Using Box–Behnken design approach to investigate benzo[*a*]anthracene formation in smoked cattle meat samples and its' risk assessment

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Abstract In present study, a novel approach was selected for benzo[*a*]anthracene determination from smoked cattle meat samples using solid phase extraction under various experimental conditions. To optimize experimental variables, a three factor, three-level Box–Behnken experimental design combining with response surface methodology and quadratic programming were employed to prevent excessive amount of benzo[*a*]anthracene formation in smoked meat based on different experimental parameters. The influence of some important process parameters including cooking time (10–30 min), fat ratio (5–25%) and distance to the cooking source (5–25 cm), which significantly affected the formation efficiency of benzo[*a*]anthracene were optimized. The analysis of variance was conducted for specifying the interactions of independent variables. The independent and the dependent variables interactions were investigated. The quadratic regression model and the response surface contour plots were used to determine optimum values for the selected variables. The results of study revealed that optimum cooking time of smoked cattle meat was determined as 24.9 min, fat ratio 7.9% and the distance to the cooking source 21.8 cm. Under optimum conditions, minimum benzo[*a*]anthracene was formed in meat and its amount was determined using high performance liquid chromatography-mass spectrophotometer as qualitative and quantitative. The limit of detection and limit of quantitation

values were calculated as $0.4 \mu\text{g kg}^{-1}$ and $1.1 \mu\text{g kg}^{-1}$, respectively.

Keywords Benzo[*a*]anthracene · Box–Behnken design · Response surface methodology · High performance liquid chromatography-mass spectrophotometer · Smoked cattle meat

Introduction

Polycyclic aromatic hydrocarbons (PAHs) form a large class of organic compounds containing multiple benzene rings (Wenzl et al. 2006). They are chemically inert and hydrophobic (Luch 2005). These compounds are well known as immanent ecotoxicants because of their harmful effects to human health (Janoszka et al. 2004; Essumang et al. 2014). The effects of PAHs on health have recently been discussed extensively in various studies. Low IQ, low birth weight, growth retardation, the disruption of endocrine systems, small head circumference, deoxyribonucleic acid (DNA) damage in unborn children are some of the effects of PAHs. In addition, reproductive-related effects including early menopause owing to ova destruction have also been identified with PAHs (Shen et al. 2008; Essumang et al. 2011). The PAHs can undergo metabolic activation with diols and epoxides in mammalian cells. They can be covalently attached to cellular macromolecules. This interaction in DNA can lead to mutations and cancer-causing problems during the replication of DNA (Lightfoot et al. 2000). Generally, the PAHs taken in the body are converted to various epoxide form including phenols and diols, and they were metabolized in the form of conjugation such as glucuronic acid and glutation. At this stage, PAHs intermediate in the form of epoxide forms

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bind to DNA and they can be initiate tumor formation (Denissenko et al. 1996). Consequently, there is a serious concern about the possible PAHs contamination effect on human health especially for newborns and fetuses (Zanieri et al. 2007).

From past to present almost all over the world, smoked or roasted foods such as fish, chicken and meat products have been consumed. Food processing techniques, especially at high temperatures, are known major sources of PAHs contamination (Phillips and Grover 1994). The PAHs have a lipophilic effect in nature and usually they tend to accumulate in the organisms' fatty tissues. The PAHs are known to occur fat pyrolysis processes at temperatures above 200 °C; their levels considerably increase at temperatures over 700 °C (Es-sumang et al. 2012). Numerous researchers reported that PAH production depends on some variables such as fat ratio, cooking time and whether the cooking resource is indirect or direct during cooking process (Kazerouni et al. 2001; Martí-Cid et al. 2008; Stumpe-Viksna et al. 2008).

Almost all health organizations including United States Environmental Protection Agency (US EPA), Joint Expert Committee on Food Additives (JECFA), International Agency for Research on Cancer (IARC) have satated about 16 PAHs including benzo[*a*]anthracene as persistent organic pollutants (WHO 2005; IARC 2012). Since benzo[*a*]anthracene is emphasized as carcinogenic and genotoxic by the Scientific Committee on Food (SCF) along with many health organizations such as World Health Organization (WHO), IARC, US EPA and it is reported as mutagens and/or potential human carcinogens (Simon et al. 2007). For this reason, benzo[*a*]anthracene was preferred in this study.

Several countries and many environmental agencies have established a legal limit for PAHs. For example, Germany has adopted a legal limit for smoked foodstuffs as 25 µg kg⁻¹ for total PAHs (Moret and Conte 2000; Chen and Chen 2005). The Brazilian National Environment Council (CONAMA), limited a maximum concentration level of benzo[*a*]anthracene as 0.05 µg L⁻¹ in fresh water (Brum and Netto 2009). Spain has adopted a legal limit for PAHs content in various oils and they defined maximum tolerable PAHs such as benzo[*b*]fluoranthene and especially benzo[*a*]anthracene, amount in the oil as 5 µg kg⁻¹ (Lage Yusty and Cortizo Davina 2005). The maximum acceptable concentration level for most PAHs is usually 0.2 µg kg⁻¹ (Ferrer et al. 1996).

The aim of this research was to identify whether there is a relationship between meat cooking conditions and benzo[*a*]anthracene formation, and optimal cooking conditions for smoked cattle meat samples, also. There are few studies about the factors which influence the

PAHs level in foods. The response surface methodology (RSM) was used for these factors optimization. The RSM provides a mathematical model and reduces the number of experiments (Azmir et al. 2014). This model is a beneficial tool to optimize experimental conditions, and it can be successfully used to perform the multiple factors effects and their interactions (Li et al. 2015).

Materials and methods

Reagents and equipments

All used solvents in high performance liquid chromatography-mass spectrophotometer (HPLC–MS) were in analytical grade. They were purchased from Sigma-Aldrich (Stockholm-Sweden), Merck (Darmstadt, Germany) and Fluka (Stockholm-Sweden).

Water purification system (ELGA LabWater's PURE-LAB) was used to obtain ultrapure water. Benzo[*a*]anthracene stock solutions were prepared in acetonitrile. After diluting benzo[*a*]anthracene solutions every week, they were stored in refrigerator until used.

Benzo[*a*]anthracene concentrations were determined using an Agilent 1200 LC/MS (HPLC–MS system with quadropole (6110), Germany). This system consists of an autosampler, a temperature-controlled column oven, a binary pump and MS detector equipped with atmospheric pressure chemical ionization (APCI). A intersil ODS-3 (4.6 mm × 150 mm, 5 µm) column was used.

Single factor experiments

Two most common methods including the HPLC with fluorescence and mass spectrometry (MS) detection and gas chromatography (GC) with MS detection have been used for PAHs analysis. The HPLC–MS is the most preferred method among those because of higher sensitivity and reliability as well as its simplicity. The HPLC–MS–APCI consisting of several parameters including flow rate of mobile phase, sample injection volume, column temperature and fragmentor potential were optimized. Throughout the optimization process, mobile phase flow rate (75% of acetonitrile solution), sample injection volume, column temperature was applied in the range of 0.5–1.0 mL min⁻¹, 20–100 µL, 20–55 °C, respectively. In order to optimize fragmentor potential, positive ionization mode was used in the MS system for the measurements. Standard solution of benzo[*a*]anthracene was studied for the fragmentor potential alterations in the range of 50–200 V.

Sample preparation

Classical and conventional methods can not depict all factors combination which effect the experiment. At the same time, they take a lot of time to identify optimum levels of variables. Such a problem can be overcome by using a statistical experimental design, which is optimizing all the effecting parameters collectively. Because for modeling of process parameters RSM that contains a small number of experiments is widely used. Experimental design technique is suitable tool for developing, improving and optimizing processes and multi-factor experiments. It researches the common relationship between various factors for the most favorable conditions of the processes, which helps understanding the interactions among parameters that have been optimized. The primary target of Box–Behnken design is to detect the optimum operational conditions for the system or to detect a region that compensates the operating specifications. The cattle meat samples were prepared according to the experimental conditions and identified using Box–Behnken experimental design. Samples were cooked on wood coal taking into consideration the cooking time, fat ratio, and distance to cooking source. To extract the benzo[*a*]anthracene from these samples, 3 g of homogenized smoked meat sample was taken and shaken for 1 h after added 50 mL of 2 M potassium hydroxide in methanol/water (9:1, v/v). Mixture was rinsed 20 mL with n-hexane two times and 10 mL of ultra pure water was added and shaken. The obtained aqueous phase was extracted using n-hexane (10 mL). This process was repeated two times, and extracts were combined. Before passed through XAD-2 column, extracts were dried up to 5 mL. This column was eluted 75 mL of 9:1 (v/v) n-hexane/dichloromethane mixture. The obtained eluent was evaporated near to dryness by a rotary evaporator and residue was taken by using 1 mL of acetonitrile and analyzed (Ince et al. 2017).

Design of RSM and statistical analysis

The effects of independent variables which are cooking time (X_1), fat amount (X_2), and distance to cooking source (X_3), on formation of benzo[*a*]anthracene (Y) were determined according to single factor experiments.

To reduce unexplained variability effects on the observed response, randomized experimental order was carried out. Table 1 consists of different parameters including variable conditions, run order and it contains experimental values besides predicted values. A final equation was obtained from RSM in terms of actual factors was used,

$$Y = -1.39 + 1.18x_1 - 1.05x_2 - 0.19x_3 + 5.50E \\ - 0.03x_1x_2 - 0.03x_1x_3 + 4.25E - 0.03x_2x_3 - 0.02x_1^2 \\ + 0.05x_2^2 + 0.02x_3^2$$

To determine the lack of fit and other parameters including the effects of quadratic, linear, and interaction variables on formation of benzo[*a*]anthracene data were evaluated by analysis of variance (ANOVA). The RSM and data analyses were carried out using the Design Expert software program (Design Expert Version 10, Stat-Ease, USA).

Results and discussion

Optimization HPLC–MS–APCI conditions

Throughout the optimization of HPLC–MS–APCI parameters, characteristic of signals including peak symmetry, peak area and abundance were taken into consideration. Under these conditions, obtained chromatogram at 0.75 mL min^{−1} mobile phase flow rate was selected due to peak area and peak symmetry was the best. The obtained chromatograms to optimize sample injection volume range from 20 to 100 µL, the peak symmetry was found to be the best when 80 µL of benzo[*a*]anthracene solution was injected, and it was selected as the optimum injection volume. In order to obtain optimum column temperature, column temperatures were investigated ranging from 20 to 55 °C using benzo[*a*]anthracene solutions at the same concentration. Because of maximum abundance at 50 °C, it was selected as optimum point and this temperature was fixed for subsequent experiments. To optimize fragmentor potential using positive ionization mode, fragmentor potential was changed from 50 to 200 V in the APCI-mass spectrometer. When considering peak areas, 130 V fragmentor voltage was found to be optimum.

Analytical performance

Standard benzo[*a*]anthracene solutions in the range from 1 to 50 µg L^{−1} at the optimum conditions were used to obtain calibration graph. Obtained calibration graph was linear in this range and calibration curve (Fig. 1) equation was found as:

$$y = 595.46x + 803.04 \quad R^2 = 0.999$$

50 µg L^{−1} of benzo[*a*]anthracene solutions' MS spectrum was presented in Fig. 2. Limit of detection and limit of quantification of method were found using 3 s of blank and 10 s of blank, respectively. They were calculated as 0.4 µg kg^{−1} and 1.1 µg kg^{−1}, respectively.

Table 1 Box–Behnken design and observed responses of benzo[*a*]anthracene levels ($\mu\text{g kg}^{-1}$)

Run	Cooking time X_1 (min)	Fat amount X_2 (%)	Distance to cooking source X_3 (cm)	Benzo[<i>a</i>]anthracene levels ($\mu\text{g kg}^{-1}$)	
				Experimental values	Predicted values
1	30	15	25	2.9	2.0
2	10	15	5	3.3	4.2
3	10	25	15	13.4	12.4
4	20	5	5	7.7	7.9
5	30	5	15	3.3	3.4
6	20	25	25	15.3	15.2
7	20	25	5	19.3	19.3
8	20	15	15	5.8	5.8
9	20	15	15	5.8	5.8
10	20	5	25	5.1	6.1
11	30	15	5	9.9	10.6
12	30	25	15	16.4	9.6
13	20	15	15	5.8	5.8
14	10	5	15	2.3	0.7
15	20	15	15	5.8	5.8
16	10	15	25	6.4	6.5
17	20	15	15	5.8	5.8

Fig. 1 Benzo[*a*]anthracene calibration curve

Second-order polynomial model

The RSM with BBD and ANOVA results of benzo[*a*]anthracene formation in smoked cattle meat samples during cooking conditions were shown in Tables 1 and 2, respectively. The applied model is statistically significant ($p < 0.0001$) and there was only a 0.01% chance that the model F value was due to error. Since the model coefficient (R^2) was obtained as 0.9998, it can be said that 99.98% of the model-predicted values matched the experimental benzo[*a*]anthracene values. Le Man et al. (2010) reported that R^2 must be greater than 0.75 in order for the model to be adequate. Results of this study revealed, lack of fit was

Fig. 2 MS spectrum of benzo[*a*]anthracene standard solution

not significant ($p > 0.05$); that's why, number of experiments were sufficient to determine the effects of the independent variables for benzo[*a*]anthracene determination (Li et al. 2015). The results expressed that using statistical model was adequate to predict the benzo[*a*]anthracene levels and was fitted to second-order polynomial equation. The effects of X_1 , X_2 , X_3 , X_1X_2 , X_1X_3 , X_2X_3 , X_1^2 , X_2^2 and X_3^2 for formation benzo[*a*]anthracene was highly significant ($p < 0.01$). The quadratic and linear coefficients introduced that all factors play vital role in the response of benzo[*a*]anthracene formation in smoked meat samples.

Table 2 Analysis of variance (ANOVA) for the quadratic polynomial mode

Source	Sum of squares	df	Mean square	F value	P value* Prob F	
model	478.84	9	53.20	3310.52	< 0.0001	significant
X_1	5.95	1	5.95	370.30	< 0.0001	
X_2	303.81	1	303.81	18903.81	< 0.0001	
X_3	6.84	1	6.84	425.91	< 0.0001	
$X_1 X_2$	1.21	1	1.21	75.29	< 0.0001	
$X_1 X_3$	25.50	1	25.50	1586.82	< 0.0001	
$X_2 X_3$	0.72	1	0.72	44.96	0.0003	
X_1^2	16.84	1	16.84	1047.95	< 0.0001	
X_2^2	105.26	1	105.26	6549.71	< 0.0001	
X_3^2	14.02	1	14.02	872.58	< 0.0001	
Residual	0.11	7	0.016			
Lack of fit	0.11	3	0.037			
Pure error	0.000	4	0.000			
Cor total	478.96	16				
R^2	0.9998					
Adj. R^2	0.9995					
Pred R^2	0.9962					
Adeq Precision	174.456					

* $p < 0.01$ highly significant; $0.01 < p < 0.05$ significant; $p > 0.05$ not significant

RSM analysis

Two-dimensional (2D) contour plots and three-dimensional (3D) response surface plots were used as they are useful for determining reaction points including maximum, minimum, and middle point. Contour plots, which give the opportunity to determine the level of the variable and to contribute at the desired level, are useful, and the variables at the same level are drawn with an equal response curve. On account of these reasons, contour plots are easy to interpret. The fat ratio and cooking time have an effect on formation of benzo[a]anthracene in meat during smoked processes as presented in Fig. 3. Benzo[a]anthracene amount increased with increasing fat ratio until the fat percentage reaches to 15% and this contributed to the level of benzo[a]anthracene. Furthermore, the cooking time and the distance to the cooking source have significant effects on benzo[a]anthracene formation. In addition, the fat ratio is very important as the other parameters in the formation of benzo[a]anthracene. The effects of distance to cooking source and fat amount in the benzo[a]anthracene formation were shown in Fig. 4. Benzo[a]anthracene amount increases with increasing fat ratio until it reaches to 25%. This has contributed to increasing the benzo[a]anthracene level, as well as having significant effects of quadratic variables ($p < 0.01$).

Applications

Peak areas were converted to concentration values using linear calibration curve. The related results were given as the average of three replicates. In the smoked cattle meat samples, benzo[a]anthracene concentrations were found to be in the range from 2.3 to 19.3 $\mu\text{g kg}^{-1}$ under cooking conditions.

Purcaro et al. (2009) optimized a microwave assisted extraction method to determine PAHs in smoked meat samples. This method was carried out using different extraction times (5, 15, 30 min), extraction temperatures (60, 90, 115 °C) and sample amounts (1, 2, 3 g). The optimum conditions were determined as 2 g for sample amount, 115 °C for extraction temperature and 15 min for extraction time. After the determination of optimum extraction parameters, the extracted samples were analyzed by HPLC–MS and benzo[a]anthracene concentrations were found in the range of < 0.05–1.0 $\mu\text{g kg}^{-1}$ (wet weight) in smoked meat samples. In another study, by a new pressurized liquid extraction was used for PAH extraction in different smoked fish samples (Veyrand et al. 2007).

Martí-Cid et al. (2008) analyzed 16 PAHs in foodstuff widely consumed in Spain. The highest benzo[a]anthracene level was found as 3.6 $\mu\text{g kg}^{-1}$. In another study, benzo[a]anthracene concentration and 11 other PAHs were analyzed in large number of commercial cured meat products and 14 home-grilled meat samples in Estonia. In

Fig. 3 Response surface and contour plot for the formation of benzo[a]anthracene as a function of fat amount and cooking time

Fig. 4 Response surface and contour plot for the formation of benzo[a]anthracene as a function of distance to cooking source and fat amount

those meat samples, the average concentrations of benzo[a]anthracene were found to be $0.7 \mu\text{g kg}^{-1}$ in smoked meat, $1.3 \mu\text{g kg}^{-1}$ in smoked chicken, $0.5 \mu\text{g kg}^{-1}$ in grilled chicken and $1.1 \mu\text{g kg}^{-1}$ in grilled meat by HPLC with fluorescence detector (Reinik et al. 2007). Chen et al. (1996) reported that benzo[a]anthracene concentrations in grilled chicken as $31.8 \mu\text{g kg}^{-1}$, stewed

chicken liver as $26.6 \mu\text{g kg}^{-1}$, stewed chicken heart and grilled duck as $10.2 \mu\text{g kg}^{-1}$, stewed pork stomach as $2.6 \mu\text{g kg}^{-1}$, smoked pork as $2.8 \mu\text{g kg}^{-1}$, smoked Frankfurt sausage as $1.6 \mu\text{g kg}^{-1}$, while the levels were found below the detection limit in other three meat products. In addition, Stumpe-Viksna et al. (2008) figured out the effect of different species of wood on

benzo[*a*]anthracene concentration in smoked meat. They found minimum benzo[*a*]anthracene concentration by apple ($8.43 \mu\text{g kg}^{-1}$), while maximum level was obtained by spruce wood ($111.80 \mu\text{g kg}^{-1}$). As seen in the literature, the concentration of benzo[*a*]anthracene is highly variable even in the same food samples. The differences in these values may be due to various variables such as the meat type, cooking time, etc. As a result, further studies are needed using reliable and sensitive methods on benzo[*a*]anthracene determinations, especially in high temperature processed food samples. In the current study, HPLC–MS was used for qualitative and quantitative analysis since it is reliable, selective and sensitive method. In this study, benzo[*a*]anthracene concentrations were found to be from 2.3 to $19.3 \mu\text{g kg}^{-1}$. However, the levels observed in the optimum cooking conditions were found to be lower than the recommended concentration of $10 \mu\text{g kg}^{-1}$ by FAO/WHO.

Conclusion

By using BBD combining with RSM, ideal smoked cattle meat cooking conditions were identified and optimized. In the optimum conditions smoked meat results revealed that the experimental benzo[*a*]anthracene value was in accordance with the predicted value. The optimum cooking time of smoked meat was determined as 24.9 min, fat ratio was 7.9% and the distance to the cooking source was 21.8 cm. In addition, study describes a reliable and sensitive HPLC–MS analytical method for the determination of benzo[*a*]anthracene in smoked meat samples. The developed method can be applied to other food samples as well as other PAHs. When considered the benzo[*a*]anthracene toxicity and the widely consumption of grilled and smoked meat and meat products in world, the data reported about benzo[*a*]anthracene amount are important. These data deduced that during food cooking process, benzo[*a*]anthracene may be exceeded the permissible concentration. Consequently, efforts should be made to minimize the benzo[*a*]anthracene formation during the smoked process to protect human health from exposed to benzo[*a*]anthracene. Moreover, further work is necessary for traditional and home-made foods to get a more complete overview about benzo[*a*]anthracene level in various cooked or grilled meat products form.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest in the research.

Informed consent Informed consent was obtained from all individual participants included in the study.

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