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Published online: 14 Jan 2020.

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ARTICLE

Polycyclic aromatic hydrocarbons in traditionally smoked Slavonska kobasica

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ABSTRACT

The traditional smoking procedure, which is the use of open fire, can lead to the formation of PAHs in sausages. The aim of this paper was to assess the types and concentrations of 16 PAHs in 30 samples of *Slavonska kobasica*, a traditional smoked sausage. In general, some samples showed high values of anthracene and acenaphthylene. In one sample, acenaphthylene reached the value of 1050 μg/kg and in another 1491 μg/kg anthracene was measured. Cancerogenic benzo(*a*)pyrene content was little above the maximum limit of 5 μg/kg in four samples, but mainly remained below the limit of quantification. PAH4 (i.c. benzo(*a*)anthracene, chrysene, benzo(*b*)fluoranthene and benzo(*a*)pyrene) were above the maximum limit of 30 μg/kg in three samples. Generally, it can be noted from the results that samples with high PAH4 and benzo(*a*)pyrene concentrations also have high PAH16 concentrations.

Introduction

The evolving need of contemporary consumers to return to original and organic products results in the expansion of such products on the market. Most of these are produced by small family businesses and are sold at the local market. For traditional meat products the demand is high and so is the supply. *Slavonska kobasica* is a traditional smoked dry meat fermented sausage that is currently under consideration to receive the protected geographical indication (PGI), according to the EU legislative recommendation. Since *Slavonska kobasica* is a product that has to undergo a smoking procedure in order to obtain its traditional properties, like the characteristic aroma, taste, and smell, it can be assumed it might contain a certain amount of PAHs. PAHs are well-known food contaminants and are especially investigated and monitored in traditional meat products in other European countries as well, such as Portugal, Spain, and Greece (Farhadian et al. 2010; Wretling et al. 2010; Roseiro et al. 2011; Gomes et al. 2013; Ledesma et al. 2016; Hokkanen et al. 2018). They are a result of incomplete combustion of wood during the smoking process and in significant amounts, i.c. higher than the legislative limits, some of them can display carcinogenic, teratogenic, toxic, and mutagenic properties (Falcó et al. 2003; Reinik et al. 2007; IARC 2010; Kim et al. 2013). Food submitted to grilling,

cooking, smoking, or roasting also has higher chances to be contaminated with PAHs (Šimko 2002; Ledesma et al. 2015; Babić et al. 2018).

Where EU law regulated 4 PAHs being hazardous to human health via food, the US Environmental Protection Agency (US EPA 2008) identified a total of 16 PAHs (PAH16) as priority environmental pollutants: naphthalene (Nap), acenaphthylene (Anl), acenaphthene (Ane), fluorene (Flu), anthracene (Ant), phenanthrene (Phen), fluoranthene (Flt), benz(*a*)anthracene (BaA), pyrene (Pyr), chrysene (Chry), benzo(*b*)fluoranthene (BbF), benzo(*k*)fluoranthene (BkF), benzo(*a*)pyrene (BaP), dibenz(*a,h*)anthracene (DahA), benzo(*g,h,i*)perylene (BghiP) and indeno(1,2,3-*cd*)pyrene (InP). This due to the fact that PAH concentrations are a direct consequence of not only the smoking procedure (type of wood and its moisture content, casing), but they also stem from the environmental contamination throughout diverse sources, like exhaustion fumes, wildfire, and other combustion-prone processes (Ciganek and Neca 2006). Regarding this, the PAH content found in meat products can also be related to the contamination of feed used in pig breeding.

Even though the research is still ambiguous, the European Food Safety Authority (EFSA 2008) decided that the concentrations of BaP and the sum of the concentrations of four PAHs, BaP, BaA, BbF, and Chry (PAH4) will be considered as a reference for the

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determination of PAHs in food. According to European Commission regulations (EU) No. 1881/2006 and 835/2011 (EC 2006, 2011a), the maximum limit of BaP in meat products is 2 μg/kg and the sum of the PAH4 concentrations should not exceed 12 μg/kg. However, according to European Commission regulation (EU) No. 1327/2014 (EC 2014), for traditional meat products produced and marketed in some EU countries, including Croatia, the maximum BaP level is set to 5 μg/kg and the sum of the PAH4 must not exceed 30 μg/kg.

Therefore, this survey aimed to assess the types and concentrations of 16 PAHs in 30 samples of *Slavonska kobasica*, a traditionally smoked sausage, marketed at the local market and labelled as traditional food. It was conducted in order to get an insight into the amounts and types of PAHs an average consumer is exposed to when consuming a traditional *Slavonska kobasica* bought at the local market. Since the samples of *Slavonska kobasica* are made at small family farms, certain hygienic demands have to be fulfilled. However, PAH contamination is inevitable, since the traditional smoking method purports the exposure of sausages to open fire. Some authors report that the composition and concentration of PAHs in smoked meat products are in correlation with wood type and its moisture content, oxygen concentration in the smoking chamber, wood combustion temperature, etc., and that concentrations of PAHs in traditionally smoked meat products can reach alarming high concentrations (Purcaro et al. 2009; Wretling et al. 2010; Roseiro et al. 2011; Babić et al. 2017), whereas other authors (García-Falcón and Simal-Gándara 2005; Santos et al. 2011; Pöhlmann et al. 2012; Gomes et al. 2013; Hitzel et al. 2013; Škaljac et al. 2014; Fasano et al. 2016; Malarut and Vangnai 2018) reported that diffusion and deposition of smoke components into smoked foods depend on the presence of obstacles such as casing (natural, collagen, cellulose).

Materials and methods

Sampling

In order to carry out this study, 30 samples of *Slavonska kobasica*, manufactured by representative homemade producers, were chosen. The sausages were made in a non-industrial environment, characterised by small-scale batch production with a limited degree of mechanisation, using traditional techniques without any additives (nitrites or ascorbic acid), strongly defined by the climate and the region of origin. Once collected, the samples were transported to the laboratory in a very short time and stored in a refrigerator (below 4°C). The traditional production lasts from November until February or March. Sausages are made from the pigs that are at least 12 months old and over 150 kg of weight. Only high-quality parts of the pig, such as the thigh, the back and shoulder are used in the production of traditional *Slavonska kobasica*. The meat is cut into stripes of ca. 30 cm long, 10 cm wide and 3 cm thick. The stripes are then left on a pierced inox/stainless steel plate and exposed to low temperatures until the temperature of the meat reaches −2 to −5°C. The meat is then ground trough a grinding plate with holes of 6 mm in diameter. Optimum pH value of the minced meat is below 5.9. According to the traditional recipe, the ground meat is then mixed with 1.8% salt, 1% sweet red paprika, 0.6% of hot paprika, and 0.2% garlic. Then, the mixture is stuffed into a pig's thin intestine (lat. intestinum tenue). The raw *Slavonska kobasica* is put into the smokehouse and smoked with dry hardwood (hornbeam, beech, and its sawdust) every second day for 2 weeks. The temperature and relative humidity at this stage vary from 18°C to 20°C and 70% to 90%. After smoking, the *Slavonska Kobasica* is left to undergo the ripening stage. This stage is the longest part of the production and it lasts more than 2 months. During that period, the sausages are kept in a darkroom with the temperature ranging from 14°C to 17°C and with relative humidity reaching 70–80%. Thereafter, the *Slavonska kobasica* sausage is ready for consumption.

PAH analysis

Standard solutions of PAHs were prepared with a PAH mix of 16 polycyclic aromatic hydrocarbons (Ultra Scientific, North Kingstown, USA) of 500 ± 0.2 μg/mL. To minimise the matrix influence, a calibration by using a matrix blank sample was performed. Retention times of the peaks and the target ions were obtained from the standard solution.

Sample preparation

Samples were prepared using the quick, easy, cheap, effective, rugged, and safe method (QuEChERS; Anastassiades et al. 2003) as adapted from the Association of Analytical Communities (AOAC) Official method 2007.01 for extraction and clean-up, described and adjusted by Novakov et al. (2017) and Mastanjević et al. (2019). In short, the method included extraction using acetonitrile (ACN, Sigma-Aldrich, St. Louis, Missouri, USA) in the presence of anhydrous magnesium sulphate ($MgSO_4$) and anhydrous sodium acetate (CH_3COONa), both obtained from Merck (Darmstadt, Germany). Three grams of the sample were transferred into the centrifuge tube where a mixture of 3 mL of acetonitrile and 3 mL of water was added. After

intensive stirring on a vortex mixer for 1 min, 3 g of anhydrous magnesium sulphate and 1 g of anhydrous sodium acetate were added. The sample was then centrifuged for 5 min at 3000 rpm and 1 mL of upper layer of the acetonitrile extract was transferred into the 5-mL tube, along with 150 mg of anhydrous magnesium sulphate, 100 mg of Primary and Secondary Amine (PSA) and 50 mg of C18, obtained from Merck (Darmstadt, Germany). The content was centrifuged again for 5 min at 3000 rpm, ensuring a clear and pure extract. Half a mililitre of the extract was subjected to evaporation under nitrogen gas and reconstituted with hexane, resulting in a sample ready for analysis on a GC-MS (Agilent 7890B/5977A, Santa Clara, CA, USA).

GC-MS analysis

GC-MS parameters were adjusted as described by Petrović et al. (2019) and Mastanjević et al. (2019). In short, a DB-5MS column (30 m × 0.25 μm × 0.25 mm; Agilent J&W, Santa Clara, CA, USA) was used to separate the individual PAHs. A sample volume of 4 μL (splitless mode) was injected at the constant pressure of 11.36 psi and a flow through the column of the carrier gas of 1.2 mL/min. The target and qualifier abundances were determined by injection of a mixture of PAH standards under the same chromatographic conditions. A full scan with the mass/charge ratio ranging from 60 to 500 m/z was employed. In order to minimise the matrix effect, standard solutions were prepared in blank matrix extracts. With the aim of obtaining more reliable results, further PAH quantification is performed in SIM mode and the obtained data were processed using Mass Hunter Software (Agilent, Santa Clara, CA, USA). Method performance was validated in the calibration range from 0.005 to 0.1 mg/kg. The standard solution of the PAH mixture served as the base for the quantification, using matrix calibration curves. The coefficients of determination (r^2) for the PAH standard calibration plots were above 0.99.

An Agilent 7890B/5977A GC-MS system was used for the analysis. The GC operating conditions were the following: a fused silica column [30 m x 0.25 μm film of HP-5M (thickness)] at an injection temperature of 280°C, using splitless mode and the volume injected was 4 μL. The column temperature was programmed as follows: hold at 50°C for 0.4 min; 50–195°C at 25°C/min, hold for 1.5 min; 195–265 at 8°C/min; 265–315°C at 20°C/min and maintained at 315°C for 12 min. The MSD temperature was 280°C. The verification of peaks was done comparing retention times and target ions with standard values. Procedural and solvent blanks were analysed, but no PAHs were found.

Data handling

Experimental data were subjected to the analysis of variance (ANOVA) and Fisher's least significant difference (LSD) tests, with significance defined at $p < .05$. Statistical analysis was carried out using Statistica 12.7 (StatSoft Inc. Tulsa, 2015, OK, USA).

Method validation

Validation referred to the determination of precision, reproducibility, accuracy, linearity (r^2), LOQ (limit of quantification), LOD (limit of detection), and uncertainty, according to ISO 17025. The method precision was evaluated by repeatability using smoked meat spiked with PAHs (50.0 μg/kg, n = 20) and analysed in triplicate. Accuracy was calculated using recovery values. Linearity of the detector was tested (5 to 500 μg/kg) and was satisfactory for all ranges.

Results and discussion

Method validation data are given in Table 1. The LOD and the LOQ values appeared to be somewhat higher than the levels set by Regulation No.836/2011 (EC 2011b). The method used in this study was applied in a proficiency test (FAPAS 2018), where the z-score for PAH4 was 0.4, at an assigned value of 39.4 μg/kg, indicating good analytical method performance. Table 2 gives the results of PAH4 and PAH16 content of all samples. In most samples, Nap, Anl, Ane, Flu, Phen, Ant, and BaA were quantified. Nap was found in 29 samples, with values ranging from 3.30 μg/kg in sample SK10 to 190 μg/kg in sample SK15. Anl and Ant were the most abundant light PAHs in all of the samples and showed high concentrations only in several samples. Anl in samples SK2, SK4, SK5, SK12, SK15, and Ant in SK2, SK4, SK5, SK8, SK12, and SK19. The highest concentration of Anl was determined in sample SK12 (1080 μg/kg) while the highest concentration of Ant was found in SK5 (1491 μg/kg). Ane was quantified in 14 samples and its values ranged from 2.36 in SK22 to 29.5 in SK13. Flu was detected in all samples with values ranging from 9.10 μg/kg in sample SK29 to 386 μg/kg in SK5. Phe was determined in 28 samples, while it remained below the level of quantification in 2 samples. Its values ranged from 7.60 μg/kg in SK16 to 386 μg/kg in SK5. BaA was also detected in 28 samples, with the lowest value in SK16 (0.58 μg/kg) and the highest in sample SK2 (23.7 μg/kg). Flt was quantified in 26 samples. The highest value was recorded for sample SK5, 348 μg/kg and the lowest value was 2.00 μg/kg in SK16.

Table 1. Method validation data.

PAHs	Precision (%)	Reproducibility (%)	Accuracy (%)	Linearity (r^2)	LOQ ($\mu g\ kg^{-1}$)	LOD ($\mu g\ kg^{-1}$)
Nap	11.3	6.33	95.0	0.99	1.20	0.30
Anl	7.91	7.82	99.0	0.99	1.30	0.29
Ane	8.52	8.32	99.3	0.99	1.05	0.32
Flu	2.82	10.2	100	0.99	1.11	0.30
Ant	3.53	3.73	98.7	0.99	1.10	0.30
Phen	4.31	11.4	85.9	0.99	1.18	0.35
Flt	3.61	3.72	95.3	0.99	1.15	0.30
BaA	9.44	8.6	89.7	0.99	1.30	0.37
Pyr	4.74	6.91	91.1	0.99	1.21	0.32
Chry	5.33	8.20	92.5	0.99	1.13	0.34
BbF	8.52	14.3	86.4	0.99	1.30	0.36
BkF	3.51	3.32	94.3	0.99	1.21	0.32
BaP	3.23	3.81	96.8	0.99	2.00	0.53
DahA	8.72	11.3	91.2	0.99	1.99	0.51
BghiP	9.71	11.3	81.5	0.99	1.90	0.45
InP	9.51	10.3	85.3	0.99	1.91	0.53

Table 2. PAH4 and PAH16 contents (µg/kg) in *Slavonska kobasica*.

Sample	PAH4	PAH16
SK1	13.7h	733i
SK2	42.6a	2299c
SK3	16.5f	748hi
SK4	30.8c	1811d
SK5	38.7b	3729a
SK6	8.67j	766hi
SK7	13.6hi	765hi
SK8	12.8i	392kl
SK9	26.2e	686i
SK10	14.8g	724i
SK11	14.5g	587j
SK12	28.8d	3050b
SK13	15.9f	1634e
SK14	16.2f	823gh
SK15	1.74op	883g
SK16	1.03p	101pq
SK17	2.19no	412kl
SK18	5.50k	396kl
SK19	1.57op	559j
SK20	12.7i	1042f
SK21	2.07no	214no
SK22	<LOQ	69.1q
SK23	1.82o	391kl
SK24	3.26m	209no
SK25	1.99o	459k
SK26	2.97m	177op
SK27	4.46l	335lm
SK28	2.80mn	91.1pq
SK 29	<LOQ	245no
SK 30	2.77mn	295mn

Means within rows with different letters are significantly different ($p < 0.05$). LOQ – limit of quantification; ∑ PAH4: BaA – benzo(*a*)anthracene; Chry – chrysene; BbF – benzo(*b*)fluoranthene; BaP – benzo(*a*)pyrene; ∑PAH16: Nap – naphthalene; Anl – acenaphthylene; Ane – acenaphtene; Flu – fluorene; Ant – anthracene Phen – phenanthrene; Flt – fluoranthene; BaA – benzo(*a*) anthracene Pyr – pyrene; Chry – chrysene; BbF – benzo(*b*)fluoranthene; BkF – benzo(*k*)fluoranthene; BaP – benzo(*a*)pyrene; DahA – dibenzo(*a,h*)anthracene; BghiP – benzo(*g,h,i*)-perylene; InP – indeno(1;2;3-*cd*)pyrene.

BaP was found in 15 samples and 4 samples showed elevated concentrations when compared with Regulation (EU) No. 1327/2014, so above 5 µg/kg, with samples SK4 and SK5 being almost identical in results (6.88 and 6.87 µg/kg). Bogdanović et al. (2019) found only the low concentration of 0.49 µg/kg of BaP in Croatian dry homemade sausages. This can be explained by the unstandardised smoking procedures in traditional production of these sausages. BaP content in Serbian *Petrovska klobasa* was below the limit of detection (Škaljac et al. 2014, 2018) and Portuguese, Italian, and Spanish traditionally smoked sausages showed somewhat higher values for BaP content (García-Falcón and Simal-Gándara 2005; Purcaro et al. 2009; Santos et al. 2011; Gomes et al. 2013). Rozentale et al. (2018) reported higher BaP levels of 0.05 to 166 µg/kg in 77 traditionally smoked meat samples in Latvia, Lithuania, and Estonia.

The PAH4 data shown in Table 2 give an insight into samples which are more hazardous for human health. According to EU regulation No 1327/2014, the sum of PAH4 in Croatian traditional meat products should not exceed 30 µg/kg. In this survey, only 3 samples (SK2, SK4, and SK5) do not comply with this regulation, with SK2 having the largest value of 42.6 µg/kg. Slámová et al. (2017) reported higher levels for BaP and PAH4, exceeding 2–50 times the limits in EC 1881/2006 for Cambodian fish smoked from 1 up to 4 or 5 days. Babić et al. (2018) reported results of a comparison between carp meat smoked in traditional conditions without filter and with different types of filters, showing that any filter lowered PAH levels. While the PAH4 level without a filter was 2.83 µg/kg, the filtered values were below 1 µg/kg.

The sums of PAH16 shown in Table 2 seem high, but since no legal regulation for PAH16 has been set regarding limiting concentrations in food, it is impossible to estimate whether those concentrations are harmful for human health. However, it can be noted from the results that samples with high PAH4 and BaP concentrations also have high PAH16 concentrations. This is probably due to the smoke intensity, i.e. duration and temperature, as these factors have a major influence on PAH concentrations in smoked meat products. Prolonged smoking or smoking with an unfitting wood type (oak, pine, or other types of softwood) can lead to elevated PAH levels in meat products.

Lorenzo et al. (2010, 2011) reported lower values for PAH16 content for traditionally smoked Spanish dry

sausages "Chorizo gallego", "Chorizo de cebolla", "Botillo" and "Androlla", probably due to the different casing used for sausage production and different wood used for smoke production resulting in a different surface/mass ratio, which is an important factor for PAH contamination.

Slavonska kobasica showed a large surface per unit of volume, being smaller in diameter than most European-smoked dry sausages, thus favouring adsorption of PAHs.

Conclusions

Nap, Anl, Ane, Flu, Phen, Ant, and BaA were found in most samples. Nap was quantified in 29 samples and Anl and Ant showed high concentrations. Phen and BaA were detected in 28 samples and Ane in only 14 samples. Flu was found in all samples. BaP was detected in 15 samples, but only 4 showed concentrations above 5 µg/kg. PAH4 concentrations were above 30 µg/kg in 3 samples, meaning they are not in accordance with regulation (EU) No 1327/2014. Although not legally regulated, the sum of PAH16 showed a certain pattern. Samples with high PAH4 and BaP levels also showed higher PAH16 concentrations. In order to reduce the PAH concentration in traditional meat products, application of the ALARA (as low as reasonably achievable) principle is under discussion. However, legislative recommendations that would help to standardise smoking procedures should be issued as well, regarding the type of wood used for smoking, minimal heights for meat products hung in smokehouses during exposure to (open fire) smoking, ventilation regulations, and casings used for filling.

Disclosure statement

No potential conflict of interest was reported by the authors.

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