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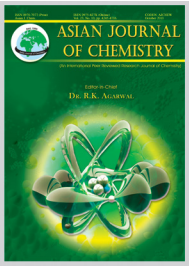

High Performance Liquid Chromatography-Mass Spectrometry for Determination of Benzo[*a*]pyrene in Grilled Meat Foods

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The concentrations and varieties of benzo[*a*]pyrene were determined in Turkish foodstuffs including grilled meat and chicken samples by high performance liquid chromatography-mass spectrometry (HPLC-MS). The parameters that are thought to affect the response in the HPLC-MS analysis were optimized. The optimized conditions were found to be 0.75 mL min^{-1} for flow rate of mobile phase, 45 µL for injection volume, 55 ºC for column temperature and 140 V for fragmentor potential. The optimized method was applied for the determination of benzo[*a*]pyrene concentrations in grilled meat and chicken foods. The obtained concentrations were found in the range of 2.7-8.3 µg kg^{-1} for the normal grilled meat and chicken samples and 72-138 µg kg^{-1} for over-grilled samples. The results showed that the concentrations of benzo[*a*]pyrene were significantly dependent on the kind of meat and the cooking time and source. The limits of detection and quantitation were found to be 0.7 and 2.33 µg L^{-1}, respectively.

Key Words: Benzo[*a*]pyrene, High performance liquid chromatography, Mass spectrometry, Grilled meat, Chicken, Food.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of over 100 different chemicals that are formed during the food-processing procedures such as frying, grilling and cooking due to incomplete burning of coal, fat and gas. The other factors such as the cooking temperature, time and materials, distance from the heat source and amount of fat also affect the PAHs formation in the grilled foods[1,2]. Data from animal studies indicate that PAHs may associate with various health effects and can even cause cancer[3]. As a result, U.S. Environmental Protection Agency (EPA) classified 16 of PAHs as priority pollutants and a number of individual PAHs are classified as probably (group 2A) or possibly (group 2B) carcinogenic to humans[4]. Benzo[*a*]pyrene (B[*a*]P) is involved in this priority group and it was established as a probable human carcinogen by International Agency for Research on Cancer (2004)[5]. Further, Benzo[*a*]pyrene seems to be a good marker for other PAHs in food samples[6]. The correlation coefficient between total PAHs and benzo[*a*]pyrene concentration has been reported[7] as 0.87 and between the carcinogenic PAHs and benzo[*a*]pyrene as 0.98. It is also known that benzo[*a*]pyrene can be formed commonly in foods such as charcoal-broiled meat, smoked or grilled foods, fats, oils, plant materials and seafoods, as a result of incomplete combustion of organic materials[3]. In 1987, Food and Agriculture Organization (FAO)/World Health Organization (WHO)-Expert Committee on Food Additives declared that the concentration of benzo[*a*]pyrene should not exceed 10 µg kg^{-1} in foods[4]. In 2006, benzo[*a*]pyrene was upgraded from group 2A (probable human carcinogens) to group 1 (known carcinogens in humans) by the International Agency for Research on Cancer (IARC)[8]. Maximum acceptable concentration of 5 µg kg^{-1} has also been established, in recent years, by European Union Commission in smoked meats and smoked meat products for benzo[*a*]pyrene[9]. Thus, the Scientific Committee on Food (SCF) of the European Commission requires further investigations of the levels of benzo[*a*]pyrene and other PAHs in foods, in particular, those highlighted to be carcinogenic.

The most common methods to measure PAHs are by high performance liquid chromatography (HPLC) with fluorescence[10] and mass spectrometry (MS)[11] detection and gas chromatography with mass spectrometry detection (GC-MS)[4,12,13]. However, highly selective and specific method is required for determination of benzo[*a*]pyrene because of the complexity of cooked food matrices, which contain numerous aromatic and heterocyclic aromatic compounds in addition to PAHs. Although variable excitation and emission wavelengths may be chosen for detection of different PAHs by HPLC with fluorescence detection, the method still lacks specificity and false

positive results may occur. High performance liquid chromatography-mass spectrometry (HPLC-MS) is the most popular method among those methods because of higher sensitivity and reliability than the others as well as its simplicity. Various extraction methods such as, solid phase microextraction[14], microwave assisted extraction[15] and solid-phase extraction with low-pressure have been used for separation in the determination of benzo[*a*]pyrene by HPLC-MS. The reagents such as isooctane[7], potassium hydroxide in methanol/water[16], hexane/acetone[17], *n*-hexane and dichloromethane[4,13,18] solutions were generally used in the extraction procedure. Purcaro *et al.*[15] used a rapid microwave assisted extraction to determine benzo[*a*]pyrene in meat by reversed phase-high performance liquid chromatography (RP-HPLC). Benzo[*a*]pyrene concentration was found to be lower than 0.05 μg kg^{-1} in smoked pork and beef meat, in their study. They reported that a slightly cleaner extract was produced by using hexane and extraction of polar interferents was minimized. As a result, there are requirement more studies on benzo[*a*]pyrene determinations in foods processed at high temperatures by using reliable, selective and sensitive method such as HPLC-MS.

In Turkey, grilled meats on charcoal fire are commonly consumed foods. Therefore, this study was focused on the determination of benzo[*a*]pyrene in Turkish grilled meats (namely, Adana kebab, Kusbasi and Doner) available in almost every restaurant in Turkey. The samples were extracted by KOH in methanol/H_2O (9/1; v/v) and hexane. To determine benzo[*a*]pyrene by HPLC-MS, the parameters such as flow rate of mobile phase, injection volume, column temperature and potential fragmentor were optimized. The optimized conditions were applied to the determination of benzo[*a*]pyrene at different levels in various grilled meat foods.

EXPERIMENTAL

Benzo[*a*]pyrene and acetonitrile were obtained from Fluka and Sigma-Aldrich (Stockholm-Sweden), respectively. Potassium hydroxide, hexane and dichloromethane were obtained from Merck (Darmstadt, Germany). Amberlite XAD-2 resin (Sigma, surface area: 330 m^2/g, pore diameter 90 Å) was used in cleaned up step. All of the solvents used were of HPLC grade and the other chemicals used were of analytical grade. Ultrapure distilled water (18.2 ohm^{-1}) was obtained from a Milli-Q water purification system (Millipore Direct-Q, France). The stock benzo[*a*]pyrene solutions were prepared in acetonitrile. Diluted solutions of benzo[*a*]pyrene were freshly prepared in every 2 weeks with acetonitrile and stored in refrigerator. All solutions were stored at 4 ºC in the dark for up to 2 weeks.

An Agilent 1200 HPLC-MS system (6110 Quadropole LC/MS, Germany) was used for the determinations. The HPLC-MS system consists of an autosampler, a binary pump, a temperature-controlled column oven that is coupled to an Agilent 1200 MS detector equipped with atmospheric pressure chemical ionization (APCI). The column used was Zorbax Eclipse XDB-C 18 (4.6 mm × 150 mm, 5 μm).

Optimization of HPLC-MS parameters: Before the measurements, flow rate of mobile phase, injection volume, column temperature and fragmentor potential were optimized. In each one of the studied parameters, other parameters were chosen at optimum value by using benzo[*a*]pyrene solutions of the 0.5 mg L^{-1}. For optimization of the flow rate of mobile phase, the flow rates were changed in the range of 0.5-1.0 mL min^{-1}. For optimization of injection volume, the volumes changed in the range of 5-100 μL were injected. For optimization of column temperature, the column temperatures were changed in the range of 25-60 ºC. In the MS system, the benzo[*a*]pyrene solutions were studied by changing the fragmentor potential in the range of 90-180 V for optimization of fragmentor potential. The positive ionization mode and selected ion monitoring (SIM) were chosen in the APCI-MS measurements. Other conditions were applied as follows; drying gas flow: 6.0 L min^{-1}, drying gas temperature: 300 ºC, nebulizer pressure: 60 psi, vaporizer temperature: 500 ºC. In the HPLC system, 75 % of acetonitrile solution was used as mobile phase. In the optimization studies, each experiment was repeated, at least three-times.

Preparation of samples: Grilled meat samples (namely, Adana kebab, Doner and Kusbasi) were purchased from local restaurants and then stored at 4 ºC. Twenty samples were prepared by cooking on charcoal fire or flame of butane gas. Furthermore, some samples were exceedingly grilled up to darkened. Extraction of samples was carried out using the method shown in Fig. 1. The grilled samples were thoroughly homogenized with a 600 W blender and stored in dark at 4 ºC until the analysis. A 3.0 g portion of each homogenized sample was taken into a 500 mL of round-bottom flask and then 50 mL of 2 mol L^{-1} KOH in methanol/H_2O (9:1, v/v) was added into sample to remove tissue fats and transform phenols to the polar non-extractable form of phenolates. The mixture was shaken for 1 h and rinsed twice with 20 mL of *n*-hexane. After adding 10 mL of distilled water, the slurry was shaken for 5 min and then transferred to separatory funnel. The mixture was allowed to stand for separation of aqueous and organic phases. After the separation, the aqueous phase was extracted twice with 10 mL of *n*-hexane and all the hexane extracts were combined. The hexane extract was evaporated to get less than 5 mL. This extract was cleaned-up by using Amberlite XAD-2 (polymeric adsorbent) column. (20 cm × 0.5 cm i.d.). The column was rinsed with 10 mL of acetone, dichloromethane and *n*-hexane, respectively, before using. The extract adsorbed onto XAD-2 resin was eluted with 75 mL of *n*-hexane/dichloromethane (9:1, v/v) mixture. The eluent was evaporated to near dryness on a rotary evaporator at 25-30 ºC. The residue was dissolved in 1.0 mL of acetonitrile. The solution obtained was analyzed by HPLC-MS using optimum conditions described above. The recovery experiments were conducted by spiking standard benzo[*a*]pyrene solution in over grilled meat at a level of 210 ng per 3 g to determine the reliability of the results. The prepared column as described above was reused up to 4-5 folds.

RESULTS AND DISCUSSION

Optimization of parameters: In the determination of optimum conditions, the peak area, peak symmetry and abundance on chromatogram were taken into consideration by using the positive ionization mode and APCI ionization source. Furthermore, each parameter was optimized by using

the other optimum conditions. Different flow rates of mobile phase were examined to determine optimum value. It was seen that the peak area was maximum for flow rate of mobile phase at 0.75 mL min^{-1} and the peak symmetry was the best at this flow rate. Thus, the flow rate of 0.75 mL min^{-1} was chosen as optimum. From the obtained chromatograms for benzo[*a*]pyrene by using different injection volumes (in ranges of 5-100 µL), the peak symmetry was found to be the best when 45 µL of benzo[*a*]pyrene solution was injected. From the chromatograms obtained to optimize sample injection volumes, the peak symmetry was found to be the best when 45 µL of benzo[*a*]pyrene solution was injected. Furthermore, it was observed that the peak symmetry was decomposed when the injection volume was increased more than 45 µL. Therefore, 45 µL was chosen as the optimum injection volume. From the obtained peak areas for optimization of column temperature, the temperature of 55 ºC was chosen as optimum because of maximum value in abundance at this temperature. As related with the optimization of fragmentor potential, it was found that peak areas were in range of 130-150 V. Due to obtaining maximum value in abundance at 140 V, this fragmentor potential was chosen as optimum. All optimum conditions found for HPLC-MS system were given in Table-1.

Analytical performance: Although calibration curves can vary considerably from day to day on the same MS instrument, the typical calibration graph can be useful to consider dynamic range at the optimized conditions. A calibration curve was obtained by using standard benzo[*a*]pyrene solutions in the range of 10-250 µg L^{-1} at the optimum conditions described above (Fig. 2). The calibration graph was linear in this range and the equation of calibration curve was found to be as follow:

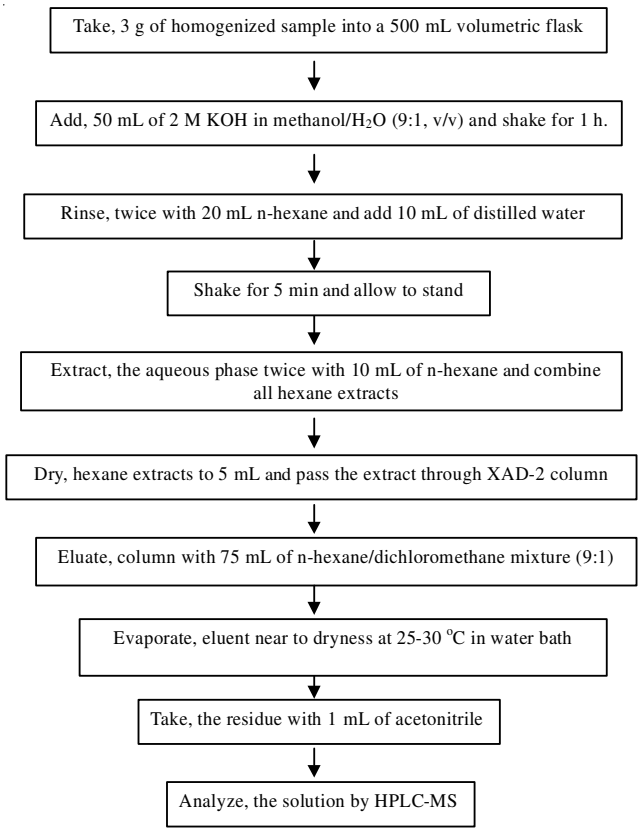

Fig. 1. Sample preparation steps for benzo[*a*]pyrene determination

TABLE-1
OPTIMUM CONDITIONS OF HPLC-MS PARAMETERS

Mobile phase	75 % Acetonitrile
Mobile phase flow rate	0.75 mL min^{-1}
Column temperature	55 ºC
Column	4.6 × 150 mm, 5 µm ZORBAX Eclipse XDB-C_{18}
Injection volume	45 µL
Fragmentor potential	140 V
Nebulizer (N) pressure	60 psi
Drying gas flow	6.0 L min^{-1}
Drying gas temperature	300 ºC
Vaporizer temperature	500 ºC
Capillary voltage	4000 V

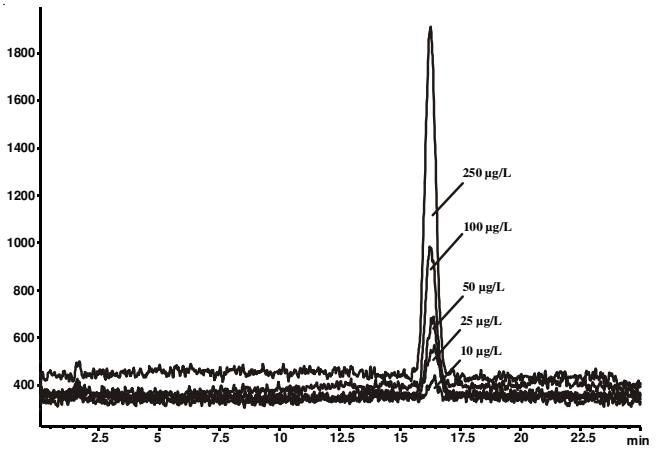

Fig. 2. Chromatograms of benzo[*a*]pyrene solutions in ranges of 10-250 µg L^{-1} at optimum conditions

$$y = 194.23 x - 1424; R^2 = 0.99$$ for 10-250 µg L^{-1} benzo[*a*]pyrene

The recoveries of benzo[*a*]pyrene from the studied samples fortified with this compound were examined to test the accuracy. Typically, the chomatograms obtained for benzo[*a*]pyrene in the sample of over grilled chicken by butane gas, in standard benzo[*a*]pyrene solution and in the sample plus standard benzo[*a*]pyrene solution were combined in Fig. 3. It was found that the recovery obtained was higher than 91 %. Furthermore, the standard additions method for the determination of benzo[*a*]pyrene in the studied samples was examined. The slope of the calibration curve was compared with the slope of the standard additions method. Because the slope of the calibration curve was identical with that of the standard additions method, it was concluded that external calibration method can be used for quantitative analysis in the further studies. Limit of detection (LOD) and limit of quantitation (LOQ) values of the method were calculated using 3s and 10s of blank, respectively. It was found that LOD and LOQ were 1.0 and 3.0 ng mL^{-1}, respectively. This LOQ value is equal to 1.0 ng g^{-1} taking into considering 3 g of sample and 1 mL of final solution. In all those studies, the results obtained were mean values of, at least, three different portions of the same sample.

Applications: The obtained SIM chromatograms of the grilled meat samples were evaluated. The observed values were transformed to concentrations by using calibration graph. The results obtained were given in Tables 2 and 3. Each of the samples was analyzed in triplicate and average of three values

was given as a mean value plus standard addition. So, each data in these Tables is the mean value of three separate portion of the same sample. From these Tables, benzo[*a*]pyrene concentrations in the grilled meat samples were found to be in the range of 2.7-8.1 μg kg^{-1} for normally grilled samples and 72-138 μg kg^{-1} for over-grilled samples, depending on differences in restaurant, kind of meat (cattle or chicken) and the cooking type of grilled sample (Adana kebab, kusbasi and doner). The maximum acceptable concentration (MAC) of 5.0 μg kg^{-1} for benzo[*a*]pyrene in smoked meat products[9] was exceeded in four normally grilled samples. The products with high benzo[*a*]pyrene levels in normally grilled meat samples were grilled cattle-three Adana kebab and one doner with concentrations of 6.5, 5.5, 8.3 and 5.5 μg kg^{-1}, respectively. Taking into considering the standard deviation of the values, only two samples among those exceed the maximum acceptable concentration level (5.0 μg kg^{-1}).

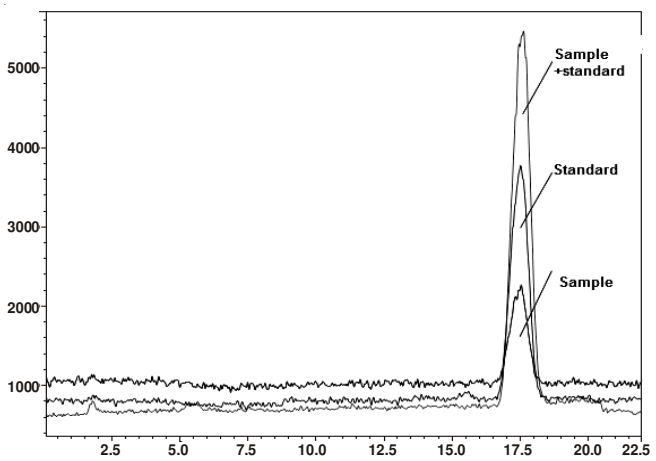

Fig. 3. Chromatograms of benzo[*a*]pyrene in the sample of over grilled chicken by butane gas, standard (10 μg L^{-1}) and sample plus standard (10 μg L^{-1})

TABLE-2
BENZO[*a*]PYRENE CONCENTRATIONS IN NORMAL GRILLED SAMPLES, μg kg^{-1}. THE RESULTS OBTAINED ARE MEAN VALUES OF, AT LEAST, FOUR DIFFERENT PORTIONS OF THE SAME SAMPLE

Sample, grill type	Benzo[*a*]pyrene ± s
Grilled cattle, Adana kebab 1	6.5 ± 1.1
Grilled cattle, Adana kebab 2	5.5 ± 1.0
Grilled cattle, Adana kebab 3	8.3 ± 1.9
Grilled cattle, Adana kebab 4	4.5 ± 1.0
Grilled cattle, Adana kebab 5	3.7 ± 0.7
Grilled cattle, Adana kebab 6	4.1 ± 0.8
Grilled cattle, doner 1	5.5 ± 1.1
Grilled cattle, doner 2	4.4 ± 1.2
Grilled cattle, doner 3	3.9 ± 1.0
Grilled cattle, doner 4	3.8 ± 1.0
Grilled cattle, kusbasi 1	2.7 ± 1.1
Grilled cattle, kusbasi 2	3.0 ± 0.7
Grilled cattle, kusbasi 3	3.5 ± 1.2
Grilled cattle, kusbasi 4	3.3 ± 1.0
Grilled chicken, kusbasi 1	4.6 ± 0.7
Grilled chicken, kusbasi 2	3.8 ± 0.9
Grilled chicken, kusbasi 3	2.7 ± 0.6
Grilled chicken, kusbasi 4	4.0 ± 0.8
Grilled chicken, kusbasi 5	3.5 ± 0.6
Grilled chicken, kusbasi 6	2.9 ± 0.4

TABLE-3
BENZO[*a*]PYRENE CONCENTRATIONS IN EXCEEDINGLY GRILLED SAMPLES UP TO DARKENED, μg kg^{-1}. THE RESULTS ARE MEAN VALUES OF, AT LEAST, THREE DIFFERENT PORTIONS OF THE SAME SAMPLE

Sample (cooking material)	Benzo[*a*]pyrene ± s
Grilled chicken (by butane gas)	138 ± 20
Grilled chicken (by charcoal)	72 ± 10
Grilled cattle, Doner (by butane gas)	96 ± 12
Grilled cattle, Adana (by charcoal)	84 ± 13

Benzo[*a*]pyrene concentrations in grilled meat samples on charcoal were examined by Aygun and Kabadayi[19] using HPLC with fluorescence detection. They found the benzo[*a*]pyrene concentrations in the grilled beef and lamb meats as 31.33 and 43.80 μg kg^{-1} for normally grilled meats and 37.60 and 62.60 μg kg^{-1} for over-grilled samples, respectively. High concentrations of benzo[*a*]pyrene in grilled meat and chicken samples can be attributed to fat dropped onto charcoal[20]. The reported benzo[*a*]pyrene concentrations of similar samples in literature were given in Table-4. Kazerouni *et al.*[7] examined benzo[*a*]pyrene levels in 200 food items by using HPLC. They found the highest levels of benzo[*a*]pyrene up to 4.8 μg kg^{-1} in grilled/barbecued very well done steaks, hamburgers and chicken with skin. In another study, the concentrations of benzo[*a*]pyrene and 11 other PAHs were analyzed from 322 commercial cured meat products and 14 home-grilled meat samples as part of the Estonian food safety monitoring program[1] during 2001-2005. In these meat samples, the average concentrations of benzo[*a*]pyrene were found to be 0.7 μg kg^{-1} in smoked meat, 1.3 μg kg^{-1} in smoked chicken, 0.5 μg kg^{-1} in grilled chicken and 1.1 μg kg^{-1} in grilled meat by HPLC with fluorescence detection[1]. Lee and Shim[21] found the highest level of benzo[*a*]pyrene in the surface part of fried chicken (5.55 μg kg^{-1}), followed by sliced dried beef (5.47 μg kg^{-1}), the inside part of fried chicken (5.225 μg kg^{-1}) and smoked chicken (4.35-4.64 μg kg^{-1}). In addition, Stumpe-Viksna and coworkers[2] figured out the effect of different species of wood on benzo[*a*]pyrene concentration in smoked meat. They found minimum benzo[*a*]pyrene concentrations by charcoal (10 μg kg^{-1}) and alder (9.4 μg kg^{-1}), while maximum level was obtained by aspen wood (35 μg kg^{-1}). Among these studies, the highest reported concentrations of benzo[*a*]pyrene have been reported in foods cooked over open flames[19]. The differences in benzo[*a*]pyrene levels in grilled meat samples[1-4,7,10-22] may be attributed from measurement methods as well as other factors during the smoking and grilling process such as type of wood and smoke generator, grilling or smoking temperature, duration of smoking or grilling, availability of oxygen and oven dimensions. Briefly, there are large variable values in the concentrations of benzo[*a*]pyrene even for the same food samples. This large variation at data can be attributed to the meat type (cattle or chicken), the cooking type (frying, Adana, Doner, Shish Kebab and similar) and the differences in restaurant as a result of grilling time and kind of charcoal.

In this study, benzo[*a*]pyrene concentrations in normally grilled meat samples using charcoal were found to be in the range of 3.7-8.3 μg kg^{-1} for grilled cattle (Adana kebab), 3.7-5.5 μg kg^{-1} for grilled cattle (doner), 2.7-3.5 μg kg^{-1} for grilled cattle (kusbasi) and 2.7-4.6 μg kg^{-1} for grilled chicken (chicken

kusbasi). The results found for normally grilled meat samples were found to be lower than the results reported by Aygun and Kabadayi[19] for Turkish grilled beef meat (31.33 μg kg^{-1}). The values for all normally grilled chicken and grilled cattle samples (kusbasi) were found to be lower than the maximum acceptable benzo[*a*]pyrene levels established by European Commission[9]. Moreover, the levels observed were found to be lower than limit concentration of 10 μg kg^{-1} suggested by FAO/WHO although higher values were obtained with over-grilled (up to darkened) samples. On the other hand, benzo[*a*] pyrene concentrations in over-grilled meat samples (up to darkened) were found to be 138 μg kg^{-1} for grilled chicken by using butane gas, 72 μg kg^{-1} for grilled chicken by using charcoal, 96 μg kg^{-1} for grilled cattle using butane gas and 84 μg kg^{-1} for grilled cattle by using charcoal. As a result, the maximum acceptable concentration of 5 μg kg^{-1} for benzo[*a*] pyrene in smoked meat products[9] was exceeded in all over-grilled (up to darkened) chicken and cattle meats.

TABLE-4
REPORTED B(*a*)P CONCENTRATIONS IN GRILLED AND SMOKED MEAT SAMPLES, (μg kg^{-1})

Sample	B(*a*)P	Reference
Normally grilled beef and lamb	31.33-43.80	Aygun and Kabadayi[19]
Over grilled beef and lamb	37.60-62.60	
200 food items, including Grilled/barbecued steaks, hamburger and chicken with skin	Up to 4.8	Kazerouni *et al.*[7]
Grilled lamb meat	0.32-2.81	Mottier *et al.*[22]
Total food	1.28	Marti-cid *et al.*[3]
Estonian home-grilled meat products, during 2001-2005	0.3-1.8	Reinik *et al.*[1]
Grilled chicken	4.6	Chen *et al.*[4]
Fried chicken	5.23-5.55	Lee and Shim[21]
Smoked portk and beef meat	< 0.05	Purcaro *et al.*[15]

Conclusion

This study describes a reliable and sensitive HPLC-MS analytical method for the determination of benzo[*a*]pyrene in protein-rich food samples such as grilled cattle and chicken meats. In the measurement steps, all of the parameters were optimized to obtain good S/N ratio for benzo[*a*]pyrene. The optimized conditions were found to be 0.75 mL min^{-1} for flow rate of mobile phase, 45 μL for injection volume, 55 ºC for column temperature and 140 V for fragmentor potential. The method may be also applied for determination of benzo[*a*]pyrene in other foods. Considering the toxicity of benzo[*a*]pyrene and the widely consumption of grilled and smoked meat products in Turkey, the data reported on the levels of carcinogenic benzo[*a*]pyrene in food is clearly important. These data suggest that during the grilling or cooking process of foods, benzo[*a*]pyrene may be exceeded the permissible concentration. Consequently, efforts should be made to minimize the formation of benzo[*a*]pyrene during the grilling process to protect human health from exposure to benzo[*a*]pyrene, a known human carcinogen. Again, further work is necessary in order to get a more complete overview about benzo[*a*]pyrene contents in other kind of grilled or cooked meat products from traditional and home-made foods.

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