New simplified procedure for isolation of benzo[*a*]pyrene from smoked sausages

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Summary

The aim of the work was to develop and verify a new simplified procedure for isolation of benzo[*a*]pyrene (BaP) from smoked sausages. The procedure uses extraction and clean-up operation in one step, thus avoiding loss of analyte and saving time, solvents and chemicals. In order to eliminate post-extraction clean-up step, silica gel as sorbent was applied directly to the accelerated solvent extraction (ASE) extraction cell to prevent the extraction of interfering compounds into BaP fraction. The procedure was studied at three content levels – 0.4 $\mu g \cdot kg^{-1}$, 5 $\mu g \cdot kg^{-1}$ and 10 $\mu g \cdot kg^{-1}$. The spiked sample was placed into extraction cell on a sorbent layer and extracted with *n*-hexane at 100 °C and 10 MPa for 10 min. The flush volume was 60% and the purge time 120 s. One static cycle was accomplished three times. The extracts were evaporated to dryness, residue was dissolved in methanol and analysed by HPLC. Based on the experimental data, parameters of the procedure are as follows: coefficient of determination (R^2) was 0.99; limit of detection and the limit of quantification were 0.1 $\mu g \cdot kg^{-1}$ and 0.2 $\mu g \cdot kg^{-1}$, respectively; precision: values of Horrat coefficients $HORRAT_R$ and $HORRAT_r$ were lower than 2, and recovery varied between 93% and 103%.

Keywords

accelerated solvent extraction; polycyclic aromatic hydrocarbons; benzo[*a*]pyrene; smoked sausages; HPLC

Polycyclic aromatic hydrocarbons (PAH) belong to hazardous food contaminants due to their known or suspected carcinogenicity and/or mutagenicity. Some of them, even though not carcinogenic, may act as synergists [1]. In general, PAH are formed by incomplete combustion of fossil fuels and other forms of organic matter. For these reasons, they can be found in all parts of the environment as well as in foods [2]. PAH are also formed at thermal processes during food production or preparation, such as baking, grilling, roasting, frying and smoking [3, 4]. Benzo[*a*]pyrene (BaP) is regarded as a marker for the occurrence, and genotoxic and carcinogenic effects of PAH in food. EU has established a maximum level for BaP of 5 $\mu g \cdot kg^{-1}$ wet weight for smoked meat and smoked meat products [5].

A large number of studies were published on isolation of PAH from smoked meat products by extraction that is a crucial step in the quantitative analysis of PAH [3, 6, 7]. Various papers discussed various extraction techniques, such as saponification [8–16], Soxhlet extraction [7, 17–19], ultrasonic extraction [7, 18, 20–23], supercritical fluid extraction [7], accelerated solvent extraction, matrix solid-phase dispersion [24] and microwave-assisted extraction and their combinations [7, 19, 22, 25].

Accelerated solvent extraction (ASE) is a new extraction procedure that significantly simplifies isolation of PAH from the food matrix. The procedure is based on the extraction of sample under elevated temperature and pressure, so reducing the time of isolation. Then, the extract is trans-

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ferred from the extraction cell to a standard collection vial for clean-up or analysis. The entire extraction process is fully automated, performed in minutes and consumes low amount of the solvent [26]. Various authors studied the efficiency of ASE at extraction of PAH from environmental matrices [27–32]. However, less attention was paid to optimization of ASE of PAH from biological matrices and foodstuffs [33–37]. After the ASE procedure, various additional clean-up procedures can be applied to remove interfering compounds from extracts. The aim of our study was to develop and verify a new simplified one-step procedure for simultaneous isolation and clean-up of BaP fractions from smoked sausages, suitable for direct analysis by HPLC.

MATERIALS AND METHODS

BaP was of analytical grade, purchased from Supelco (Bellefonte, Pennsylvania, USA) in solid state.

Poly(acrylic acid), partial sodium salt-graft-poly(ethylene oxide) was of analytical grade, purchased from Sigma Aldrich (Munich, Germany) in solid state.

Silica gel 40 was of analytical grade, purchased from Merck (Darmstadt, Germany).

Solvents

n-hexane of analytical grade and methanol of HPLC grade were purchased from Merck. The solvents were rectified just before use in a distillation apparatus.

Sample

Twenty samples of traditional Slovak smoked home-made sausages were obtained from local shops in Bratislava, Slovakia.

Experiments

About 1 g of smoked sausages homogenized in the knife mill Grindomix (Retsch, Haan, Germany) were mixed with the same amount of the drying material poly(acrylic acid), partial sodium salt-graft-poly(ethylene oxide). Amount of 10 g of silica gel was added into the extraction cell before adding the sample to prevent the extraction of unwanted lipids. A 33-ml extraction cell was plunged with cellulose microfibre filter at the outlet to prevent washing out the sorbent. The extraction was performed in ASE (Dionex, Sunnyvale, California, USA) using *n*-hexane at 100 °C and 10 MPa at a static time of 10 min. The flush volume was 60%, the purge time was 120 s, and one static cycle was repeated three times. Then, the extract was evaporated in a water bath to dryness (40 °C) using a nitrogen stream. Finally, the residuum was dissolved in methanol and analysed by HPLC with fluorescence detection (HPLC-FLD).

HPLC conditions

HPLC analyses were carried out on the Shimadzu equipment (Kyoto, Japan) consisting of solvent delivery module LC-20AD, autosampler SIL-20A, degasser DGU-20A5, column ovens CTO-20A, communications bus module CBM-20A, diode array detector SPD-M20A and fluorescence detector RF-10AXL. The analytical separation was performed on Zorbax Eclipse XDB-C18 (100 mm × 4.6 mm, 1.8 μm) column connected to guard column Zorbax Eclipse PAH (12,5 mm × 4,6 mm × 5 μm) (Agilent, Santa Clara, California, USA) using isocratic elution with methanol at a flow rate 0.7 ml·min^{-1} and at 35 °C. Fluorescence detector operated at excitation wavelength 300 nm and emission wavelength 410 nm.

RESULTS AND DISCUSSION

Quantification

The external standard method was used to determine BaP content in the samples. Linearity of the method was checked by injection of BaP standard solutions ranging from 0.1 ng·ml^{-1} to 15 ng·ml^{-1}. Linear regression was applied to construct a calibration curve reporting peak area versus BaP content. HPLC chromatograms of BaP standard solutions and spiked samples of smoked sausages are shown in the Fig. 1–4.

Verification of the novel procedure to reach criteria of EU Regulation 2007/333/EC

The procedure was optimized and verified according to the performance criteria required by EC for the analysis of benzo[*a*]pyrene and described in Commission Regulation 2007/333/EC [38]. The requirements are shown in Tab. 1.

Linearity of the detector response was checked through the calibration curve, which was obtained by linear regression of the peak area versus concentration of BaP in the injected solution ranging from 0.1 ng·ml^{-1} to 15 ng·ml^{-1}. The calculated coefficient of determination (R^2) value was above 0.99 for BaP. As mentioned in Regulation [38], the limit of detection (*LOD*) and the limit of quantification (*LOQ*) values recommended for BaP must be lower than 0.3 μg·kg^{-1} or 0.9 μg·kg^{-1}, respectively. *LOD* and *LOQ* were calculated from calibration data by using software QC Expert (TriloByte

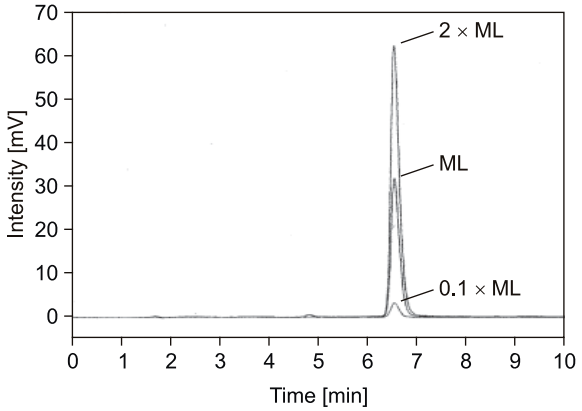

Fig. 1. HPLC-FLD chromatograms of 3 BaP standard solutions.

ML – maximum level = 5 $\mu g \cdot kg^{-1}$.

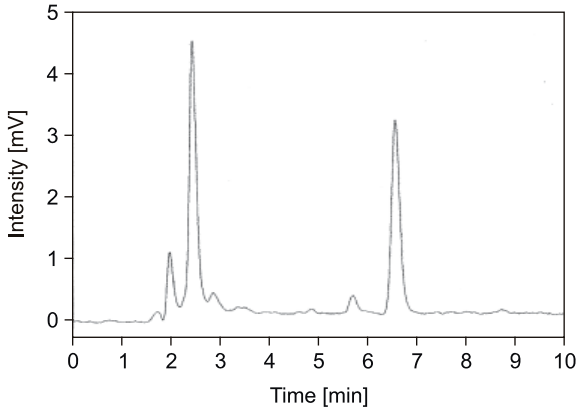

Fig. 2. HPLC-FLD chromatogram of smoked sausage spiked with 0.5 $\mu g \cdot kg^{-1}$ BaP standard solution.

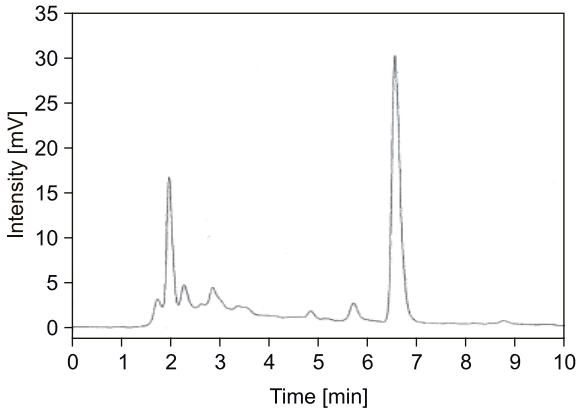

Fig. 3. HPLC-FLD chromatogram of smoked sausage spiked with 5 $\mu g \cdot kg^{-1}$ BaP standard solution.

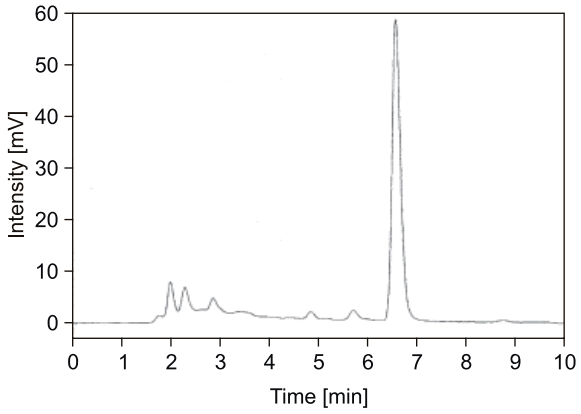

Fig. 4. HPLC-FLD chromatogram of smoked sausage spiked with 10 $\mu g \cdot kg^{-1}$ BaP standard solution.

Statistical Software, Pardubice, Czech Republic).

Targeted spiking levels, mean introduced contents (average of the real contents introduced), *LOD*, *LOQ*, precision, recovery and linearity are indicated in Tab. 2.

Precision has been assessed by calculating the Horrat coefficient (2007/333/EC) for BaP at three contents, in repeatability (*r*) and reproducibility (*R*) conditions (2007/333/EC). Blank smoked sausage was spiked with the standard solution to obtain samples at BaP content of 0.4 $\mu g \cdot kg^{-1}$, 5.0 $\mu g \cdot kg^{-1}$ and 10.0 $\mu g \cdot kg^{-1}$ in six replicates per level for repeatability and six replicates plus another six analyses by another technician, instrument (another detector was used) and on a different day for reproducibility. Both $HORRAT_R$ and $HORRAT_r$ met the performance criterion (< 2), demonstrating the validity of the procedure for the whole analytical range tested.

The mean recovery for BaP for the assessment of accuracy was then calculated. According to the performance criteria for methods of analysis for BaP in foods, recovery must be between 50% and 120%. As shown in Tab. 2, recovery varied between 93% and 103%. Specificity was evaluated by analysing BaP in 20 injections of blank smoked sausage samples and checking for any interference in the position where BaP was expected to elute.

As mentioned, several authors studied application of ASE for isolation of PAH from smoked meat products [33, 36, 37, 39, 40]. All these ASE extractions were followed by additional post-extraction steps, such as gel permeation chromatography and solid phase extraction [33, 36, 37], or extracts were treated with sulphuric acid and Florisil [40], which prolonged the duration of the isolation procedure and consumed additional solvents, chemicals and sorbent. Our new procedure

Tab. 1. Perfomance criteria required for analysis of benzo[*a*]pyrene (Commission Regulation 333/2007/EC [38]).

Parameter	Value/Comment
Applicability	Food specified in Regulation (EC) No 1881/2006 [5]
Limit of detection	Less than 0.3 $\mu g \cdot kg^{-1}$
Limit of quantific*ation*	Less than 0.9 $\mu g \cdot kg^{-1}$
Precision	$HORRAT_r$ or $HORRAT_R$ values less than 2
Recovery	50% to 120%
Specificity	Free from matrix or spectral interferences, verification of positive detection

Tab. 2. Validation parameters and linearity (R^2) of the HPLC-FLD method for quantification of BaP in smoked sausages.

	Targeted spiking content [$\mu g \cdot kg^{-1}$]	Mean introduced content [$\mu g \cdot kg^{-1}$]	R^2	*LOD* [$\mu g \cdot kg^{-1}$]	*LOQ* [$\mu g \cdot kg^{-1}$]	Precision		Recovery [%]
						$HORRAT_r$	$HORRAT_R$	
BaP	0.4	0.41	0.99	0.1	0.2	0.3	0.2	103
	5.0	4.33				0.2	0.1	87
	10.0	9.33				0.2	0.3	93

R^2 – coefficient of determination, *LOD* – limit of detection, *LOQ* – limit of quantification.

simplified the isolation and clean-up procedure combining both operations in one step, which accelerated the procedure of BaP extraction from smoked meat matrix, while meeting all requirements set by Regulation [38].

CONCLUSIONS

On the basis of experimentally obtained data and their statistical treatment, it is possible to postulate conclusions as follows:

- ASE procedure is a suitable procedure to obtain PAH fraction from smoked meat sausage matrix.
- It is possible to combine ASE extraction and clean-up procedure in one step to minimize consumption of solvents, chemicals and other laboratory materials, and also to save time.
- This new simplified procedure shortens considerably the duration of determination and reduces handling the sample.
- Another advantage of the procedure is greater protection of the analyte against decomposition by light.
- This new procedure meets all requirements set by Regulation (EC) No 2007/333.

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