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# Polycyclic aromatic hydrocarbons in retail Turkish yogurts

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**RESEARCH ARTICLE**

## Abstract

The level of 4 EU marker polycyclic aromatic hydrocarbons (4PAHs) was determined in various types of yogurt samples consumed in Turkey. Seventeen kinds of retail yogurt were purchased from supermarkets and local dairy firms in Turkey. A high-performance liquid chromatography method involving a liquid-liquid extraction and a pre-concentration step was applied for the determination of 4PAHs. The method was validated according to single laboratory validation guidelines with parameters such as selectivity, precision, limit of detection, limit of quantitation, linearity, accuracy, and also measurement uncertainty. The highest concentration for sum of the 4PAHs was found 0.59 µg/kg and 0.95 µg/kg in yogurt samples with low and high fat content, respectively. The present paper is the first study concerning the levels of polycyclic aromatic hydrocarbons in yogurts from Turkey.

**Keywords:** 4 EU marker PAHs, Turkish yogurt, yogurt, polycyclic aromatic hydrocarbons, HPLC

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) can be defined as a large class of organic compounds, each of them containing two or more aromatic rings. PAHs are formed and released to environment via the pyrolysis (burning) or incomplete combustion of organic materials e.g. garbage, wood, petrol, oil products and coal, as well as during industrial food processes such as smoking, frying, drying, baking, roasting and charcoal barbecuing/grilling (CCFAC, 2005; FAO/WHO, 2005; SCF, 2002a,b). According to the International Agency for Research on Cancer (IARC), PAHs are very harmful for laboratory animals, and especially for humans. Some of them can be classified as mutagenic, carcinogenic, and genotoxic (IARC, 2010, 2012).

In the past decades, International Organisations such as EFSA (European Food Safety Authority), SCF (the Scientific Committee on Food), IPCS (the International Programme on Chemical), JECFA (the Joint FAO/WHO Expert Committee on Food Additives), IACR (the International Agency for Research on Cancer) and also EPA (United States Environmental Protection Agency) extensively evaluated the PAHs in terms of the occurrence, sources, toxicity, exposure and carcinogenicity (CCFAC, 2005; EC, 2005; EFSA, 2008a,b; FAO/WHO, 2005; IARC, 2010, 2012; SCF, 2002a,b). Accordingly, European Commission (EC) selected a set of the four PAHs compounds (chrysene [CHR], benzo[a]pyrene [BaP], benz[a]anthracene [BaA], benzo[b] fluoranthene [BbF]) as the most appropriate indicator for total PAH content in foodstuffs. These PAHs were then entitled as PAH4 or as 'the four EU marker PAHs'. Valid legal regulation was presented by European Commission (EC) in 2011 (EC, 2011a). The regulation specified the maximum limits for some key food commodities such as cocoa beans and derived products, oils and fats, smoked meat and products, fish and products as between 1.0 µg/kg and 35.0 µg/kg. Maximum limits are also reported for infant formula and follow-on formula, including infant milk and follow-on milk as 1.0 µg/kg by Commission (EC, 2011a). However, because of the inadequate data about PAH levels in milk and dairy products, maximum limits are not established until now.

Human beings can be exposed to PAHs through natural resources such as soil, water, air, but the primary sources is human diet (Falcó *et al.,* 2003). The way of food processing such as smoking, frying, barbecuing and environmental food contamination can affect the quantity coming from the diet (Bansal and Kim, 2015; Bansal *et al.,* 2017; Zelinkova and

Wenzl, 2015). Studies for human exposure to PAHs carried out in the entire world reported many food categories as significant sources, especially meat and meat products (Jira *et al.,* 2008; Ledesma *et al.,* 2016), oils (Barranco *et al.,* 2003; Wen *et al.,* 2017), cereals (Kacmaz, 2016; Kacmaz *et al.,* 2016), vegetables (Shi *et al.,* 2016; Tfouni *et al.,* 2014) and also milks (Aguinaga *et al.,* 2008; Chung *et al.,* 2010; Girelli *et al.,* 2014; Naccari *et al.,* 2011). Among these food sources, particular attention is given to milk and dairy products especially to the yogurt due to its high nutritional importance component in human daily diet (Visioli and Strata, 2014). Therefore, it is important to demonstrate the effect of exposure of humans to PAHs by PAH-containing yogurt consumption. Even if various reports confirmed PAHs contaminations of milk, data in yogurts are lacking. Their presence and level in yogurts is not known very well due to the limited number of studies (Aguinaga *et al.,* 2007; Battisti *et al.,* 2015). So, this study focused to survey the contamination levels of 4EU marker PAHs (4PAHs; benz[a] anthracene, chrysene, benzo[b]fluoranthene and benzo[a] pyrene) in various yogurts commercialised in Turkey.

For this purpose, a high-performance liquid chromatography (HPLC) method involving a liquid-liquid extraction and a pre-concentration step was used. The used method was in house validated with some analytical parameters such as linearity, recovery, precision, limits of detection (LOD) and quantification (LOQ) and measurement uncertainty.

## 2. Materials and method

### Samples

The study was carried out for commercially packed yogurt samples that manufactured by small-and large-scale dairy firms in Turkey. During the period between March 2017 and April 2017, 17 yogurt samples, 3 samples with low fat (from 0.15 to 1.4%) and 14 samples with high fat content (from 3.0 to 4.7%) were selected from widely consumed commercial products. They were purchased from local Turkish markets in the Black Sea region of Turkey. The size of the sample packs was between 200 and 1,500 g. All dairy product samples were produced from cow's milk. All samples stored at 4 °C and analysed within the shelf life of the product. Five or more batches of each brand were homogenised and analysed in triplicate.

### Chemicals and reagents

The all reference standard solution of BaP, CHR, BaA and BbF (10 µg/ml in acetonitrile) were supplied by the Institute for Reference Materials and Measurements (IRMM; Geel, Belgium). All stock solutions of PAHs were prepared by diluting a pure reference standard at concentrations over a range of 0.4-85 µg/kg. They were kept at 4 °C in amber flasks because of beware light exposure if not in use.

All of the reagents, acetonitrile (99.9%), cyclohexane (>99.5%) ethanol and NaOH used were of analytical grade were purchased by Sigma Aldrich (Steinheim, Germany). Polytetrafluoroethyleneacrodisk (25 mm i.d., 0.45 µm) syringe filters were from Sigma Aldrich. Purified water was obtained in the laboratory by using a Milli-Q system (Mili-Pore) (Bedford, MA, USA).

### Extraction and clean up

An extraction and clean up method was used as stated by Battisti *et al.* (2015). Following the homogenisation of yogurt samples using ultra sound for 15 min, 2±0.1 g of sample mixture were accurately weighed into a vial. They were saponificated using NaOH ethanolic solution (4.0 ml of 0.4 M). Saponification was made in a water bath at 60 °C for 30 min. The mixture was vortexed for 5 min by adding 2.0 ml cyclohexane. After collecting the supernatant by pipetting, the remaining saponificated phase was re-extracted with 2 ml cyclohexane with 2 more times. The supernatant solutions were filtered through 0.45 µm disk syringe filters. Solvents were evaporated under nitrogen until dryness. Then it was re-dissolved with 100 µl acetonitrile. 20 µl of clear filtrate was injected into the HPLC chromatographic system for the analysis.

### Chromatographic equipment and conditions

All measurements were performed with the HPLC system, using a liquid chromatographic LC module (Agilent 1260 infinity, Agilent Technologies, Santa Clara, CA, USA), a 7125 injector that has 20 µl samples loop, and a 110 series fluorescence detector (Agilent Technologies). PAHs separation was done by a LiChrospher C18 (250×4.6 mm × 5 µm) column with a gradient elution. The gradient elution was applied using solvent A (100% $H_2O$) and solvent B (100% $CH_3CN$) as shown in Table 1. At room temperature, the flow rate was set at 1.8 ml/min. In these conditions, PAHs separated satisfactorily within 30 min. As seen in Figure 1, a typical HPLC-fluorescence detection chromatogram, there is a good agreement between retention times in the observed two chromatograms for a standard PAH solution

**Table 1. Mobile phase gradient elution program to separate 4 EU marker polycyclic aromatic hydrocarbons.**

| Time (min) | %A (water) | %B (acetonitrile) |
|---|---|---|
| 0 | 40 | 60 |
| 20 | 37 | 63 |
| 24 | 28 | 72 |
| 25 | 15 | 85 |
| 27 | 0 | 100 |
| 28 | 0 | 100 |
| 35 | 40 | 60 |

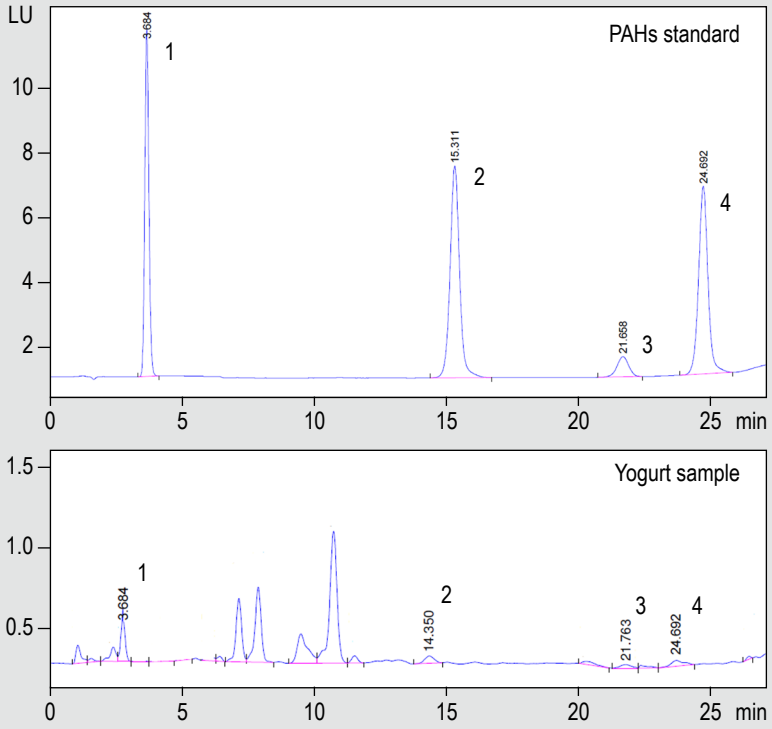


**Figure 1. Chromatogram of a standard polycyclic aromatic hydrocarbons (PAHs) solution at 5 µg/kg and of a yogurt sample. Compounds: 1 = benz[a]anthracene; 2 = chrysene; 3 = benzo[b]fluoranthene; 4 = benzo[a]pyrene.**

and a yogurt sample. Fluorescence detection was made 260 and 420 nm as the emission and excitation wavelength.

## 3. Results and discussion

### Analytical method validation

The applied method was a single laboratory validated for yogurt matrices in view of the internationally accepted guidance documents (Citac and Eurachem, 2000; Eurachem, 2014) and the Commission Decision which implementing Council Directive 96/23/EC related to the performance of methods and the review of analytical results (EC, 2011a). Selectivity, linearity, LOD, LOQ, precision, recovery, and measurement uncertainty were evaluated establishing criteria and procedures for the validation of analytical methods to be sure the comparability and quality of results produced by official laboratories.

The accuracy, as provided by recovery of 4PAHs, ranged from 92 to 106% and from 80 to 88% for low-fat (0.15%) and high-fat (4.7%) yogurt samples, respectively. Recoveries were greater than 80% for all PAHs analysed. The precision, as provided by the relative standard deviation under repeatability and intermediate precision conditions, was lower than 20% for all analytes.

### Selectivity and linearity

The linearity of the method was evaluated by Mandel's fitting test using calibration curve that obtained from eight calibration points' signals (Mandel, 1964). Mandel's tests passed and any trends observed for the four target analytes. Calibration functions of each PAH were linear for all analyte in the range between 0.4 µg/kg and 85 µg/kg.

The selectivity of the method was evaluated using the procedural blank. It was controlled in terms of interferences at the retention time of each analyte. For this purpose, whole extraction and analysis procedure was simultaneously performed to the blank sample that consisted of 2 ml extraction solvent.

Selectivity can be defined acceptable in case of nonexistence of peaks in the chromatogram of the procedural blank at the retention time of the analytes ± 0.1 min whether peaks did not pass over 30% of the height of the native analyte in the chromatogram of the lowest calibration point (Kacmaz *et al.,* 2016). The data as presented in Table 2, spectral interference at the retention time of each analytes was between 11 and 28% of the peak heights of analyte peaks at the lowest point of calibration. All of the data were within acceptable range.

**Table 2. Correlation coefficient, linearity range, limit of detection (LOD) and limit of quantification (LOQ) of four polycyclic aromatic hydrocarbons (PAHs).**

| PAH | $R^2$ | Linearity (µg/kg) | Mandel's test | LOD (µg/kg) | LOQ (µg/kg) | Height PB/CS1 (%)[1] |
|---|---|---|---|---|---|---|
| Benz[a]anthracene | 0.9999 | 0.4-85 | passed | 0.07 | 0.25 | 24 |
| Chrysene | 0.9992 | 0.4-85 | passed | 0.06 | 0.19 | 17 |
| Benzo[b]fluoranthene | 0.9980 | 0.4-85 | passed | 0.06 | 0.20 | 28 |
| Benzo[a]pyrene | 0.9998 | 0.4-85 | passed | 0.05 | 0.17 | 11 |

[1] Difference for percentage of height compared to the peak of the lowest calibration point (CS1) of the peaks that elute at the retention time of the analytes in the procedural blank (PB) samples.

### Limit of detection and limit of quantification

The LOD and LOQ shows the sensitivity of the system. In this study, LOD and LOQ was determined for each PAHs using a signal-to-noise ratio of 3 and 10, respectively. The LOD and LOQ were found between 0.05-0.07 µg/kg and 0.17-0.25 µg/kg for all PAHs, respectively (Table 2).

### Precision (repeatability and intermediate precision)

The precision of analytical method was assessed in terms of repeatability and intermediate precision which were done by ANOVA and stated as relative standard deviation (RSD%). Repeatability and intermediate precision were determined at the lowest point of working range from naturally low contaminated yogurt sample, which were spiked with a 0.25 µg/kg of standard PAH solution. Each spiked sample was analysed on three different days as triplicate under repeatability conditions such as same operator, laboratory and equipment. The obtained relative standard deviation for repeatability ($RSD_r$ %) were found between 5.9 and 10.5% that lower than 15% for all analytes.

Intermediate precision stated as relative standard deviation ($RSD_{IP}$ %), was assessed by applying three independent sequences as triplicate analysis spread over one month. The obtained data were found between 7.5 and 16.8% that lower than 20% for all analyte. Table 3 present all obtained results for repeatability and intermediate precision.

Additionally, precision was assessed in terms of Horwitz ratio (HorRat) values that were defined as the method's precision performance criteria in the EC regulation No. 836/2011 (EC, 2011b). Accordingly, $HorRat_r$ and $HorRat_R$ values can be estimated from the Horwitz equation by dividing the relative standard deviation to repeatability ($RSD_r$ %) and reproducibility ($RSD_{IP}$ %) values, respectively (EC, 2011a). It is concluded that, for a precise analysis, both values should be less than 2. All of the PAHs fulfilled these criteria (Table 3).

### Recovery

The accuracy of analytical method was evaluated with recovery studies. Five replicate samples of 2 g fluid yogurt with low (0.15%) and high fat (4.7%) were spiked with a known amount of PAHs (0.25 µg/kg). The average recoveries (Table 4) were calculated using the differences of the measurement results between spiked and unspiked samples. Results are reported as percentage recovery and standard deviation (SD). Recoveries of each PAHs were in the range of 92-106%, with SDs between 5 and 8% for low-fat yogurt (0.15%), and in the range of 80-88%, with SDs between 8 and 10% for high-fat yogurt (4.7%), respectively. Recoveries were greater than 80% for all PAHs analysed.

**Table 3. Relative standard deviation of repeatability ($RSD_r$), relative standard deviation of intermediate precision ($RSD_{IP}$), Horwitz ratio (HorRat) values for repeatability ($HorRat_r$) and reproducibility ($HorRat_R$), and relative expanded measurement uncertainty (U) for four polycyclic aromatic hydrocarbons (PAHs) in yogurt samples which were spiked with 1 µg/kg of each of the four analytes.**

| PAH | $RSD_r$ % | $RSD_{IP}$ % | $HorRat_r$ | $HorRat_R$ | U (k=2) % |
|---|---|---|---|---|---|
| Benz[a]anthracene | 6.1 | 9.0 | 0.4 | 0.4 | 11 |
| Chrysene | 10.5 | 14.6 | 0.7 | 0.7 | 18 |
| Benzo[b]fluoranthene | 5.9 | 16.8 | 0.4 | 0.8 | 20 |
| Benzo[a]pyrene | 7.5 | 7.5 | 0.5 | 0.3 | 9 |

**Table 4. Mean recoveries and standard deviations (SD %) of four polycyclic aromatic hydrocarbons (PAHs) in yogurts spiked with 0.25 µg/kg.**

| PAH | Low-fat yogurt (0.15%) | | High-fat yogurt (4.7%) | |
|---|---|---|---|---|
| | Recovery (%) | SD (%) | Recovery (%) | SD (%) |
| Benz[a]anthracene | 92 | 7 | 84 | 9 |
| Chrysene | 96 | 6 | 86 | 8 |
| Benzo[b]fluoranthene | 102 | 8 | 80 | 10 |
| Benzo[a]pyrene | 106 | 5 | 88 | 10 |

### Measurement uncertainty

The uncertainty of the analytical method was evaluated according to the Eurachem/Citac Guidelines (Citac and Eurachem, 2000). Measurement uncertainty was estimated based on the law of error propagation. These results were calculated as a combined uncertainty taking into account the following factors: the uncertainty of the preparation of PAH standard solutions for instrument calibration, uncertainty of spiking solutions, the uncertainty contribution arising from calibration curve, the uncertainty from the precision of the analyses and the uncertainty of bias (Citac and Eurachem, 2000; Kacmaz *et al.,* 2016). With a confidence level of 95%, the expanded uncertainty (U) was calculated by using combined uncertainty multiplying a coverage factor (k) of 2. The calculations of measurement data, content level of 0.1 µg/kg were used. As seen in Table 3, the highest expanded uncertainty was found 20%.

### PAHs content in retail yogurt samples

Liquid-liquid extraction, a pre-concentration and HPLC-fluorescence detection was applied in order to determine the concentration of the 4 EU marker PAHs in totally 17 commercials yogurts with low and high fat content, purchased from the Turkish market. PAH concentrations detected in all yogurt samples are shown in Table 5 and 6. The highest concentration for sum of the 4 PAHs in low and high-fat yogurt samples were found 0.59 and 0.95 µg/kg, respectively. The data indicated that CHR was the most widespread PAH in yogurt samples with the highest average concentration of 0.60±0.08 µg/kg. BaA concentration was in the range between 0.08 and 0.20 µg/kg whereas BaP varied from 0.06 to 0.30 µg/kg. BbF was not detected in any kind of analysed samples.

The results showed that PAHs occurs at rather low concentration levels in retail Turkish yogurts. All results were found to be lower than 1.00 µg/kg. However, it was appeared that PAH distributions and levels were in parallel with the studies of the literature (Aguinaga *et al.,* 2007; Battisti *et al.,* 2015) and it was also very similar to that reported in milk samples (Aguinaga *et al.,* 2008; Chung *et al.,* 2010; Girelli *et al.,* 2014; Naccari *et al.,* 2011).

According to the findings given in Table 5 and Table 6, PAH concentrations are affected by fat amount while PAH distribution did not change in high and low-fat yogurts. This could be attributed to more triglycerides amount resulting higher PAHs level in high fat yogurts. In addition, heat treatment applied in dairy processing might affect PAHs formation, as reported by Aguinaga *et al.* (2007), Girelli *et al.* (2014) and Battisti *et al.* (2015).

**Table 5. Mean polycyclic aromatic hydrocarbons concentrations (µg/kg ± SD) in retail low-fat yogurts (0.15-1.40%).**

| ID | Benz[a]anthracene | Chrysene | Benzo[b]fluoranthene | Benzo[a]pyrene | Total 4 EU PAHs |
|---|---|---|---|---|---|
| Y1 | 0.20±0.03 | 0.14±0.02 | nd[1] | 0.25±0.04 | 0.59 |
| Y2 | 0.16±0.02 | 0.08±0.01 | nd | nd | 0.24 |
| Y3 | 0.24±0.04 | 0.06±0.01 | nd | nd | 0.24 |
| mean | 0.20±0.04 | 0.11±0.04 | nd | 0.25 | 0.36±0.04 |
| min | 0.16 | 0.08 | nd | 0.25 | 0.24 |
| max | 0.24 | 0.14 | nd | 0.25 | 0.59 |

[1] nd = not detectable.

**Table 6. Mean polycyclic aromatic hydrocarbons concentrations (µg/kg ± SD) in retail high-fat yogurts (3.0-4.7%).**

| ID | Benz[a]anthracene | Chrysene | Benzo[b]fluoranthene | Benzo[a]pyrene | Total 4 EU PAHs |
|---|---|---|---|---|---|
| Y4 | 0.10 ± 0.02 | 0.10±0.03 | nd[1] | nd | 0.20 |
| Y5 | 0.13 ± 0.02 | 0.20±0.05 | nd | nd | 0.33 |
| Y6 | 0.12 ± 0.02 | nd | nd | nd | 0.12 |
| Y7 | 0.20 ± 0.03 | nd | nd | nd | 0.20 |
| Y8 | nd | nd | nd | nd | nd |
| Y9 | 0.20 ± 0.05 | nd | nd | nd | 0.20 |
| Y10 | 0.08 ± 0.01 | 0.60±0.08 | nd | 0.30±0.05 | 0.95 |
| Y11 | nd | nd | nd | nd | nd |
| Y12 | nd | 0.20±0.06 | nd | nd | 0.20 |
| Y13 | 0.10 ± 0.01 | nd | nd | 0.07±0.01 | 0.17 |
| Y14 | 0.08 ± 0.01 | 0.20±0.04 | nd | 0.10±0.02 | 0.36 |
| Y15 | 0.08 ± 0.01 | nd | nd | nd | 0.08 |
| Y16 | nd | 0.40±0.11 | nd | 0.06±0.01 | 0.43 |
| Y17 | nd | 0.50±0.09 | nd | 0.30±0.05 | 0.80 |
| mean | 0.09 ± 0.04 | 0.31±0.18 | nd | 0.16±0.18 | 0.33±0.18 |
| min | 0.08 | 0.10 | nd | 0.06 | 0.08 |
| max | 0.20 | 0.60 | nd | 0.30 | 0.95 |

[1] nd = not detectable.

## 4. Conclusions

This study focused to determine the concentration of 4 EU marker PAHs in totally 17 retail yogurt samples consumed in Turkey. A sensitive HPLC method with fluorescence detection was applied. The method was validated for yogurt matrices according to single laboratory validation guideline. The validation parameters such as accuracy, linearity, precision, LOD and LOQ were found within acceptable range.

Although the CHR were the most prevalent PAHs in these kinds of samples, all obtained data showed that PAHs occurs rather low levels (lower than the 1.00 µg/kg) in yogurts consumed in Turkey.

The present study was the first attempt concerning the levels of PAHs in yogurts from Turkey. However, this survey might be useful for health management of the consumers in Turkey, because it provides baseline information about potential health risk of PAHs-containing yogurts.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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