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*Original paper*

# Research on The Detection of Polycyclic Aromatic Hydrocarbons (PAHs) from Fish and Smoked Fish Samples, The Values Obtained and The Significance of Their Presence on Human Health

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**Abstract**

A total of 42 samples of fish and smoked fish products from 22 assortments were analyzed to detect Polycyclic Aromatic Hydrocarbons (PAHs). The sample collection was performed in accordance with the provisions of EC Regulation 333/2007 with its subsequent modifications and additions, and the used detection method was the HPLC (High Performance Liquid Chromatography), validated and accredited by the National Accreditation Body. The obtained values were between undetectable/non-quantifiable and 3.57 ± 7.67 μg/kg for benzo[a]pyrene and between undetectable/non-quantifiable and 25.65 ± 10.660.31 μg/kg for the PAH4. The values are below the maximum permitted levels of Regulation (EC) 1881/2006 with its subsequent amendments and additions.Even if the obtained values are within the tolerance limits, fish and smoked fish products must be monitored and examined by laboratory examinations, and the establishment of maximum levels for benzo[a]pyrene and PAH4 are extremely important for protecting the public health. Until now, no data have been submitted in Romania referring to the expertise of fish and smoked fish products for the detection of PAHs.

**Keywords** Polycyclic Aromatic Hydrocarbons (PAHs), benzo[a]pyrene, PAH4, expertise.

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# Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are a class of organic compounds containing two or more combined (condensed) aromatic rings. (I.A. DOGARU [1]). They include a number of carcinogenic and genotoxic substances, of which the most important are benzo[a]pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, Benzo[*ghi*]perylene, Chrysene, Cyclopenta[*cd*]pyrene, dibenzo[ah]anthracene, dibenzo[ae]pyrene, dibenzo[ai]pyrene, dibenzo[al]pyrene, indeno[1,2,3-cd]pyrene and 5-methylcrisene (G.K. ONTIZAAN & al, [5], FOOD STANDARDS AGENCY [14] [15]).

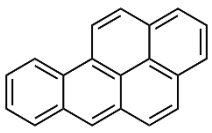
PAHs have a pronounced carcinogenic potential, which has been described since 200 years ago. In 1775, for the first time, English physicist Percival Pott described a correlation between the incidence of scrotum cancer at chimney sweepers and continuous contact with PAH-rich ash (G. PUCHIANU & al, [8]).

The formation of PAH in food depends on the thermal processing methods, correlated with the chemical composition of the product, the way it is packaged, plus the structure of the coating. In this regard, it should be noted that most food preparation processes are performed at temperatures between 370-390°C, but also at temperatures higher than 400-600°C (eg in the case of fat frying). At temperatures between 400 and 1000°C, the PAHs content increases linearly, and the phenols and fatty acids reach the maximum amount at 600°C. The smoking temperature between 20 and 55°C does not significantly affect the production of PAHs, and 40°C temperature allows for superior flavors than the 20°C or 55°C.

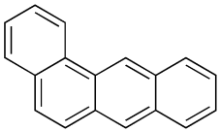
In roasted meat (roasting with charcoal grill, frying) PAHs are formed during the incomplete combustion process or by the pyrolysis of the fat, when oxygen and carbon are released, which lead to the formation of polycyclic compounds. These compounds are transported in the resulting smoke and then deposited on meat (A. FARHADIAN & al, [2]). PAHs concentration in meat roasted at a higher distance from coal is lower than in meat roasted closer to coal (M.D. GUILLEN & al, [4]). In order to avoid the formation of large amounts of PAHs, grilled meat should not be in contact with the flame, the process should last longer and the fat should be kept as low as possible (W. STALL & al, [9], S. TAO & al, [10].

Another PAHs-generating process is smoking, a type of food preservation, which has been correlated with the increased incidence of pharyngeal cancer, observed in Icelandic fishermen, large smoked fish consumers, even if PAHs were detected in low concentrations. Accumulation of PAHs in smoked food depends on how food was smoked, the type of the fuel, the temperature and duration of smoking. Wood quality plays an important role, so pyrolysis of hardwood generates poorer smoke in PAHs than soft woods that contains resins (G.. GRIMMER [3]),. PAHs are mainly formed from the carbohydrates found in food, at high temperatures and in the absence of oxygen.

As a contaminant, the marker is considered benzo(a)pyrene and the PAH4: benzo(a) pyrene (C20H12), benzo(a)anthracene (C18H12), benzo(b)fluoranthene (C20H12) and chrysene (C18H12) (http://ro.wikipedia.org/wiki/, Benzo [a] piren [22], http://ro.wikipedia.org/wiki/, Benz [a] antracen [23], http://ro.wikipedia.org/wiki/, Benzo [b] fluoranten [24], http://ro.wikipedia.org/wiki/, Crisen [25]).



$C_{20}H_{12}$



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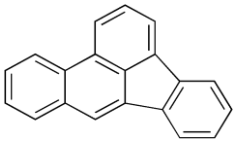

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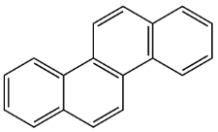

$C_{18}H_{12}$

Depending on the number of rings, hydrocarbons can be divided into:

– light PAHs (2-4 aromatic rings): chrysene; benz(a)anthracene;

– heavy PAHs (with 5 or more aromatic rings): benzo(a)pyrene; benzo(a)fluoranthene; dibenzo(a)anthracene.

Benzo[a]pyrene is a polycyclic aromatic hydrocarbon found in coal tar. Its metabolites are mutagenic and highly carcinogenic (classified as a Carcinogenic Group 1 by IARC), being a result of the transformation of starch, amino acids and fatty acids during incomplete combustion at temperatures between 300-600°C, following. (IARC [17]). The content is expressed in micrograms / kg (μg / Kg = ppb). The carcinogenic action of benzoprene can be obtained by direct contact (dermal), and especially by ingestion (A.R. NAGHIU & al, [7]).

# Materials and Methods

Within the Sanitary Veterinary and Food Safety Laboratory in Brasov, a number of 42 samples of fish and smoked fish products from 22 assortments were analyzed for the detection of Polycyclic Aromatic Hydrocarbons (SPECIFIC PROCEDURE [20]).

Sampling was carried out in accordance with the provisions of EC Regulation 333/2007 which determines the sampling methods and methods of analysis for the official control of the levels of Pb, Cd, Hg, 3-MCPD

and PAHs in food, with subsequent amendments and additions (EU REGULATION no. 333/2007 [13]).

Thus, sampling was carried out by trained persons, with due regard for measures designed to eliminate the risk of accidental contamination of the prelevated samples and to avoid any changes that could have affected contaminant levels, which would have had a negative effect on analytical determination or who would have made the evidences unrepresentative.

The overall sample was about 1 kg, unless this was not possible, for example, when the sample consisted of a package or a unit.

The minimum number of incremental samples taken from the lot or sublot was established as follows: (Table 1).

If the batch or sublot was made up of separate packages or units, the number of packages or units to be sampled to form a bulk was determined according to Table 2.

**Table 1.** The minimum number of incremental samples to be taken from the batch or sublot.

| Lot / sublot weight or volume (in kg or liter) | Minimum incremental number of samples to be sampled |
|---|---|
| < 50 | 3 |
| ≥ 50 şi ≤ 500 | 5 |
| > 500 | 10 |

**Table 2.** Number of packages or units (incremental samples) to be taken to constitute the bulk sample if the consignment or sublot was made up of separate packages or units.

| Number of packages or units in the lot/sublot | Number of packages or units to be taken |
|---|---|
| ≤ 25 | at least 1 pack or 1 unit |
| 26-100 | about 5%, at least 2 packages or units |
| > 100 | about 5%, up to 10 packages or units |

After sampling, each sample was placed in a clean and inert container (aluminum foil) which provides adequate protection against contamination or adsorption of substances on the inside of the container and damage during transport. The taken samples were sealed and identified, keeping records of each sampling.

The detection method used was high performance liquid chromatography (HPLC), a qualitative analysis method used in biochemistry and analytical chemistry for the separation, identification and quantification of compounds, validated and accredited by the National Accreditation Body (RENAR) (SR EN ISO 15753 / 2007 [21], A.D. NAGHIU & al, [6], M.A. QUILLIAM [11]).

The principle of the method is that PAHs are extracted in an acetonitrile/acetone mixture followed by two purifications on the C18 reverse phase cartridge and on the florisil cartridge. After separation, liquid chromatography is determined and fluorescence measured at different excitation and emission wavelengths, according to the standardized and optimized working method. In order to obtain repeatable results, the ambient temperature in the laboratory was stable (≤ 20°C), higher temperatures, increasing the solubility of short chain fatty acids. The detection program is presented in Table 3.

**Table 3.** Detection program

| Component | Duration min | Excitation wavelength nm | Emission wavelength nm |
|---|---|---|---|
| ***Benz[a]anthracene*** | 12.5 | 270 | 385 |
| ***Chrysene*** | 14.0 | | |
| ***Benzo[b]fluoranthene*** | 21.8 | 256 | 446 |
| ***Benzo[a]pyrene*** | 31,2 | 292 | 410 |
| ***Dibenz[a,h]anthracene*** | 44.1 | | |

After weighing exactly 2 gramms of the sample, 5 steps (0-4) were carried out for the analytical process, followed by the actual determination (optimization of the apparatus, calibration of the calibration curve, reading, calculation and expression of the results).

**Step 0**

The sample to be analyzed, chopped and well homogenized, was extracted with methanol and then with chloroform, in two steps. The last bottom layer was collected in the same vial and then evaporated to dryness (Figures 1 and 2).

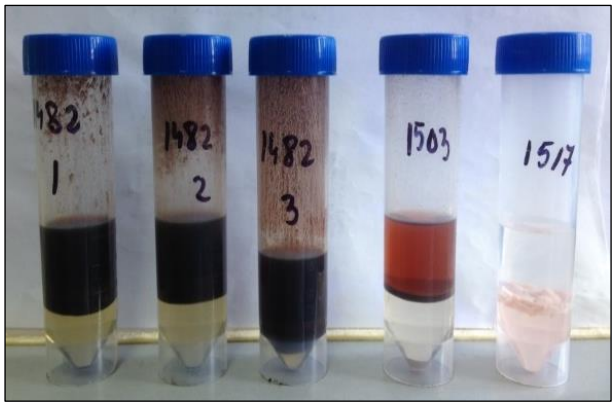


**Figure 1.** Extraction of solid samples.

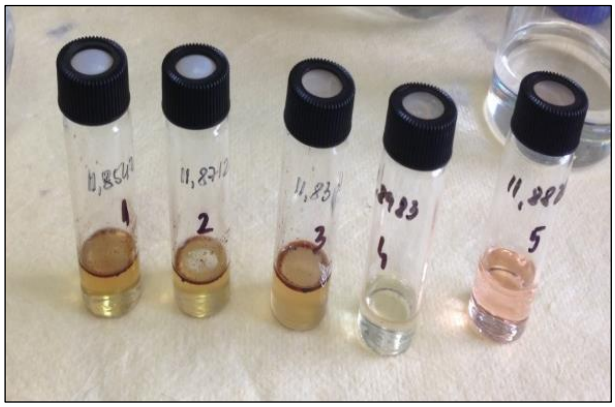

**Figure 2.** Collecting lowest layer.

Thus, the fat was obtained.

**Step 1**

The sample to be analyzed was subjected to a three-step extraction with 'Mixture1', the supernatant (3 x 4 ml) being collected in the same vial, then evaporated to dryness. The fatty residue was thus obtained (Figure 3).

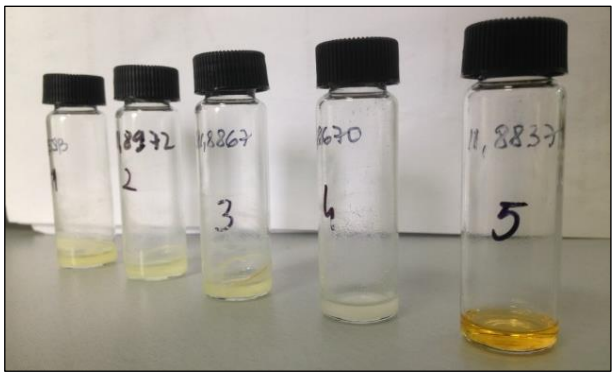


**Figure 3.** Samples after evaporation to dryness – fatty residue.

**Step 2**

The fat residue was purified by successive extractions with the same 'Mixture1' in three steps, the supernatant (3 x 2 ml) being collected in the same vial, and then purified on the C18 cartridge. Prior to passing through the cartridge, it was conditioned according to the manufacturer's operating instructions (Figure 4).

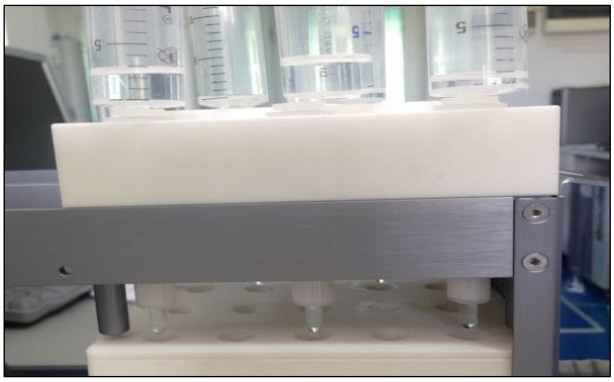

**Figure 4.** Conditioning the cartridge.

After passing the eluate, 'Mixture 1' was added to elute all the particles of interest. All the eluate thus obtained was evaporated to dryness. The resulting oily residue was treated with hexane and thus the hexane extract was obtained, which was kept for 24 hours at -18°C.

**Step 3**

The hexane extract was subjected to successive extractions with 'Mixture 3' in three steps, the supernatant (3 x 2 ml) was collected in the same vial and then purified on the florisil cartridge (Figure 5)

**Figure 5.** Purification of the supernatant through the fluorisyl cartridge.

Prior to passing through the florisil cartridge, it was conditioned according to the manufacturer's instructions. After passing the eluate, Mixture 3 was added to elute all the particles of interest.

**Step 4**

All the eluate thus obtained was concentrated to 1 ml. It was then treated with 0.5 ml of toluene and evaporated to 50 µl, internal standard was added and completed up to the volume of interest (250, 500 µl) with acetonitrile. Note that

all of the fat residues were weighed and the evaporation to dryness was done with nitrogen in a water bath at 350°C.

**Figure 6.** Varian LC MS

**Determination Steps:**

***Optimizing the device***

The machine was started before the determination and it started the optimization of the working parameters (Figure 6).

***Marking the standard curve***

PAHs were prepared at concentrations of 1, 2, 3, 4, 5 ppb, with which the calibration curve was drawn. The curve shall be linear and pass through the origin, and the correction coefficient shall be at least 0.995.

***The readout***

After adjusting the device, the identification data of the samples to be analyzed was entered into the program (Figure 7).

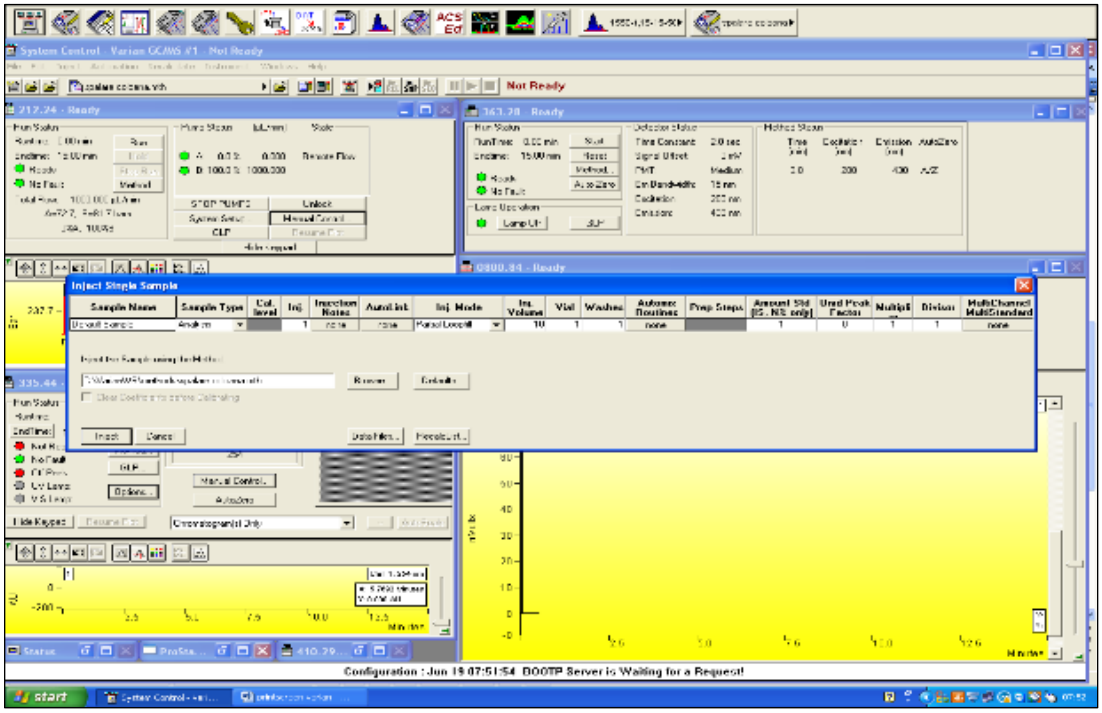

**Figure 7.** Inserting sample identification data in the software.

After the device was initialized, the samples were injected, following all the steps required by the machine's reading program. The reading was completed by the individual chromatogram for each sample and by an analysis report, which identifies the concentration of each substance at the retention times specific to the analyzed PAHs.

To the determination set it was also added a fortified sample at the maximum allowed according to the legislation in force (EC Regulation 1881/2006, with subsequent amendments and completions) (EU REGULATION no. 1881/2006 [12]).

**Calculation and results analysis**

Before the calculation, the following aspects were followed: the recovery percentage of the fortified sample to be in accordance with the regulations in force (EC Regulation 333/2007 with subsequent amendments and completions); retention times are appropriate for PAHs analyzed; the internal standard area is the appropriate one; in the case of dilutions, take account of the dilution factor; issuing the analysis report and processing the data by correcting the value with the recovery ratio for Benzo(a)pyrene and the PAH4 respectively.

## Results and Discussion

Following the laboratory examination of 42 samples of fish and smoked fish products, the following results were obtained: (Table 4).

**Table 4.** The type of analyzed samples, their number and the obtained results.

| Sample | Number of probes | Results | | |
|---|---|---|---|---|
| | | benzo[a]pyrene µg/kg | benzo[a]pyrene recuperation % | PAH4 µg/kg |
| Mackerel pastrami | 1 | Undetectable* | - | 3,24±1,35 |
| Smoked mackerel | 5 | Unquantifiable** | - | 2,83±1,18 |
| | | Unquantifiable** | - | 1,87±0,78 |
| | | Unquantifiable** | - | 1,99±0,83 |
| | | Undetectable | - | 3,92±1,53 |
| | | Undetectable | - | Unquantifiable** |
| Smoked mackerel fillet | 2 | 2,29±0,91 | 84,9 | 3,09±1,28 |
| | | 2,11±1,23 | 86,8 | 1,98±0,86 |
| Smoked Trout | 3 | Unquantifiable** | - | 2,99±1,24 |
| | | 0,75±0,30 | 88,3 | 7,61±3,16 |
| | | Unquantifiable** | - | 1,99±0,83 |
| Smoked Sprat | 3 | Unquantifiable** | - | 6,30 ±2,62 |
| | | 0,87±0,35 | 88,3 | 7,64±3,17 |
| | | 1,37±0,4306 | 86,8 | 11,7813 |
| Smoked Sprat in Sunflower Oil | 7 | Unquantifiable** | - | Undetectable* |
| | | Undetectable* | - | 2,27 ±0,94 |
| | | Unquantifiable** | - | 6,54±2,72 |
| | | Undetectable* | - | 0,25 ±0,10 |
| | | Unquantifiable** | - | 2,23±0,93 |
| | | Unquantifiable** | - | 2,11±0,96 |
| | | Undetectable* | - | 3,67 ±1,52 |
| Smoked Sprat in Rapeseed Oil | 1 | Undetectable* | - | 2,57 ±1,07 |
| Sprat smoked with beech and alder smoke | 2 | Undetectable* | - | 18,46 ±7,67 |
| | | Undetectable* | - | 4,96 ±2,06 |
| Frozen Smoked Sprat | 1 | 3,57 ±7,67 | - | 25,65 ±10,66 |
| Mussels in sunflower oil | 2 | 0,76±0,30 | 98,32 | 8,17±3,39 |
| | | 0,84±0,33 | 98,32 | 9,73±4,04 |
| Smoked Herring | 1 | Unquantifiable** | - | 9,88±4,10 |
| Sliced smoked salmon | 1 | Unquantifiable** | - | 2,32 ±0,96 |
| Smoked salmon filet | 2 | Undetectable* | - | 1,39±0,58 |
| | | Undetectable* | - | 1,39±0,58 |
| Smoked Shells in oil | 2 | Unquantifiable** | 98,32 | 7,84 ±3,26 |
| Smoked Bighead Filet | 1 | Undetectable* | - | 0,80±0,33 |
| Vaccumed Smoked Tuna Filet | 1 | Undetectable* | - | 0,69±0,29 |
| Smoked Pontic Shad | 2 | Unquantifiable** | - | 2,3128 ±1,3749 |
| | | Unquantifiable** | - | 2,32 ±0,96 |
| Smoked Herring Filet | 1 | Undetectable* | - | 1,72±0,71 |
| Doripesco Fish Rod | 1 | Unquantifiable** | - | 1,92±0,61 |
| Frozen mussels pasteurized in vacuum | 1 | Undetectable* | 98,32 | 2,89 ±1,20 |
| Mussels in smoke flavored oil | 1 | 1,38±0,55 | 69,4 | 6,94±2,88 |
| Smoked Mussels in Sunflower Oil 'Franz' | 1 | 0,79±0,31 | - | 5,54±2,30 |

* Undetectable < LOD (Limit of detection, smallest measured content, from which it is possible to deduce the presence of the analyte with reasonable statistical certainty.)

** unquantifiable < LOQ (Limit of quantification, lowest content of the analyte which can be measured with reasonable statistical certainty.)

**Table 5.** Maximum allowable limits for PAHs in fishmeal and smoked fish products (EU REGULATION no. 1881/2006 [12]).

| **Maximum limits *(µg/kg)*** | |
|---|---|
| **Benzo[*a*]pyrene** | **Sum of Benzo[*a*]pyrene, Benz[*a*]anthracene, Benzo[*b*]fluoranthene and Chrysene** |
| 5.0 µg/kg before 1.09.2014<br>2,0 µg/kg after 1.09. 2014 | 30.0 µg/kg before 1.09.2014<br>12,0 µg/kg after 1.09.2014 |

The obtained values were between undetectable/non-quantifiable and 3.57 ± 7.67 µg / kg for Benzo(a)pyrene and between undetectable/non-quantifiable and 25.65 ± 10,660.31 µg / kg for PAH4 (corrected with recovery). All these values are below the maximum permitted levels specified in Regulation (EC) 1881/2006 with subsequent amendments and additions (Table 5).

There is an exception for smoked sprats and canned smoked sprats *(sprattus sprattus)*; bivalve molluscs (fresh, chilled or frozen); heat treated meat and heat treated meat products sold to the final consumer, where the admissibility limit for Benzo[*a*]pyrene is 5.0 µg/kg, and for PAH5 is 35.0 µg/kg. Also for bivalve mollusks, the admissibility limit is 6.0 µg/kg, and for PAH4 is 35.0 µg/kg. (FOOUD SAFETY AUTORITY OF IRELAND [16]).

The highest value for Benzo[*a*]pyrene was detected in the Frozen Smoked Sprat samples, 3,57 ±7,67 µg/kg. It is higher than the maximum admissible value (5,0 µg/kg) with 1,43 µg/kg. Also high values were detected in the Smoked mackerel samples (2,29±0,91 µg/kg), in the Mussels in smoke flavored oil samples (1,38±0,55 µg/kg) and in the Smoked Sprat samples (1,37±0,4306 µg/kg).

As for PAH4, the highest value was detected in the Frozen Smoked Sprat samples (25,65 ±10,6667 µg/kg), higher than the maximum admissible value of 35,0 µg/kg, resulting a difference of 9,35 µg/kg. High values were also detected in the Sprat smoked with beech and alder smoke samples (18,46 ±7,67 µg/kg), in the smoked herring samples (9,88±4,10 µg/kg), in the Mussels in sunflower oil samples (9,73±4,04 µg/kg, respectively 8,17±3,39 µg/kg) and in the Smoked Shells in oil samples (7,84 ±3,26 µg/kg).

The obtained values from the other food products were lower for both parameters, the lowest values being recorded for benzo(a)pyrene in the smoked mackerel samples, smoked trout, Smoked Sprat in Sunflower Oil, and in the herring, salmon, tuna, shells specialities, and, in case of PAH4, in the smoked mackerel and Smoked Sprat in Sunflower Oil samples.

Even if the obtained values are within the admissible limits, fish and smoked fish products must be monitored and tested by laboratory examinations, and setting the maximum levels for benzo(a)pyrene and for PAH4 are very important since they are meant to protect the public health.

This is why, annually, the National Sanitary Veterinary and Food Safety Authority establishes a sampling plan for both animal and non-animal food samples in order to monitor and detect PAHs, for the purpose of preventing possible risks as follows: (Table 6).

**Table 6.** Control of contaminants in food of animal origin (ORDER OF THE N.S.V.F.S.A. 35/2016 [19], and 51/2005 [18]).

| **Substance** | **Matrix** | **Prelevation source** | **Analysis method** | |
|---|---|---|---|---|
| Benzopyrene | Smoked meat and smoked meat products | Processing units, marketing units | Screening | Confirmation |

In case of smoked fish and fish products, maintaining levels of PAHs within acceptability limits can reasonably be achieved by observing good manufacturing practices and by applying prevention measures against food contamination.

In order to ensure effective public health protection, fish and smoked fish products containing excess PAHs must not be placed on the market as such or mixed with other foods nor used as ingredients.

# Conclusion

The maximum values of PAHs were obtained from the examination of frozen smoked sprat. Thus in the case of benzo(a)pyrene the value was 3.57±7.67 µg/kg, and in the case of PAH4 the value was 25.65±10.6667 µg/kg. The values are within the admissibility limits, but they are close to the maximum admissible limits. Higher values, but within tolerance limits, were obtained for smoked mackerel for benzo(a)pyrene and for sprat smoked in beech and alder smoke in the case of PAH4.

The lowest values were recorded for benzo(a)pyrene, in smoked mackerel, smoked trout, smoked sprat in oil and herring, mackerel, salmon, tuna, novak, shells samples., and in the case of PAH4, for smoked mackerel and smoked sprat in oil samples.

The obtained results show that fish and smoked fish products have not been a hazard to the human consumer, but values close to the maximum allowable values are an

argument for the requirement of smoked food to be tested for PAHs in order to prevent the illness of consumers.

Until now, no data has been presented in Romania referring to the evaluation of fish and smoked fish products in regard to PAHs values.

## Competing interest

The authors declare no conflict of interest.

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