

PAHs	Method B	Method C
NAP	Nd	Nd
ALC	15.2±7.3	45.3±19.0
ACY	10.8±9.1	46.5±14.7
FLU	23.8±4.8	72.3±20.9
PHE	28.3±10.7	67.6±22.6
ANT	30.4±15.3	61.6±16.9
FLT	48.0±10.9	77.5±24.8
PYR	44.7±1467	72.0±25.9
BaA	70.9±16.7	80.0±14.4
CHR	59.3±15.5	95.1±27.5
BbF	93.5±21.1	80.9±11.3
BkF	45.4±11.1	72.7±22.4
BaP	127.0±35.0	85.6±7.0
DBA	78.4±17.9	92.1±18.3
BGP	64.5±15.7	86.9±21.9
IcdPy	66.3±14.7	75.0±15.7
Sum of LMW PAHs	15 ± 8 %	58.7±12.3
Sum of HMW PAHs	58 ± 21 %	81.8±8.0

Methods= B: detergent solution extraction; C: liquid-liquid extraction.

In milk samples, fat extraction method has a substantial effect on which PAHs can be identified. Recovery rates with Method C agreed with those reported for environmental pollutants in biological matrices at concentrations less than 1 µg kg^{-1}, where rates can range from -50 to +20%[29]. This recovery rate allows accurate assessment of the presence of PAHs in milk samples.

Of the four analyzed milk brands one (A) contained no detectable PAHs, whereas in the remaining three brands at least one of the sixteen compounds was detected (Table 4); that is, 75 % of samples were positive for PAHs. The compounds PHE and ANT had the highest incidence (54.5 %), followed by FLUO and DBA (45.5 %). The highest concentration was of ANT (341 µg g^{-1}), followed by PHE (20 µg g^{-1}) and DBA (12.3 µg g^{-1}). These results coincide with previous reports in which LMW PAHs occur with more frequency at higher concentrations[17,23].

Table 4: Presence of polycyclic aromatic hydrocarbons (PAHs) in milk samples (n=12)

	ACE	FLUO	PHE	ANT	PYR	BaA	CHR	BbF	BkF	BaP	IND	DBA
% Inc	36.4	45.5	54.5	54.5	9.1	27.3	27.3	27.3	18.2	9.1	27.3	45.5
Sum	1.2	5.6	20.0	341.0	3.8	0.2	0.2	2.3	1.1	0.1	6.3	12.3
Min	0.2	0.3	0.4	0.0	3.8	0.0	0.0	0.2	0.3	0.1	0.7	0.1
Max	0.6	3.6	7.5	155.0	3.8	0.1	0.1	1.3	0.7	0.1	3.0	5.6

Inc= incidence, Min= minimum, Max= maximum.

Of the four analyzed milk brands, D had the largest mean sum of four PAHs (Table 5). This concentration exceeds EU guidelines for nursing formulas (1 µg kg^{-1})[14], indicating it poses a risk to human health. Perhaps the higher concentration in this brand was due to the vegetable fat included in its formulation, which is absent in the other three milk brands.

Table 5: Mean sum of sixteen and four polycyclic aromatic hydrocarbons (PAHs) in four milk brands from Mexico City

Brands	Σ 16 PAHs µg kg^{-1}	Σ 4PAHs µg kg^{-1}
A	Nd	Nd
B	47.56	0.23
C	93.95	1.14
D	51.49	4.04

Nd= Not detected.

When extracting fat from milk samples for identification and quantification of polycyclic aromatic hydrocarbons, methods B and C preserved variable percentages of low and high molecular weight compounds. Method C exhibited the best recovery rate, although Method B could be an alternative when using gas chromatography-mass spectrometry. Three of the four (75 %) milk brands were positive for polycyclic aromatic hydrocarbons, and two brands exceeded maximum levels recommended by the European Union.

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