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THE CONTENT OF BENZO(A)PYRENE IN SLOVAKIAN SMOKED CHEESE

Rajmund Michalski[1], Robert Germuska[2]

[1] Institute of the Environmental Engineering of Polish Academy of Sciences, Zabrze, Poland;
[2]State Veterinary and Food Institute, Dolny Kubin, Slovakia

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Of polycyclic aromatic hydrocarbons (PAHs) present in food, benzo(a)pyrene (B(a)P) is the most often determined compound as a surrogate for all PAHs. Various chromatographic procedures, including gas, liquid and thin layer chromatography have been applied to isolate these substances from contaminated samples. Because of the complexity of food matrices additional clean up of extract prior chromatographic analysis is necessary.

This paper describes extraction of B(a)P from special kind of Slovakian smoked cheese by accelerated solvent extraction (ASE), clean up by gel permeation chromatography (GPC) and finally determination of B(a)P by high performance liquid chromatography (HPLC) with fluorescence detector in 430 nm. The influence of parameters such as: sample preparation as well as smoking time and temperature on the concentration of B(a)P in smoked cheese was investigated. The method allows determination of B(a)P in smoked cheese on μg/kg level.

The content of B(a)P in the analysed smoked cheese samples ranged from <0.1 μg/kg to 3.8 μg/kg and strongly depended on time and temperature of processing. In most cases, the concentration of B(a)P in samples of cheese from home-made ovens was over acceptable limit.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds that have been the subject of many concerns in recent years due to their toxic potential. PAHs are formed by the incomplete combustion of organic matter. They are widely distributed in the environment and human exposure to them is unavoidable. A number of them, such as B(a)P, are carcinogenic and mutagenic, and they are widely believed to make a substantial contribution to the overall burden of cancer in humans.

It is a reason that determination of carcinogenic substances in the environment requires the involvement of an experienced laboratory and in most cases also expensive equipment in order to achieve the specificity, accuracy and sensitivity needed.

The presence of PAHs in food is a matter for concern and requires continuous monitoring. Reviews of the literature have been written by Bartle [1991] and Phillips [1999].

Although several hundreds of PAHs exist, most of the studies determine just a limited amount of them, usually 16. This includes both carcinogenic and compounds considered being non-carcinogenic. The other approach has used B(a)P measurement as a marker and surrogate for all PAHs.

The first approach gives a truer picture of the overall burden of PAHs in food, because the relative amounts of each can vary widely. However, it is commonly the case that more than half of the total PAHs are accounted for the pyrene and fluoranthene, which are non-carcinogenic and very weakly carcinogenic, respectively. Thus, it can be argued that B(a)P provide information about the source of contamination.

The metabolism of B(a)P and other PAHs is very complex and fairly rapid. A variety of metabolites and their conjugates are formed in the body and excreted in urine and bile [Larsen, 1995]. Sensitive immunological methods have been developed to determine B(a)P in plasma and urine by Hutcheon [Hutcheon *et al.*, 1983].

Several epidemiological studies have reported an association between preference for intake of well-done food and a higher incidence of cancer in humans [Melikian *et al.*, 1999; Stavric & Klassen, 1994].

Due to the complexity of food samples, measurement of PAHs is difficult. The methods of analysis adopted generally consist of a solvent extraction procedure followed by gas or liquid chromatography [Biggs & Fetzer, 1996].

Reference standards of PAHs are used not only to identify the peaks detected in the chromatograms, but also to determine the efficiency of the methods for extracting of compounds from samples.

Extraction of PAHs from food samples can be achieved with 100% recovery if the materials are soluble in organic solvents such as cyclohexane. Insoluble remains require saponification before extraction. Because of the complexity of food matrices, the presence of substances that interfere with the extraction process or with the separation and identification of the PAHs may necessitate additional clean up of extracts prior to analysis.

Author's address for correspondence: Instytut Podstaw Inżynierii Środowiska PAN, ul. M. Skłodowskiej-Curie 34, 41-819 Zabrze, tel.: (48 32) 271 64 81 ext. 218; fax: (48 32) 271 74 70; e-mail: misiek@ipis.zabrze.pl

More recently other extraction techniques have been developed reducing extraction time and the amount of solvents. Some of these methods like the supercritical fluid extraction (SFE) [Saim *et al.*,1997; Hawthorne *et al.*, 2000; Castro & Jimenez-Carmona, 2000], microwave assisted extraction (MAE) [Zuloaga *et al.*, 1998; Shu *et al.*, 2000; Camel, 2000], and accelerated solvent extraction (ASE) [Richter, 2000; Popp *et al.*, 1997] have successfully been applied for the determination of PAHs in samples with complex matrix.

Various exposure estimates indicate that the daily intake of B(a)P from cooked meat products can only be in the range of 1–20 μg per person [Sexton *et al.*, 1992].

This paper describes the application of accelerated solvent extraction followed by gel permeation chromatography and high-performance liquid chromatography with fluorescence detector for the determination of B(a)P in special kind of smoked cheese from Slovakia, the so-called "korbaciki", which is manufactured from sheep milk. The first part of this work concerns determination of B(a)P in smoked cheese originated from private producers and made in home-made ovens. The second part of the study describes the influence of temperature and smoking time on final concentration of B(a)P in smoked cheese obtained from commercial smoke oven.

MATERIAL AND METHODS

Apparatus. The extraction of B(a)P from smoked cheese samples was performed on Dionex Accelerated Solvent Extractor (ASE® 200, Dionex, USA) equipped with 22 mL stainless extraction cells. The following equippment was used: for sample preparation: TurboVap II (Zymark, USA) with 40 mL vials, end point – 1 mL, compatible with Dionex ASE® 200, preparative liquid pump with 0.5 mL loop, (ISCO 2350, USA) and rotary evaporator (Buchi, Switzerland).

Samples clean-up were carried out on gel permeation stainless steel column (Bio Beads S X-3, 500 x 8 mm, containing styrene-divinylbenzene copolymer with particle size of 37–75 μm, Tessek, USA). The final separations of B(a)P were performed on Supelcosil LC PAH column, (250 mm × 4.6 mm, 5 μm, Supelco, USA) using HPLC system with fluorescence detector (Unicam, UK).

The cheese was smoked in a commercial oven (AUF UD 120 021 00, Strojobal, Prague, with water filter). The rest of the samples were smoked in home-made ovens by private producers.

Chemicals. Analytical-grade acetonitrile for HPLC (Merck, Germany), chloroform, acetone and anhydrite sodium sulphate (Mikrochem, Slovakia), Hydromatrix® (Varian, USA) were used for sample and eluents preparation.

B(a)P standard solutions were prepared using mussel homogenate consisting of polychlorinated biphenyls, chlorinated pesticides, and 15 PAHs (CRM IAEA-142, Monaco).

Water used in the experiments was purified using Millipore equipment (Bedford, USA) and its electrical conductivity was < 0.05 μS cm^{-1}.

Sample preparation and extraction. Samples were dried and ground before filling the extraction cells. The samples containing water (more than 10%) were mixed in equal proportion with sodium sulphate or Hydromatrix®. A sample (1 g) of smoked cheese was mixed with 5 g of sodium sulphate or 2 g of Hydromatrix® in a mortar and filled to 22 mL extraction cell. Dead volume was filled by sea sand. Residues of PAHs were extracted in ASE® 200 by means of chloroform/acetone (2:1, v/v) for 5 min. The temperature of an extract purged with nitrogen was 100°C.

Then, the extract was collected to 40 mL extraction vial and after that evaporated to 1 mL volume by TurboVap II with a bath temperature of 40°C and nitrogen pressure of 0.5–1.0 bar. The volume was arranged to 2 mL with chloroform.

Clean-up procedure. The extract (0.5 mL) was undergoing the clean-up procedure using gel permeation chromatography. The extract (0.5 mL) obtained from ASE was injected into Bio Beads S-X3 column and flushed with chloroform of 0.6 mL/min flow rate.

Then 17–26 mL chloroform fraction was collected and evaporated by Rotary evaporator (at 40°C). The residues were dissolved in 0.5 mL solution of acetonitrile: :water (80:20).

Fats and oils easily soluble in organic solvents are simply transferred into chloroform prior to GPC clean-up step. No more then 300 mg of fat should be loaded onto the column described. To avoid plugging of GPC system, only clean, free and without any solid particles samples can be applied.

HPLC analysis. HPLC separation of B(a)P was performed on Supelco Sil LC PAH column with the following eluents gradient programme: water/acetonitrile (60:40, v/v) held for 5 min and an increase in acetonitrile content up to 100% in 30 min. The total eluents flow rate was 1.5 mL/min and sample loop volume 20 μL. The fluorescence detector was set to 430 nm. The wavelengths of excitation and emission were set to 290 and 430 nm, respectively.

An example of chromatogram of B(a)P in the analysed smoked cheese obtained in this experiment is given in Figure 1.

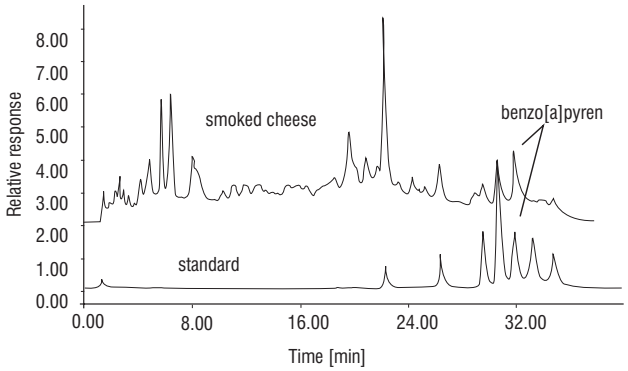

FIGURE 1. An example of chromatogram of B(a)P in analysed smoked cheese.

During this experiment, each sample of smoked cheese was weighted, extracted, cleaned, and analysed separately three times.

Method performance and validation. There were prepared 10 calibration solutions of B(a)P in the range of 0.5–5.0 μg/L. Calibration experiment was carried out under

analytical conditions described above. Figure 2 shows the calibration curve obtained. The statistical data calculated according to DIN 32 645 Standard (DIN 32 645, 1995) are

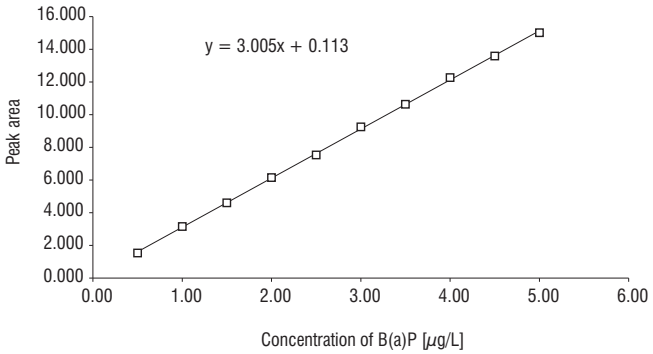

FIGURE 2. Calibration curve of B(a)P in the range of 0.5 μg/L–5.0 μg/L (r = 0.989).

TABLE 1. Selected statistical data obtained in calibration experiment.

Statistical data	-
Standard deviation μg/L	0.03
Coefficient of variation %	1.11
Limit of quantification μg/L	0.25

presented in Table 1.

Precision and recovery experiments were performed by spiking the cheese sample DK 14 (with B(a)P concentration below limit of detection) with different levels of standard B(a)P. The selected statistical data calculated according to ISO 8466-1 [ISO 8466-1, 1990] are given in Table 2. Calculated limit of detection is on 0.1 μg/kg level.

TABLE 2. The method's precision and recovery.

Sample	Number of replicates	Mean concentration [μg/kg]	RSD [%]	Recovery [%]
Smoked cheese DK 14	3	< 0.1	-	-
Smoked cheese DK 14 spiked with 0.5 [μg/kg] of B(a)P	3	0.4(8)	4.0	96
Smoked cheese DK 14 spiked with 2.0 [μg/kg] of B(a)P	3	1.9	5.0	95
Smoked cheese DK 14 spiked with 5 [μg/kg] of B(a)P	3	4.7	6.0	94

Analysis of smoked cheese. Two different experiments were carried out. In the first one, B(a)P were analysed in sixteen samples of home-made smoked cheese obtained from private producers. The results of analyses and processing conditions are described in Table 3.

In the next experiment, use was made of a commercial oven (AUF UD 120 021 00, Strojobal, Prague, with water filter) and as before of beech wood for smoking. Results are given in Table 4.

TABLE 3. Concentration of B(a)P in the analysed home-made smoked cheese samples.

Sample	Mean concentration [μg/kg]	Smoke temperature [° C]	Processing time [min]
ZA1	1.1	35	165
ZA2	1.1	35	165
LM3	3.5	22	200
DK1	3.6	30	90
DK2	1.6	30	120
DK3	2.6	28-35	180
DK5	0.9	25	120
DK6	2.4	13	120
DK7	0.3	28	150
DK8	1.0	32	180
DK9	2.6	25	360
DK10	1.7	25	180
DK11	2.7	25	150
DK12	1.5	23	150
DK13	3.8	24	180
DK14	< 0.1	27	90

TABLE 4. The effect of smoke temperature, processing time and kind of wood used on concentration of B(a)P in smoked cheese.

Sample	Mean concentration [μg/kg]	Smoke temperature [°C]	Processing time [min]
1/I	< 0.2	13	30
2/I	0.9	18	60
3/I	1.0	20	90
4/I	1.3	21	120
5/I	1.5	22	180
		Series 2	
1/II	0.2	14	30
2/II	0.7	19	60
3/II	1.0	21	90
4/II	1.1	23	120
5/II	1.7	25	180
		Series 3	
1/III	0.3	20	30
2/III	0.5	23	60
3/III	0.6	27	90
4/III	1.0	30	120
		Series 4	
1/IV	< 0.2	19	30
2/IV	0.2	22	60
3/IV	0.4	25	90
4/IV	1.0	29	120

RESULTS AND DISCUSSION

A lot of studies have been carried out to determine the levels of exposure to PAHs (including benzo(a)pyrene) from representative human diets and the proportion of the overall burden of environmental exposure to PAHs that is attributable to the diet. In many cases, it is concluded that diet is the major source of human exposure to PAHs.

According to literature [Phillips, 1999; Tomaniova *et al.*, 1997], the major dietary sources of B(a)P are cereals and vegetables as well as smoked food.

In accordance with Slovak regulations [Direction 981, 1996], the limits for B(a)P in food samples are: 1.0 μg/kg for smoked meat and fish products and 3.0 μg/kg for paprika, pepper and sausages.

In most cases, the concentrations of B(a)P in smoked cheese obtained from home-made ovens are over acceptable limit. The conditions used by private producers are non--repeatable and strongly diverse. In fact, the smoking process is out of control and the most important information for producers is the colour of the smoked cheese obtained.

In the second experiment, 50 pieces of raw cheese were inserted into a commercial oven with water filter and subjected to the smoking process. After 30 min, 10 pieces of smoked cheese were taken out (Table 4, series 1, sample 1/I) and after mixing analysed using ASE, GPC, and HPLC methods. In another 30 min, next 10 pieces of smoked cheese were taken out from the oven and analysed (Table 4, series 1, sample 2/I). The last part of samples was smoked for 180 min (Table 4, series 1, sample 5/I). This experiment was repeated 4 times (Table 4, series 2, 3 and 4). In series 3 and 4, the maximum temperature was set at 120°C.

Smoke curing of food (usually meat and fish) is now largely a highly industrialised process involving modern technology. Nevertheless, traditional smoke-houses are still used fairly widely.

Gomaa *et al.* [1993] described measurements of PAHs in smoked food. Total PAHs concentrations ranged from 2.6–29.8 μg/kg (smoked meat) and 9.3–86.6 μg/kg (smoked fish). A study on smoked food commercially available and home-made in Canada [Panalkas, 1976], UK [Dennis *et al.*, 1983], Italy [Lodovici *et al.*, 1995] and Scandinavian countries [Nordholm *et al.*, 1986] produced similar findings.

CONCLUSIONS

According to the obtained results, conclusions are as follows:

1. ASE combined with safety evaporation technique (TurboVap II), GPC and HPLC techniques offer fast, convenient and sensitive analytical results for samples with complex matrix. The detection limits as well as precision and recovery coefficient are quite satisfactory for the determination of B(a)P in food samples on μg/kg level.

2. The concentration of B(a)P strongly depends on the time and temperature of processing.

3. The optimal temperature is 25°C–30°C.

4. Time processing should not exceed 2 h.

5. Beech tree is a suitable kind of wood.

6. Cheese should be dried before smoking.

7. When the light brown colour is appearing, the process should be finished.

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ZAWARTOŚĆ BENZO(A)PIRENU W SŁOWACKIM SERZE WĘDZONYM

Rajmund Michalski[1], Robert Germuska[2]

[1]Instytut Podstaw Inżynierii Środowiska Polskiej Akademii Nauk, Zabrze, Polska;
[2]State Veterinary and Food Institute, Dolny Kubin, Slovakia

Spośród wielopierścieniowych węglowodorów aromatycznych (WWA) obecnych w żywności, benzo(a)piren (B(a)P) jest najczęściej oznaczanym surogatem wszystkich węglowodorów z tej grupy. Do wydzielania WWA stosuje się wiele różnych metod chromatograficznych, np.: chromatografię gazową, cieczową i cienkowarstwową. Z powodu złożonej matrycy próbek żywnościowych, konieczne jest dodatkowe oczyszczanie ekstraktu przed analizą chromatograficzną.

Niniejsza praca opisuje ekstrakcję benzo(a)pirenu ze specjalnego rodzaju wędzonego sera Słowackiego za pomocą przyspieszonej ekstrakcji rozpuszczalnikowej (ASE), oczyszczanie próbki metodą chromatografii żelowej (GPC) oraz końcowe oznaczanie (B(a)P techniką HPLC z detekcją fluorescencyjną przy długości fali 430 nm. Wpływ takich parametrów jak: sposób przygotowania próbki, jak również czas i temperatura wędzenia na zawartość B(a)P został przebadany. Metoda pozwala na oznaczanie B(a)P w wędzonym serze na poziomie μg/kg.

Zawartość B(a)P w analizowanych próbkach wędzonego sera wahała się od <0,1 μg/kg do 3,8 μg/kg i zależała w znaczący sposób od czasu i temperatury wędzenia (tab. 4). W większości przypadków zawartość B(a)P w próbkach sera otrzymywanych domowym sposobem wędzenia przekraczała dopuszczalny limit.