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Determination of benzo[a]pyrene levels in smoked and oil fried *Lates niloticus*

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds included in priority pollutant lists because of their mutagenic and carcinogenic properties. Several studies have shown that exposure to benzo[a]pyrene (BaP), a member of the PAHs increases the risk of cancer. This study investigated the effects of firewood smoking and oil frying on the BaP levels in Nile perch (*Lates niloticus*) sold in Western Kenya. The methodology involved BaP extraction with cyclohexane and dimethylformamide-water, clean up on silica gel column and determination by high performance liquid chromatography (HPLC) using fluorescence detection. Variable levels of BaP were detected ranging from 7.46 to 18.79 µg/kg in smoked fish and 4.17 to 11.26 µg/kg in oil fried fish. These levels were further compared with the regulatory limits. All smoked fish samples were found to exceed the acceptable Maximum residual limit (MRL) of 5 µg/kg while 20% of the oil fried samples were within the acceptable limit. BaP was not detected in raw fish samples analysed. It was concluded that firewood smoking as practiced in the study areas resulted in higher levels of BaP contamination compared to oil frying.

Introduction

Benzo[a]pyrene (BaP) is a member of polycyclic aromatic hydrocarbons (PAHs) which are a large group of organic compounds with mutagenic and carcinogenic properties (Ramalhosa *et al*., 2009). Benzo[a]pyrene is the only polycyclic aromatic hydrocarbon with sufficient toxicological evidence to allow the setting of a guideline (Moret *et al*., 2005; Yusty and Davina, 2005).

Most individuals are predominantly exposed to PAHs from dietary sources (Bordajandi *et al*., 2008). Polycyclic aromatic hydrocarbons PAHs are bio-available to marine species via the food chain, as water borne compounds and contaminated sediments. Due to being lipophilic compounds, they can easily cross lipid membranes and have the potential to bio-accumulate in aquatic organisms. Despite fish and sea food representing only a small part of the total diet in most human diets, their contribution to the daily intake of PAHs in some individuals may be comparatively important (Domingo *et al*., 2007).

Contamination of foodstuffs by PAHs can occur at source through atmospheric deposition on crops (Culotta *et al*., 2002), or from preservation of food by drying and cooking procedures. This contamination is enhanced during smoking and intense thermal processing (Chen and Chen, 2001). During intense thermal processing the contamination occurs by direct pyrolysis of food nutrients (Orecchio and Papuzza, 2008). The PAHs are also deposited from smoke produced through incomplete combustion of different thermal agents. There has been considerable evidence which indicated that meat processing by frying, boiling, barbecuing, roasting among others, produces traces of compounds with mutagenic or carcinogenic potential (Oosterveld *et al*., 2003).

In western Kenya, smoked fish is a common source of protein in the diets of many households (Bille and Shemkai, 2006). Artisanal fish processors (AFPs) prepare dried and smoked fish mostly for local market. Industrial fish processors (IFPs) freeze or chill fish for export and to a lesser extent, for consumption in Kenya's urban areas (Abila, 2003).

The maximum acceptable level for benzo[α] pyrene for the European Union market in smoked fish is 5 µg/kg, (EC, 2005). Safety of food is a growing concern worldwide and PAHs residues if present in food above the maximum residual limit (MRL) pose a serious threat to the public health. In Kenya there is no national program for routine monitoring of benzo[A]pyrene in foods and there are no standards

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set for these residues in smoked and oil fried fish. This study investigated the presence of benzo[a] pyrene in smoked and oil fried fish sold in Western Kenya. It aimed to determine the residual levels of benzo[a]pyrene levels in smoked and oil fried *Lates niloticus*.

Materials and Methods

Samples

The study site was done on the shorelines of Lake Victoria in Nyanza and Western provinces of Kenya (geographic coordinates Latitude: 0° 06' South Longitude: 34° 45' East). Ten fish markets were randomly selected and from each market ten fish dealers identified randomly. From the selected fish dealers, five samples of raw (500 g each), smoked and oil fried samples each weighing 200 g were sourced and placed in clean, sterilized containers. Four kilograms of freshly landed *Lates niloticus* control samples were bought from an aquaculture farm which acted as a control. The control samples were washed at the sampling site and packed in perforated polythene bags and loaded into an ice box filled with crushed ice.

The sampling was repeated in triplicate to ensure homogeneity and representation of the fish process practice. All samples were refrigerated at 4^{o}C before analysis. Twenty-five grams of flesh was extracted from each fish sample and analyzed. All reagents used were of analytical grade sourced from Sigma Chemical Company (St. Louis, MO, USA).

Benzo[a]pyrene standard

Benzo[a]pyrene standard with a purity of 99.9% was sourced from Sigma Ltd through their agent Kobian Scientific in Kenya. Stock solutions containing 100 mg/L and 100µg/l of BaP were prepared and stored at 4^{o}C in volumetric glass flasks and wrapped in aluminum foil to avoid possible light degradation. Benzo[a]pyrene standards were prepared by dilution of the stock solution.

Benzo[a]pyrene extraction

A homogenized portion of 25 g of each smoked or oil fried fish sample was heated (100^{0}C) for 4 hours under reflux with 50 ml of a 2% solution of potassium hydroxide in methanol. The saponified materials were transferred into 250 ml separating funnels. The flasks were rinsed with 100ml methanol/ distilled water (1:1, v/v). The mixtures were then individually extracted twice for 2 min with 100 ml of cyclohexane. The organic layer was washed, first with 50 ml methanol/water (1:1, v/v) and finally with 100ml distilled water. The organic layer was then transferred into a 250 ml round bottom flask. The volume of the samples was reduced to 50 ml at 40ºC using a vacuum rotary evaporator. The cyclohexane was extracted with three aliquots of N,N-dimethylformamide–water (5:1, v/v) (50, 25 and 25 ml). The combined dimethyl-formamide extract was diluted with 100ml of a 1% sodium sulfate solution and re-extracted with aliquots of 50, 35 and 35 ml of cyclohexane. The combined solution was washed twice with 40ml distilled water, dried with anhydrous sodium sulfate (5 g) and concentrated on a rotary evaporator to 5ml at 40ºC.

Post- extraction cleanup

The concentrated 5ml extracts were purified by column chromatography on silica gel, as described by Hossain *et al*. (2009). In brief, a clean up column with an internal diameter of 1cm (i. d.=1cm) was filled with cotton in the bottom. An activated silica gel (17 g) soaked with dichloromethane was then loaded into the clean up column (5cm), which was thereafter topped with 1.5 cm of anhydrous sodium sulphate. A volume of 5 ml of dichloromethane was added to wash the sodium sulphate and the silica gel. The dried 1ml sample was then transferred into the glass column packed with deactivated silica gel; the vessel was rinsed twice with 2 ml dichloromethane, which was also added to the glass column. A volume of 50 ml of dichloromethane was then added to the glass column and allowed to flow through the column at a rate of 5 ml/minute, and the eluent was then collected. The collected eluent from the clean-up procedure was re-concentrated further to 0.5 ml with a K-D concentrator.

HPLC analysis

The analysis for BaP in the fish samples was carried out using a Waters® HPLC apparatus equipped with a Model 600 controller pump, an in-line degasser, a Model 717 plus auto-sampler, a Model 474 fluorescence detector with an excitation wavelength of 290nm and emission wavelength of 430nm and a Millennium 32 data processor. For separation, a C18 column stable at 30ºC was used. The mobile phase consisted of 75% acetonitrile and 25% water at a flow rate of 1ml/minute. The injection volume used was 30 µl. The signal due to benzo[a]pyrene was identified by comparison of sample chromatograms with the chromatogram of the benzo[a]pyrene standard.

BaP quantification

The external standard plot method was used. Duplicate injections of 0.2 µl-12.8 µl BaP standard

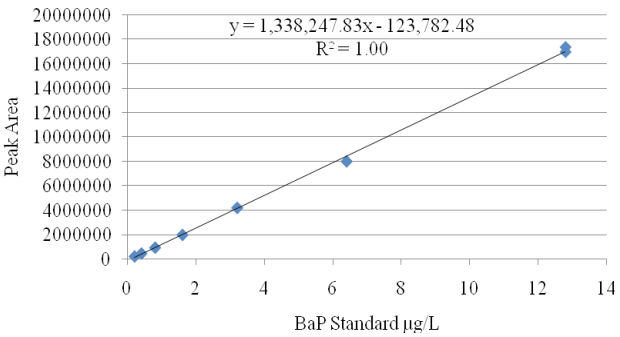

Figure 1. External standard plot

solutions were used to construct linear regression lines which are peak area ratios versus BaP concentration. The response was highly linear (Figure 1). Conversion of these values to µg of BaP per kg of sample was done by division of the results obtained by the mass of the sample analysed.

Recovery study

In order to verify the accuracy and precision of the analytical procedure, recovery experiments were carried out by spiking the smoked and oil fried samples (n=6) (n= number of samples spiked) with three different concentrations of the BaP standards ranging from 0.1 to 5 µg/l. The spiked samples as well as the unspiked controls were analysed in triplicate using the HPLC. Recoveries were calculated from the differences in total amounts of BaP between the spiked and the unspiked samples. The repeatability of the method was evaluated through the coefficients of variation (CV) associated to measurements of the BaP performed during the recovery tests.

Data analysis

The data from the three sampling times were pooled and the mean values for BaP levels in smoked, oil fried and fresh fish were analyzed for each of the treatments. The means of BaP levels of the selected smoked, oil-fried and fresh fish at each sampling occasion were subjected to analysis of variance (ANOVA). The least significance difference (LSD) procedure was used to test for the difference between the treatments means with significance being defined at $p \leq 0.05$. The correlations between the different parameters in the study were calculated using SPSS version 15 (SPSS Inc., Chicago, Illinois).

Results

The average recovery for benzo[α]pyrene was 95% with a standard deviation of 1.3% and a coefficient of variation of 4.23 as shown in Table 1. BaP was identified by comparison of HPLC retention times of the benzo[α]pyrene standard and those of spiked or unspiked, smoked and oil-fried fish samples. The BaP standard and sample chromatographs obtained from HPLC are as shown in Figure 2, 3 and 4. The BaP standard chromatogram with a retention time of 27.01 min and an instrument response of 100mv which gave a peak area of 214654

Table 1. Detection limits and mean recovery levels of BaP in spiked fish sample

PAH	R^a (%)±STDEV	CV (%)	Retention Time (min)
BaP	95±1.3	4.23	27.01

R, mean recovery, [a] Average of three different concentrations, CV, coefficient of variation, STDEV, standard deviation

Table 2. Mean BaP concentration in smoked fish at different sampling occasions

MARKETS	BaP levels (µg/kg) in smoked fish samples		
	1st Occasion	2nd Occasion	3rd Occasion
Asat	10.65±0.01[a]	7.22±0.47[a]	14.14±1.20[b]
Luanda Kotieno	15.89±0.41[a]	17.22±0.12[a]	17.05±0.05[a]
Usenge	24.91±1.01[a]	12.08±0.19[b]	16.15±0.15[b]
Uyoga	19.36±0.11[a]	19.04±0.08[a]	17.98±0.10[a]
Kamagogo	14.45±0.36[a]	16.01±0.33[a]	16.05±0.91[a]
Marenga	12.81±0.41[a]	12.05±0.71[a]	13.21±0.64[a]
Bukama	19.08±0.58[a]	18.41±0.04[b]	18.75±0.75[b]
Osieko	12.25±2.01[a]	15.16±0.01[b]	14.48±0.51[b]
Busoma	7.1±0.22[a]	8.01±0.26[a]	7.28±0.31[a]
Sialala	7.51±0.41[a]	11.84±0.20[b]	10.39±3.11[b]

Mean values in the same row with the same superscript are not significantly different (p>0.05)

Table 3. Mean BaP concentration in oil fried fish at different sampling occasions

MARKETS	BaP levels (µg/kg) in oil fried fish samples		
	1st Occasion	2nd Occasion	3rd Occasion
Asat	5.38±0.10[a]	4.22±0.12[b]	6.01±0.31[a]
Luanda Kotieno	5.45±0.05[a]	3.20±0.20[b]	3.90±0.50[b]
Usenge	5.98±1.02[a]	5.05±0.12[a]	5.47±0.30[a]
Uyoga	6.01±0.15[a]	4.81±0.61[b]	4.99±0.24[b]
Kamagogo	12.4±0.06[a]	10.50±0.31[b]	10.88±0.55[b]
Marenga	4.09±0.10[a]	4.31±1.24[a]	4.11±0.35[a]
Bukama	8.58±0.01[a]	7.98±0.44[b]	8.20±0.22[a]
Osieko	7.61±0.04[a]	8.21±0.05[a]	7.74±0.26[a]
Busoma	6.23±0.00[a]	4.05±0.10[b]	5.02±0.05[b]
Sialala	6.70±0.30[a]	8.55±0.47[b]	8.14±0.63[b]

Mean values in the same row with the same superscript are not significantly different (p>0.05)

Table 4. Mean BaP concentration (µg/kg) in smoked and oil fried *Lates niloticus* samples

Sampled Markets	Mean BaP concentration in smoked fish (µg/kg)±STDEV	BaP concentration in oil fried fish (µg/kg)±STDEV
Asat	10.67 ± 3.46[a]	5.2 ± 0.91[b]
Luanda Kotieno	16.72 ± 0.72[c]	4.18 ± 1.15[b]
Usenge	13.71 ± 6.56[a]	5.5 ± 0.47[b]
Uyoga	18.79 ± 0.72[c]	5.27 ± 0.65[b]
Kamagogo	15.5 ± 0.91[c]	11.26 ± 1.01[a]
Marenga	14.69 ± 0.59[c]	4.17 ± 0.12[b]
Bukama	18.75 ± 0.34[c]	8.25 ± 0.30[d]
Osieko	13.96 ± 1.5[a]	7.85 ± 0.32[d]
Busoma	7.46 ± 0.48[d]	5.10 ± 1.09[b]
Sialala	9.91 ± 2.2[d]	7.78 ± 0.97[d]

BaP, benzo[A]pyrene, STDEV, standard deviation Mean values with the same superscript are not significantly different (p>0.05)

Table 5. Frequency distribution (%) of BaP concentration in analyzed samples

BaP (µg/kg)	Smoked fish samples	Oil fried fish samples
<5	0	20
5-10	20	70
>10	80	10

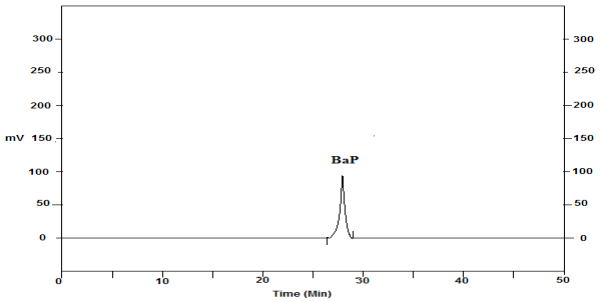

Figure 2. BaP standard chromatogram

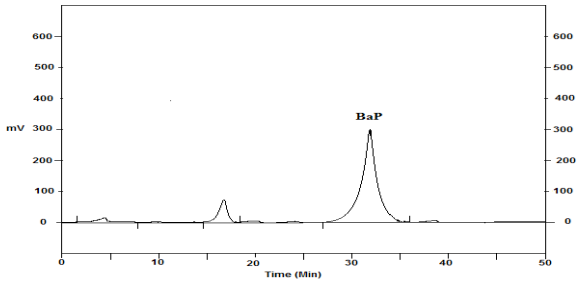

Figure 3. BaP fraction isolated from smoked *Lates niloticus*

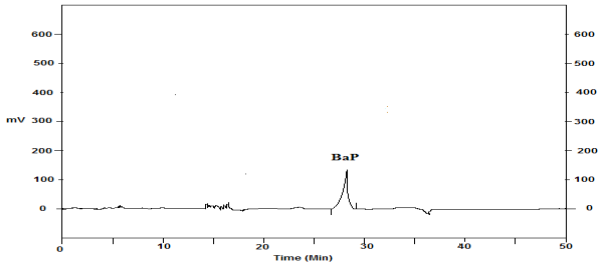

Figure 4. BaP fraction isolated from oil fried *Lates niloticus*

was obtained from Figure 2. No other peaks were observed in this chromatograph since it represented a chromatogram of BaP standard in pure solution. Figure 3 and 4 indicate the chromatograms from the smoked and oil fried fish samples, respectively. In these two chromatographs several other peaks which represented unidentified PAHs with different retention times were observed. BaP in raw fish samples from the lake and from the farm was not detected. Figure 3 which represents BaP concentration in smoked fish had the highest instrument response of (280 mV) and hence the largest peak area for BaP.

The means and standard deviation for BaP levels in smoked and oil fried fish during the three sampling occasions were as indicated in Table 2 and Table 3, respectively. Benzo[A]pyrene concentration in smoked Lates niloticus ranged from 7.46 to 18.79 µg/kg among the sampled markets as shown in Table 4. The least BaP concentration was recorded in smoked fish samples from Busoma while the highest was recorded in samples from Uyoga. There was a significant difference ($p<0.05$) in the mean level of BaP in smoked fish samples across the sampled markets.

The difference in BaP concentration in oil fried fish among the sampled markets was not statistically significant ($p>0.05$). The levels of BaP in oil fried fish samples ranged from 4.17 to 11.26 µg/kg, the highest value was reported in fish samples from Kamagogo while the least was reported in fish samples from Marenga. These levels were significantly lower as compared to the levels in the smoked fish samples as presented in Table 4. The difference between smoked and oil fried groups was significant ($p<0.05$)

All the smoked fish samples (90) examined in this study had BaP levels which exceeded the acceptable limit of 5 µg/kg as specified by the European Commission (E.C, 2005). Twenty percent (18 samples) of the oil fried fish samples had BaP levels within the acceptable limit set by the European Commission (Table 5).

Discussion

Smoked fish constitute an important and significant part of the human diet. Nutritionally this is due to their desirable sensory properties, high nutritional value and abundance of lipids rich in n-3 fatty acid residues. The findings from the present study indicate that the wood smoke used in smoking of fish contributes to contamination of the fish with BaP.

This study recorded an average recovery of 95% for BaP which is much higher than the 66.5% recovery for BaP analysis in meat samples reported by Janoszka *et al*. (2004). Moreover, Wu *et al*. (1997) also reported a lower recovery (75%). The value fulfills the performance criteria for methods of analysis for BaP, according to which the recovery for this compound should be (EFSA, 2008). The higher recovery could be due to the the integrator on and off times of the HPLC equipment. In this study this was set at between 0 and 40 min resulting in the elimination of integrator peaks. These peaks are undesirable since the retention time of the analyte had been determined from the injection of the BaP standard. Setting too large a range could have resulted in a lot of extraneous information while setting too small a range could have led to the possibility of incomplete integration due to slight shifts in analyte retention times.

The variation in the levels of BaP observed among sampled smoked fish from the selected markets could be attributed to the different processing, differences in the type of wood used for smoking or even differences in construction of smoking kilns. Concentrations of BaP in home prepared meat dishes are known to depend on many factors such as the method of

thermal treatment, the type of heat source, cooking time and even fat contents in the meat (Kazerouni *et al*., 2001). The findings from this study indicate that levels of BaP in smoked fish come from the wood smoke findings to work done elsewhere (Stolyhwo and Sikorski (2005). This was satisfactorily confirmed in this study. However, BaP was not detected in raw fish samples from both the lake and the farm in this study.

BaP in the oil fried fish samples could have originated from the over head dispersion of smoke from the wood used as fuel as well as the oil used for frying. The lower levels observed in oil fried fish sample could be due to diffusion of BaP from the fish fractions, which could reduce the level of BaP in fish.

BaP concentration of charcoal cooked meat samples has been observed to be much higher than gas cooked meat (Kazerouni *et al*., 2001; Anderson *et al*., 2002). However, Rivera *et al*. (1996) detected BaP concentrations of 4 to 19 µg/kg in charcoal grilled meat. The levels of BaP in different types of smoked foods has been observed to differ between species (Gomaa *et al*. (1993). According to Dennis *et al*. (1984), the concentration of BaP detected by HPLC-FL in grilled sausage was 0.4 µg/kg, in grilled pork chops 8.2 µg/kg, smoked herring 8.5 µg/kg, smoked ham 0.2 µg/kg and in hard grilled sausage 191 µg/kg. Binnemann (1979) found BaP concentrations of 0.6 to 100 µg/kg in sausages and special products while Terzi et al. (2008) found mean levels of BaP to be 24 µg/kg for charcoal fire cooked meat samples and 5.7 µg/kg gas fire cooked meat samples.

BaP formation is largely determined by the cooking method and degree to which the meat is cooked (Kazerouni *et al*., 2001). PAH compounds tend to form on or near the surface of the meat rather than in the interior. These compounds are generated through pyrolysis during the charcoal broiling of meat and when fat from the meat falls onto the hot coals. Significant levels of PAH will be produced during when meat is cooked directly on charcoal or wood fuel (Lijinsky, 1991; Wu *et al*., 1997; Phillips, 1999; Kazerouni *et al*., 2001).

Conclusion

Firewood smoking as practiced in the study areas resulted in higher levels of BaP contamination compared to oil frying. The levels of BaP in smoked and oil fried fish determined in this study indicate that the BaP levels are above the maximum residual levels recommended and pose a health risk to consumers.

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