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# Polycyclic aromatic hydrocarbons (PAHs) in smoked fish from three smoke-houses in Braşov county

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***Abstract***

Fifteen samples of smoked fish were analysed for BaP and other polycyclic aromatic hydrocarbons (PAHs). The high resolution gas chromatography–mass spectrometry (HRGC–MS) method employed was elaborated and validated for the control programme. The method complies with the criteria for official control according to Commission Regulation (EC) No 333/2007. Six samples of smoked fish had BaP levels exceeding 5.0 µg/kg, the concentrations ranging from 0,6 to 8.4 µg/kg. These samples were produced by traditional smoking, where the food is directly exposed to hot smoke from a burning log fire. Samples of fish smoked by indirect technique, using smoke from an external smoke generator, all had BaP levels below the limit of quantification, i.e., 0.3 µg/kg.

***Keywords***: Polycyclic aromatic hydrocarbons, Benzo[a]pyrene, Smoking process, Fish, Food safety

## 1. Introduction

From Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds consisting of three or more condensed aromatic rings. PAHs are formed during incomplete combustion processes, which occur in varying degree whenever wood, coal or oil is burnt. They can therefore be found in complex mixtures throughout the environment, including also a variety of foodstuffs. Food can be contaminated from environmental sources, industrial food processing and during home food preparation. As PAHs represent an important class of carcinogens, their presence in food has been intensively studied. Particular attention has been paid to the highly carcinogenic benzo [a]-pyrene. The EU Scientific Committee on Food (SCF) has identified 15 PAHs as genotoxic carcinogens, namely benz [a] anthracene, benzo [b ]fluoranthene, benzo [j ]fluoranthene, benzo [k]-fluoranthene, benzo [a] pyrene, benzo[g,h,i]perylene, chrysene, cyclopenta [c,d] pyrene, dibenz [a,h] anthracene, dibenzo [a,e] pyrene, dibenzo [a,h] pyrene, dibenzo [a,i] pyrene, dibenzo [a,l] pyrene, indeno [1,2,3-cd] pyrene and 5-methylchrysene. For benzo[g,h,i]perylene, however, clear evidence was found for genotoxicity but not for carcinogenic effects [3]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has nominated a 16th compound, benzo[c]fluorene, for further observation in food [7]. According to SCF, benzo[a]pyrene can be used as a marker for the occurrence and effect of carcinogenic PAHs in food. Maximum levels of benzo[a]pyrene in a range of foodstuffs are now specified in a Commission Regulation (Regulation (EC) No 1881/2006). In 2008, however, a new scientific opinion adopted by the European Food Safety Authority [2] concluded that BaP alone is not a suitable indicator for the occurrence and toxicity of PAHs in food and that eight specified PAHs (PAH8), for which oral carcinogenicity data are available, and/or a subgroup of these, PAH4, are more suitable markers.

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It is well known that PAHs occur in curing smoke and that they can deposit on the surface of, and migrate into, the food item being smoked. A number of factors in the smoking process influence the composition of the curing smoke and the uptake of PAHs in the food being smoked [9,10]. The combustion temperature during the generation of smoke seems particularly critical. According to [11], the formation of PAHs in the smoke increases linearly with increasing combustion temperature in the temperature range 400-1000$^0$C.

Since April 2005 the EU maximum level for benzo[a]pyrene (BaP) in smoked meat and meat products and muscle meat of smoked fish and smoked fishery products is 5.0 µg/kg [4,5]. Where methods in use may cause high levels of PAH contamination, alternative or optimized methods should be investigated with the producers.

## 2. Materials and methods

*Sampling.*The sampling method used complies with the Commission Directive [5].The sampling included retail samples from shops as well as samples from smoke-houses. Normally three incremental samples of at least 100 g were taken to obtain an aggregate sample of at least 300 g. The samples were packed in aluminium foil and then placed in polyethylene bags. The samples were transported in cold-boxes to Haki laboratories.

*Sample preparation.* Edible parts of smoked fish (head, bones and removable skin were removed) were homogenized in a food mixer. Incremental samples were mixed and homogenized together to obtain an aggregate sample. Each prepared aggregate sample was divided into three equal portions for enforcement, trade and reference purposes and stored in a freezer.

*Reagents.* All reagents were of analytical grade and the water of Millipore-Q quality. Methanol, cyclohexane, n-hexane, tert-butyl-methylether and potassium hydroxide pellets were from Merck (Darmstadt, Germany). Other solvents, n-pentane and 2-propanol were from Labscan (Ireland) and Riedel-de-Haen (Seelze, Germany), respectively. PAH reference standards PAH-Mix 15 (SL-30015) and PAHs in toluene (NIST-2260A) were obtained from LGC Promochem (Boras, Sweden). Surrogate perdeuterated compounds, perdeuterated PAH II (NIST-2270) and dibenzo[ai]pyrene-D14 (CIL-DLM-3740-1.2), were purchased from LGC Promochem (Boras, Sweden) and chrysene-D12 (20670100) from Dr Ehrenstorfer-Schäfers (Augsburg, Germany), respectively, to be used as internal standards. Strata SPE silica 5 g columns, reservoir 20 mL, were obtained from Phenomenex (Allerod, Denmark). Isolute SPE PAH HC 1 g columns, reservoir 6 mL, were obtained from Sorbent AB (Sollentuna, Sweden).

*Extraction and sample clean-up.* All samples were analyzed in duplicate. Aliquots of 10 g homogenised smoked fish were weighed into a 250 mL Erlenmeyer flask and spiked with 1.00 mL of a perdeuterated PAH internal standard mixture with individual d-compounds at concentrations in the range of 0.02-0.05 µg/mL. Saponification was achieved by adding 60 mL of 3.5 M methanolic KOH solution (methanol/water 9 + 1), thoroughly sealing the flask and keeping it at 70$^0$C in a drying cabinet for 2 h (flask was shaken after 1 h). Then 50 mL of cyclohexane was added to the flask, which was shaken and then cooled to ambient temperature. The contents were transferred to a 250 mL separatory funnel, the flask was rinsed with 30 mL methanol/water (4 : 1) and the rinsings added to the separatory funnel. This was shaken vigorously. After separation, the aqueous layer was transferred to a second 250 mL separatory funnel and washed with 30 mL cyclohexane.

The cyclohexane phases were combined and washed with 30 mL MeOH/$H_2O$ (4 :1), then with 30 mL MeOH/$H_2O$ (1 : 1) and finally with 30 mL water. The cleaned cyclohexane phase was decanted into a 100 mL round-bottomed flask, while carefully avoiding transferring any aqueous droplets, and concentrated to 1 mL in a rotary evaporator. This solution was transferred to a silica SPE column, which had been pre-conditioned with 15 mL of cyclohexane. The round-bottomed flask was rinsed with 1 mL cyclohexane and the rinsing was added to the column. The column was washed with 10 mL of cyclohexane. The column was eluted with 5% tert-butyl-methyl-ether in cyclohexane while applying vacuum to give an elution rate of 5 mL/min. The first 10 mL were discarded. The fraction 10-25 mL, containing the PAHs, was collected and concentrated to 0.5 mL at 40$^0$C in a rotary evaporator. The sample solution was further purified on a PAH HC SPE column that had been pre-conditioned with 6 mL n-hexane. After adding the sample solution, the column was washed with 3 mL n-pentane. The PAHs were eluted with 6 mL n-hexane containing 3.4% 2-propanol and collected in a 10 mL sample tube.

The volume was further concentrated under a nitrogen flow to 100-200 µL and transferred to a GC sample vial with a conical glass insert.

*HRGC–MS analysis.*The Agilent 6890 gas chromatograph equipped with an autosampler 7683 was connected to an Agilent 5975 mass selective detector. 1 mL of sample solution was injected in the pulsed splitless mode onto a 30m x 0.25mm i.d. DB-17 ms coated fused silica column with a film-thickness of 0.15 µm. Other operating conditions were: pulse pressure 45 psi, pulse time 0.90 min, purge flow 50mL, purge time 1.00 min; helium carrier gas constant flow 1.1 mL/min; temperatures: injector 300 $^0$C, oven programme 70$^0$C for 1min, rate 20$^0$C/min-160$^0$C, rate 3$^0$C/min-210$^0$C, rate 5$^0$C/min-final temperature 320$^0$C, hold for 5min, transfer line 310$^0$C. MS conditions: electron impact positive ion mode, detection SIM.MS Source 260$^0$C and MS Quad 170$^0$C. The GC-retention times and MS quantifying ions of PAHs and internal standards are listed in Table 1.A calibration curve for each PAH was obtained by running standards 5, 10, 20, 50, 100 and 200 ng of each PAH and 200 ng of the appropriate internal standard per mL cyclohexane.

The area ratios PAH compound/internal standard were plotted against mass ratios PAH compound/internal standard to obtain a linear graph

$Y = mx + b.$

The concentration of each PAH in the sample was calculated using the following equation: PAH (µg/kg) = (response PAH/response I:S: x b) x mass of added I:S: (µg/kg) / sample weight (g) x 1000

## 3. Results

The levels of BaP in 15 samples of smoked fish, are given in Table 2. The table also shows the smoking process and fuel used for each sample where such information has been available. Six out of 15 smoked fish samples showed BaP levels exceeding 5.0 µg/kg.

Of the six samples of smoked fish with BaP levels exceeding 5.0 µg/kg, five were produced with direct technique. However, in Table 2 it can be seen that there are some of fish samples produced by direct smoking that still have low or moderate levels. Eight samples processed by true indirect smoking showed levels below 0.3 µg BaP/kg.

***Table 1.*** GC-retention times and quantifying ions of PAHs and internal standards.

| PAH compound or internal standard | Abbreviation | GC-retention time (min) | Ouantifying ion (m/z) | Internal standard used |
|---|---|---|---|---|
| *Pyrene D10* | *PyrD10* | *22.83* | *212* | |
| Phenanthrene | Phe | 13.76 | 178 | PyrD10 |
| Anthracene | Ant | 13.91 | 178 | PyrD10 |
| Fluoranthene | Flu | 21.11 | 202 | PyrD10 |
| Pyrene | Pyr | 22.96 | 202 | PyrD10 |
| Benzo[c]fluorene | BcL | 25.50 | 216 | PyrD10 |
| *Chrysene D12* | *CHRD12* | *30.53* | *240* | |
| Benz[a]anthracene | BaA | 30.26 | 228 | CHRD12 |
| Cyclopenta[c,d]pyrene | CPP | 30.51 | 226 | CHRD12 |
| Chrysene | CHR | 30.68 | 228 | CHRD12 |
| 5-Methylchrysene | 5MC | 32.97 | 242 | CHRD12 |
| *Benzo[a]pyrene D12* | *BaPD12* | *37.43* | *264* | |
| Benzo[b]fluoranthene | BbF | 35.74 | 252 | BaPD12 |
| Benzo[k]fluoranthene | BkF | 35.86 | 252 | BaPD12 |
| Benzo[j]fluoranthene | BjF | 36.00 | 252 | BaPD12 |
| Benzo[e]pyrene | BeP | 37.33 | 252 | BaPD12 |
| Benzo[a]pyrene | BaP | 37.54 | 252 | BaPD12 |
| Perylene | Per | 38.09 | 252 | BaPD12 |
| *Benzo[ghi]perylene D12* | *BgPD12* | *42.97* | *288* | |
| Indeno[123-cd]pyrene | IcP | 41.88 | 276 | BgPD12 |
| Dibenzo[ah]anthracene | DhA | 41.98 | 278 | BgPD12 |
| Benzo[ghi]perylene | BgP | 43.07 | 276 | BgPD12 |
| Anthantrene | ATR | 43.58 | 276 | BgPD12 |
| *Dibenzo[ai]pyrene D14* | *DiPD14* | *49.67* | *316* | |
| Dibenzo[al]pyrene | DlP | 47.43 | 302 | BaPD12 |
| Dibenzo[ae]pyrene | DeP | 48.94 | 302 | BgPD12 |
| Coronene | Cor | 49.13 | 300 | DiPD14 |
| Dibenzo[ai]pyrene | DiP | 49.90 | 302 | DiPD14 |
| Dibenzo[ah]pyrene | DhP | 50.45 | 302 | DiPD14 |

The italics are used in order to distinguish the internal standards used from the PAH compounds.

***Table 2.*** Levels of benzo[a]pyrene in samples of smoked fish

| Sample no. | Smoked product | Smoke-house/ producer no | Smoking process | Fuel | BaP (µg/kg) |
|---|---|---|---|---|---|
| 1 | Rainbow trout | 1 | Direct | Sallow logs | 8.4 |
| 2 | Rainbow trout | 1 | Direct | Alder logs | 1.4 |
| 3 | Rainbow trout | 2 | Indirect | Alder chips | n.d.[a] |
| 4 | Brook trout | 2 | Indirect | Alder chips | n.d. |
| 5 | Rainbow trout fillets | 2 | Indirect | Alder chips | n.d. |
| 6 | Brook trout fillets | 2 | Indirect | Alder chips | n.d. |
| 7 | Rainbow trout | 3 | Indirect | Alder chips | <0.3[b] |
| 8 | Rainbow trout | 2 | Indirect | Beech chips | n.d. |
| 9 | Rainbow trout | 1 | Direct | Beech logs | 1.0 |
| 10 | Brook trout | 1 | Direct | Beech chips | 0.8 |
| 11 | Rainbow trout fillets | 1 | Direct | Alder logs | 0.9 |
| 12 | Rainbow trout | 3 | Indirect | Alder logs | 0.6 |
| 13 | Carp | 3 | Indirect | Alder chips | <0.3 |
| 14 | Silver carp | 3 | Indirect | Alder chips | <0.3 |
| 15 | Brook trout | 3 | Indirect | Beech chips | 0.4 |

[a] n.d. = not detected, below limit of detection (0.1mg/kg)
[b] <0.3 = not quantified, below limit of quantification (0.3mg/kg).

***Table 3.*** Levels of all 15 carcinogenic PAHs (µg/kg) found in samples of smoked fish on benzo[a]pyrene. Description of samples, see Table 2.

| Sample No | BaA | CPP | CHR | 5MC + TP | BbF | BkF | BjF | BaP | IcP | DhA | BgP | DlP | DeP | DiP | DhP | ∑15PAHs | ∑15PAHs/ BaP | PAH4 | ∑15PAHs/ PAH4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 12.9 | 19.0 | 12.0 | n.d. | 5.5 | 4.2 | 4.2 | 8.4 | 5.4 | 0.8 | 4.2 | n.d. | 0.7 | <0.5 | <0.5 | 77.3 | 9.2 | 38.8 | 2.0 |
| 2 | 5.0 | 6.0 | 4.1 | n.d. | 1.1 | 0.6 | 0.8 | 1.4 | 0.6 | <0.3 | 0.5 | n.d. | n.d. | n.d. | n.d. | 20.1 | 14.4 | 11.6 | 1.7 |
| 3 | <0.3 | n.d. | <0.3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | - | - | - | - |
| 4 | n.d. | n.d. | n.d. | n.d. | <0.3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | - | - | - | - |
| 5 | <0.3 | <0.3 | <0.3 | n.d | n.d. | n.d | n.d. | n.d. | n.d | n.d. | <0.3 | n.d. | n.d | n.d. | n.d | - | - | - | - |
| 6 | <0.3 | n.d. | <0.3 | n.d. | <0.3 | n.d | n.d. | n.d. | n.d | n.d. | <0.3 | n.d. | n.d | n.d. | n.d | - | - | - | - |
| 7 | 0.4 | 0.5 | 0.5 | n.d. | <0.3 | n.d. | <0.3 | <0.3 | n.d. | n.d. | <0.3 | n.d. | n.d. | n.d. | n.d. | 1.4 | - | - | - |
| 8 | <0.3 | n.d. | <0.3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | - | - | - | - |
| 9 | 3.0 | 3.9 | 2.8 | n.d. | 0.8 | 0.4 | 0.6 | 1.0 | 0.4 | <0.3 | 0.4 | n.d. | n.d. | n.d. | n.d. | 13.3 | 13.3 | 7.6 | 1.8 |
| 10 | 2.5 | 3.2 | 2.3 | n.d. | 0.7 | 0.3 | 0.4 | 0.8 | 0.3 | <0.3 | 0.3 | n.d. | n.d. | n.d. | n.d. | 10.8 | 13.5 | 6.3 | 1.7 |
| 11 | 1.3 | 2.6 | 1.1 | n.d. | 0.6 | 0.3 | 0.5 | 0.9 | 0.5 | <0.3 | 0.4 | n.d. | n.d. | n.d. | n.d. | 8.2 | 9.1 | 3.9 | 2.1 |
| 12 | 1.1 | 1.5 | 1.1 | n.d. | 0.4 | <0.3 | 0.3 | 0.6 | 0.3 | <0.3 | 0.3 | n.d. | n.d. | n.d. | n.d. | 5.6 | 9.3 | 3.2 | 1.8 |
| 13 | <0.3 | <0.3 | <0.3 | n.d. | <0.3 | n.d. | n.d. | <0.3 | n.d | n.d. | n.d. | n.d. | n.d | n.d. | n.d | - | - | - | - |
| 14 | 0.6 | <0.3 | 0.5 | n.d. | <0.3 | <0.3[a] | - | <0.3 | <0.3 | n.d | n.d. | n.d | n.d. | n.d | n.d | 1.1 | - | - | - |
| 15 | 1.7 | 0.8 | 1.7 | n.d. | 0.5 | 0.5[a] | - | 0.4 | <0.3 | n.d. | <0.3 | n.d. | n.d. | n.d. | n.d. | 5.6 | 14.0 | 4.3 | 1.3 |

CHR+ TP = the sum of chrysene and triphenylene.
n.d. = not detected, below limit of detection (0.2µg/kg for DlP, DeP, DiP and DhP; 0.1µg/kg for the other carcinogenic PAHs).
[a] Value given for BkF includes BjF (sum of BkF and BjF). Sample run on a GC column, DB-5, which does not separate BkF and BjF.

In Table 3 the levels of all 15 carcinogenic PAHs found in the smoked samples are given together with ∑15 PAHs, ∑15 PAHs/BaP, PAH4 and ∑15 PAHs/PAH4. In the calculation of ∑15 PAHs only PAH levels >LOQ were included. PAH4 (BaP + CHR + BaA + BbF) was calculated for samples where all four compounds reach levels >LOQ.

The sum of 15 PAHs ranged from not detected to 77.3 µg/kg. The ratios of ∑15 PAHs/BaP were calculated to test the conclusion by SCF [3] that BaP alone can be used as an indicator for the total levels of carcinogenic PAHs. The ratio of ∑15 PAHs/BaP among 15 samples of smoked fish with BaP levels >LOQ ranged from 9.1 to 14.4, with an average of 11.8. PAH4, as suggested by EFSA (2008) as a more suitable marker for the occurrence and toxicity of PAH in foods, varied from not detected to 38.8 µg/kg [2]. The ratio of ∑15 PAHs/PAH4 among 15 samples of smoked fish varied between 1.3 and 2.1, with an average of 1.77.

## 4. Discussion

Compared to the HRGC–FID method used in the study of smoked fish, several improvements/ simplifications have been elaborated and introduced into the new HRGC–MS method used in the control on PAHs in smoked fish reported here. Such improvements are:

- The saponification step is carried out in closed flasks in a drying cabinet instead of using reflux boiling.
- The manual solvent extraction procedure has been simplified, in that the DMF cleaning step has been omitted. Thus less solvent and time are spent.
- The silica column clean-up is carried out using commercially available pre-packed SPE columns instead of manually prepared columns.
- An additional clean-up on a commercially available pre-packed SPE PAH HC column facilitates the removal of any remaining interferences of aliphatic nature and thus further improves the purity of the sample solution.
- The use of the more polar DB-17 GC column, instead of the SE-54, improves the separation of the three benzofluoranthenes.
- The use of perdeuterated internal standards in connection with GC–MS and selective ion monitoring (SIM) improves quantitation.

Some separation problems between certain isomers remain (i.e., chrysene/triphenylene) and a few more difficult separations and quantifications have been introduced in the analysis of some of the ”new” target PAHs on the combined SCF/JECFA list (e.g. benzofluorenes and dibenzopyrenes). The GC–MS method used here shows good separation for most peaks of interest and the validation data indicate good accuracy for BaP and for the majority of PAHs. The method uses no expensive extraction equipment, it uses no chlorinated solvents and it uses a relatively simple ”low-cost” GC–MS.

The levels of BaP found in smoked fish are more scattered and, for samples subjected to direct smoking methods, often considerably higher than those reported in the Danish study on smoked fish and meat products [1] and in the German study on smoked meat products [8]. One sample of black-smoked belly pork ham in the latter study though, had a BaP content of 18 µg/kg, a level comparable to those high BaP levels found in traditional smoked fish reported in this study.

However, as can be seen from the data in Table 2, all traditional smoked samples had high BaP and PAH levels (5 out of 5 samples exceeded the EU maximum level of 5.0 µg/kg for BaP).

It was demonstrated that a burning log fire may produce large amounts of PAH and, when used as the source of heat in the grilling of food, very high levels of PAH and BaP could be found in the grilled product. When the grilling was carried out over the embers, i.e., when flames no longer emerged from the fire, the level of contamination was largely reduced. It seems probable that the use of glowing embers, instead of burning logs, as the source of heat and smoke, could reduce the level of PAH contamination also in traditional smoking. With reference to the Commission Recommendation (Recommendation (2005/108/EC)) regarding investigations of alternative and optimized production methods, where methods in use may cause high levels of PAH contamination, the following steps to improve traditional sauna smoking may be considered:

- As hot smoke is the only heat source, light the wood fire first and let the smoke heat up the empty smoking chamber. When the target chamber temperature is reached and the fire has burnt down to glowing embers, ventilate the chamber quickly to get rid of some smoke, and then place the food to be smoked into the smoking chamber. Maintain the fire/glow by adding small pieces of wood. Adjust the air damper so that the combustion proceeds steadily and slowly.
- Alternatively: as above, but add moistened wood chips or sawdust on top of the glowing wood pieces when the actual smoking of food starts. The moist lowers the combustion temperature and less PAHs are produced.
- Install a supplementary heat source (e.g. an electric heater), if this is needed to maintain the desired chamber temperature.

The calculated sums of 15 PAHs given in Table 3 are somewhat underestimated as all samples have a few or several PAHs at levels <LOQ, which are not included in the sum.

The ratio of ∑15 PAHs/PAH4 was less stable (RSD 28%) than ∑15 PAHs/BaP (RSD 20%) in the 15 samples of smoked fish. It should be pointed out, however, that the values reported for chrysene also includes triphenylene. As chrysene is by far the major component of PAH4, any contribution from the co-eluting triphenylene will significantly affect the value of PAH4.

## 5. Conclusions

The analytical method for the determination of BaP used in the present work complies with the performance criteria set up for BaP in a Commission Regulation (Regulation (EC) No 333/2007) [6], which has replaced Commission Directive 2005/10/EC from 1 June 2007. The method is well suited for food control purposes.

Products smoked by an indirect technique using external smoke generators have low PAH and BaP levels that are well below the maximum level of 5.0 mg/kg. Direct smoking over smouldering sawdust/wood chips, still largely used for fish smoking, generally results in low or moderate BaP levels, but occasionally the limit is exceeded. Traditional smoking of fish, however, where the food is directly exposed to smoke from a flaming log fire can be expected to give very high PAH levels with BaP well above the maximum level.

The smoke-houses differ in size and equipment, and it is not possible to make general statements on exact measures needed to lower the concentrations of PAHs in the products. It is important that the smoke-houses can study the results and that they are informed about the problem so that they can work on their own or in co-operation with the appropriate trade organisations to find solutions which are suitable to the individual smoke-house.

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