International Journal of Food Science and Nutrition
ISSN: 2455-4898, Impact Factor: RJIF 5.14
www.foodsciencejournal.com
Volume 2; Issue 4; July 2017; Page No. 83-87

Investigation of quality in fish produced by traditional processing methods in Sri Lanka

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Abstract
Concentration of residual Polycyclic Aromatic Hydrocarbons (PAHs) in fish samples processed by traditional cooking methods specially using wood smoking, in rural areas of Sri Lanka were investigated with the aim of comparing these levels with European Union standard maximum level. Furthermore, Moisture content, Water activity and Fat contents were also determined to evaluatequality of smoked fish. The methodology involved PAHs extraction using Soxhlet extraction, clean up on silica gel column and determination by high performance liquid chromatography. The sum of Benzo(a)pyrene, Benz(a)anthracene, Benzo(b)fluoranthene and Chrysene obtained for smoked fish samples were higher than the standard levels (12.0 μgKg^{-1}). Smoked fish obtained from the traditional smoking methods in Sri Lanka were of good organoleptic and keeping qualities (moisture content < 65% and water activity <0.97) smoking as practiced in the study areas resulted in higher levels of PAHs contamination and poses a health risk to consumers.

Keywords: high performance liquid chromatography, smoked fish, polycyclic aromatic hydrocarbons, water activity, moisture content

1. Introduction

Fish products mainly are a major source of animal protein in the Sri Lankan diet [1] as well as of vitamin and minerals. Fish is an extremely perishable food which becomes inedible within twelve hours at tropical temperatures.

Smoking of food products can be identified as one of the oldest food preservation technology which also used in fish processing. Smoking has become a method of offering diversified, high value added products for certain fish species where fresh consumption is not possible [2].

Smoking is a physical process which based on diffusion, absorption, dissolution and deposition in force fields. It is accompanied by chemical processes where in smoke compounds interact with food compounds [3].Wood smoke contains a number of compounds formed by the pyrolysis of wood constituents. Many of these smoke compounds can be found in smoked foods. Among them phenols, carbonyls, furans, alcohols and esters, lactones and polycyclic aromatic hydrocarbons (PAH) are the most important classes of chemical compounds [4].

In food, PAH may be accumulatethroughout processing anddomestic food preparation, such as smoking, drying, roasting, baking, frying or grilling. Moreover Pyrolysis of the fats in the meat and fish generates PAH that become deposited on fish and meat. PAH production by cooking over charcoal (barbecued, grilled) is a function ofboth the fat content of the meat/fish and the proximity of the food to the heat source [5] [6].

Amongst hundreds of PAH, the most considered compound is Benzo (a) pyrene, which is often used as anmarker for PAH in ambientair and food [7].

Mostof these PAHs have been found to be carcinogenic while some are not. However, analysis and monitoring have generally focused on BaP, or some selected or all 16 PAHs highlighted [8].Nevertheless, the 16 EPA priority PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k] flouranthene, benzo-[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i] pe-rylene, and indeno[1,2,3-c,d]pyrene) is often targeted for measurement in environmental samples.

The analysis of PAHs content in sample matrix consist of different extraction methods, concentration of extraction, post-extraction clean-up and instrumental analysis in order to obtain precise information on the extent of contamination in the sample matrix.

Water is an important basic element in foods. The terms moisture content and water content are often used interchangeably and represent a measure of the quantity of water in a product. A good understanding of water, and in particular water activity (aw), can assist in developing robust and scientifically supported food safety plans.

Mainly free water in products is responsible for the growth of undesirable organism such as bacteria or fungi, which produce “toxins” or other harmful substances and also for chemical/biochemical reactions (e.g. the Maillard reaction) gradually take place and probably change the certain factors of a product such as microbiological stability, chemical stability, Content of proteins and vitamins, Colour, taste and nutritional value. Furthermore for the stability of the compound and durability, Storage and packing, Solubility and texture of the food product [9].

Moisture content gives information about texture of a certain food product since increasing levels of moisture provide water mobility and lower the glass transition temperature. There are certain reasons to define the moisture content in food such as, storability, agglomeration in the case of powders, microbiological stability, flow properties, viscosity, dry substance content, concentration or purity, commercial grade (compliance with quality agreements), nutritional value of the product, legal conformity (statutory regulations governing

food)…etc. To determine moisture content in a food product, chemical, thermo gravimetric or loss on drying practices can be used.

Water activity represents the energy status of the water in the system. It is equal to the relative humidity of the air in equilibrium with a sample in a sealed chamber. It is defined as the vapour pressure of water in a sample divided by the vapour pressure of pure water at the sample temperature. It provides information regarding the possibility of microbiological growth on the surface. Moreover it provides valuable information about microbial spoilage, chemical stability, and physical stability. Water activity and moisture content together provide a complete moisture analysis [10].

The water activity determines the storage life of fish. Smoking decreases the water activity in fish tissue[11].Moreover the fat content of fish species could be important factors to storage. Also, the species of fish used or fat content could have been responsible for the presence of moulds

2. Materials and Methods

2.1 Sample collection

Smoked fish species namely Rohu (*L. rohita*) and Catla (*Catlacatla*) were obtained from Ampara and Smoked fish samples of Nile tilapia (Tilapia) (Oreochromis*niloticus)* was obtained from Randenigala in Sri Lanka. All the samples were packed in dark polyethylene bags and were transported in covered boxes.

2.2 Moisture content determination

For this AOAC (2000) method [12] was used. Cleaned porcelain crucibles were initially kept in the drying oven at 105ºC for 1 hour and initial weights were taken after cooling in a desiccator. Then 10.0 g of the sample was weighed into the crucible and again kept in the drying oven at 105ºC for 5 hours until it obtained a constant weight. Then the dry weight was recorded.

2.3 Water activity determination

Water activity of smoked fish samples was measured using the water activity meter. (NOVASINA Aw meter)

2.4 Determination of fat content (Bligh and dyer method)

2.4.1 Extraction of oil

The fish sample was chopped and mixed thoroughly. Then 10.0 g of the material was placed in a beaker. To this beaker 30mL of methanol (Analytical Reagent, purchased from Sigma Aldrich, USA) and 15mL of chloroform (Analytical Reagent, purchased fromSisico research laboratories (PVT) Ltd, India) was added and mixed well for 2 minutes using a homogenizer. Additional 15mL chloroform was added to the same mixture and again mixed for 30 seconds using a homogenizer. Finally 15mL of water was add to the beaker and mixed for 30 seconds.

Next the content of the flask was transferred to a centrifuge tube and was centrifuged (K-241) for 15 minutes at 3000 rpm. When the centrifugation process is completed the top layer which containing water and methanol was removed. Then the phase of solids was loosen with a spatula and filtered the solid mixture to a 50ml volumetric flask through a filter paper (whatman 110mm). Finally the volumetric flask was filled up to the mark using chloroform.

2.4.2 Fat content determination

A 25mL beaker was placed in a drying oven at 105ºC for 1hour and it was cooled in a desiccator for 20 minutes. The initial weight of the beaker was taken. Then 10 mL of the prepared mixture was pipetted out to the beaker. Beaker with the extract was placed in a fume cupboard until the chloroform has evaporated. Next the beaker was dried in a drying oven at 105ºC for 1 hour and again the weight of the beaker was taken. Calculations were done according to Eq (1).

Calculation

$$\text{Fat percentage} = \frac{g_0 \times 50 \times 100}{V \times g_s} \quad (1)$$

g_0 = Grams of oil (difference between empty beaker and beaker with dried sample)

g_s = Weight of fish sample /g

V = Volume of extract in mL brought to evaporation

2.5 Sample preparationfor PAH extraction

From the composite smoked sample of head, bones and removable skin removed fish, Fifteen grams (15.0g) was homogenised in a mortar and pestle with 15 g of anhydrous sodium sulphate (Na_2SO_4)(Analytical Reagent, 99.4% purity, purchased fromlobachemie (PVT). Ltd, India). When completely dry homogenate was obtained, it was carefully transferred to the cellulose thimble.

2.6 Extraction of PAHs (Soxhlet extraction)

A Soxhlet apparatus with 500 ml round bottom flask, condenser, an extraction chamber and water circulators were attached in temperature maintained heating mantel for the fat extraction.The sample containing extraction thimble was positioned in the extraction chamber of the Soxhlet apparatus.

For the Soxhlet extraction 200ml of dichloromethane (Analytical Reagent, 99.5% purity, purchased from Lobachemie (PVT). Ltd, India) and about 2ml of iso-octane (as the keeper) (Analytical Reagent, 99.5% purity, purchased from Merck specialties (PVT), Mumbai, India) was used in the round bottom flask. An average of 4 cycles per hour was used for the solvent circulation cycle. This extraction was carried out for 16 hrs.

After the soxhlet extraction the extract was cooled to room temperature. Then a methanol-KOH mixture was prepared in 100ml volumetric flask by dissolving 6.0g KOH pellets (Analytical Reagent, 86.1% purity, purchased from Sisico research laboratories (PVT) Ltd, India) in 12 ml distilled water and making up to the mark with methanol(HPLC grade, 99.8% purity, purchased from Sigma Aldrich, USA). This mixture was added to the round bottom flask of the soxhlet extraction apparatus and then it was refluxed for 1.5 hours at 60º C using an apparatus contained the round bottom flask and a condenser.

2.7 Separation of aqueous layer

The extract was initially cooled to the room temperature. Then aqueous layer was separated from the extract using 50 ml of distilled water using a seperatory funnel. The organic layer was washed twice with 50 ml distilled water to remove all remaining stearate. The extract was concentrated at a temperature of 45 ^{0}C using Rotavapor R - 124 until it obtained a volume about 5 mL.

The extract was taken to a 5mL vial and more concentrated to about 1 mL using a stream of an inert nitrogen gas in a sample concentrator MD-200-1

2.8 Post extraction clean-up

Silica gel column was prepared using 10 g of activated silica gel (mesh size 60- 120, purchased from Sisico research laboratories (PVT) Ltd, India) into a chromatographic column of 1cm internal diameter. About 1g of anhydrous sodium sulphate was added to the top of the column. The bottom end of the silica gel column was plugged with cotton wool. The packed silica column was pre conditioned with 20mL (1:3 v/v) dichloromethane (HPLC grade, 99.8% purity, purchased from Lobachemie (PVT). Ltd, India): distilled hexane mixture (Analytical grade, purchased from Sigma Aldrich, USA). Then the concentrated extract was loaded into a packed silica gel column. To remove n-hydrocarbons and darkest parts of the sample it was initially eluted with 20mL distilled hexane. Next the sample was eluted with 60 ml of dichloromethane (HPLC grade): distilled hexane (1:3 v/v) mixture.

The collected mobile phase was then concentrated to about 1.5 mL using Rotavapor R-124 at the temperature of 45 ^{0}C. The extract was taken to a 1.5mL vial and stream of an inert nitrogen gas was flushed to the near dryness of the sample in a sample concentrator MD-200-1.

2.9 HPLC analysis

PAH standard (PAH Mix 3 in methylene chloride: methanol (1:1) (varied), analytical standard) was used for the PAH determination. The standard was prepared by diluting 25µL of stock solution into 250 µL with methylene chloride: methanol (1:1).

For the analysis sample was dissolved in 1 mL of filtered acetonitrile (HPLC grade, 99.8% purity, purchased from Sigma Aldrich, USA)and then sample was filtered through 0.45 µm PTFE filter unit using a syringe.

HPLC analysis was performed using an Agilent 1100 – Infinity liquid chromatographic system (Agilent Technologies, Waldbronn, Germany) equipped with an Agilent 1200 diode array detector. A ZORBAX Eclipse PAH column (Agilent Technologies, USA) (4.6 x 100 mm x 5 µm particle size) maintained at room temperature was used in analysis.

The mobile phase consisted of distilled water (A) and acetonitrile (B). The total running time was 30 min with a flow rate of 2 mL min^{-1}. The elution gradient began using 60% A / 40% B and this solvent composition was continued for 20 min. From 20 to 25 min the solvent B was increased to 100% and this composition (0% A / 100% B) was continued for 20 to 25 min. From 25 to 27 min, solvent A was increased to 60% and this composition (60% A and 40% B) was continued for 27 to 30 min. Sample (10 µL) was injected into the HPLC system. PAH standard and PAHs in purified extracts of smoked fish were detected at a wavelength of 254 nm.

2.10 Statistical Analysis

Analysis of variance (ANOVA) for the confidence interval of 95% were performed to estimate the significance of difference between PAHs in smoked fish samples of raw and cooked using Minitab 17.0

3. Results and discussion

3.1 Water activity of smoked fish samples

Table 1: Water activity of smoked fish samples. Data represents mean ± SD, (n=3).

Fish Sample	Water activity ± SD
Sample 1	0.893 ± 0.005
Sample 2	0.9065 ± 0.004
Sample 3	0.891 ± 0.015
Sample 4	0.9105 ± 0.001
Sample 5	0.924 ± 0.005
Sample 6	0.9035 ± 0.006
Sample 7	0.928 ± 0.006
Sample 8	0.911 ± 0.011
Sample 9	0.9015 ± 0.011
Sample 10	0.874 ± 0.005

Considering all the smoked fish samples analyzed for water activity, all the values obtained were ranged between 0.87- 0.92. Which are below the critical level of 0.97for the formation of botulinum toxin [13].

Moreover the values obtained for smoked *Oreochromisniloticus* fish species ranged between 0.87-0.91(Sample 1 to sample 4), values for smoked *C. catla*species were between 0.89-0.91 (Sample 5 to Sample 7) and the values for water activity of smoked *L.rohita*species were ranged between 0.90-0.92(Sample 8 to Sample 10). (Table 1)

According to ANOVA (Analysis of Variances) done at 95 % confidence interval there is no significance difference between recorded water activity results for all the samples from three species.

Generally there is an inverse correlation of water activitywith NaClconcentration (salt content). Where the preservative effects are ascribed to the Salt when decreasing water activity [14] [15].

3.2 Moisture content of smoked fish samples

The values obtained for the moisture content of smoked fish samples were ranged between 24.47 ±0.09 to 51.06 ±0.94.

For *C. catla*species, the values ranged between 26.23 ±0.36 to 42.81 ±0.14, for *L.rohita* samples it is 36.64 ±0.14 to 48.34 ±0.10 and for *Oreochromisniloticus*the values are between 51.06 ±0.94 to 24.47 ±0.09. In table 2, sample 1 to sample 4 represents *C. catla*, sample 5 o sample 7 *L.rohita* and sample 8 to sample 10 represents *Oreochromisniloticus* smoked fish species. (Table 2)

According to ANOVA (Analysis of Variances) done at 95 % confidence interval there is no significance difference ($P > 0.05$) between recorded moisture contents for all the samples.

Moreover Cardinal *et al*. (2001) [15] Smoke cured fish products should be <65 percent, for the purpose of product preservation and organoleptic consequence, industrial specifications for moisture content in the flesh [16].

Table 2: Moisture content of smoked fish samples, Data represents mean ± SD (n=3)

Fish Sample	Moisture content ± SD
Sample 1	26.23 ±0.36
Sample 2	36.65 ±0.12
Sample 3	26.54 ±0.50
Sample 4	42.81 ±0.14
Sample 5	47.90 ±0.49
Sample 6	36.64 ±0.14
Sample 7	48.34 ±0.10
Sample 8	51.06 ±0.94
Sample 9	48.32 ±0.98
Sample 10	24.47 ±0.09

Table 3: Average oil percentage of smoked fish samples, Data represents mean ± SD (n=3)

Fish Sample	Oil content ± SD
Sample 1	8.98 ±0.36
Sample 2	12.46 ±0.28
Sample 3	12.41±0.29
Sample 4	9.77 ±0.48
Sample 5	5.72 ±0.36
Sample 6	5.98 ±0.29
Sample 7	5.44 ±0.21
Sample 8	5.24 ±0.36
Sample 9	5.92 ±0.42
Sample 10	5.28 ±0.31

3.3 Fat content of smoked fish samples

Fat content (%) of smoked fish samples were ranged between 5.24 ±0.36 to 12.46 ±0.28. For *C. catla*species, the values ranged between8.98 ±0.36to 12.46 ±0.28, for *L.rohita* samples it is 5.44 ±0.21 to 5.98 ±0.29 and for *Oreochromisniloticus*the values were between 5.24 ±0.36to 5.92 ±0.31. In table 3 sample 1 to sample 4 represents *C. catla*, sample 5 o sample 7 *L.rohita* and sample 8 to sample 10 represents *Oreochromisniloticus* smoked fish species. (Table 3)

3.4 PAHs content in smoked fish samples

In this study the levels of Benzo (a) pyrene found in smoked fish concentration were ranging from 35.3 to 1489.63 µg/ kg. All the smoked fish samples and cooked fish samples showed higher levels of Benzo (a) pyrene, than the recommended maximumallowable concentration of 2.0 µg/ kg fixed for Benzo (a) pyrene in smoked meat, fish and smoked meat and fishery products [17].

Table 4: PAH levels (µg/kg) in smoked fish (Mean ± SD)

Compound	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
Naphthalene	120.33	172.12	ND	178.77	900.82	438.71	ND	ND	103.09	4.77
Acenaphthyelene	598.12	ND	198.15	ND	ND	319.97	ND	ND	ND	ND
Acenaphthene	27.71	ND	26.74	8.48	ND	112.43	ND	430.94	129.34	ND
Fluorene	89.80	ND	60.08	ND	751.48	34.25	275.15	ND	623.45	ND
Phenanthrene	ND	53.03	ND	49.09	540.18	273.93	49.69	31.32	197.41	52.27
Anthracene	11.84	ND	ND	ND	254.38	52.25	ND	54.31	ND	60.53
Fluoranthene	45.685	35.91	ND	50.12	ND	ND	14.09	ND	ND	86.73
Pyrene	21.87	22.51	89.59	ND	ND	34.69	ND	62.69	83.98	ND
Chrysene	ND	ND	6.83	5.19	ND	56.62	ND	ND	ND	ND
Benz[a]anthracene	ND	14.95	61.32	ND	ND	41.19	9.39	29.13	19.16	ND
Benzo[a]pyrene	458.78	1489.63	ND	50.67	ND	35.30	453.39	414.09	1055.21	ND
Benzo[k]fluoranthene	ND	ND	149.5	ND	ND	2030.76	521.08	ND	146.14	ND
Benzo[b]fluoranthene	ND	1202.485	149	ND	276.12	ND	ND	ND	ND	ND
Indeno[1,2,3-cd]pyrene	105.77	14.39	142.3	9.74	451.18	221.96	146.79	ND	7.89	ND
Benzo[g,h,i]perylene	ND	ND	ND	ND	ND	31.87	ND	ND	ND	ND
Dibenz[a,h]anthracene	ND	ND	ND	ND	ND	ND	ND	ND	ND	7.82
Total	1479.89	3005.03	883.52	352.06	3174.16	3683.94	1469.61	1022.48	2262.54	310.51

The different smoked fish species had different PAH levels contribution from the smoking process. This could be attributed to the differences in fat and moisture contents and the nature of skin cover [18].

More recently researches reported Benzo (a) pyrene at levels ranging from 2.4 to 31.2 µg/kg wet weights smoked fish and meat samples [19].

Furthermore, variable levels of BaP were detected ranging from 7.46 to 18.79 µg/kg in smoked fish [20].

According to commission regulation (EU) no 835/2011, the maximum levels fish and smoked fishery products 5. 0 µg/ Kg until 31.8.2014 2.0 µg/ Kg as from 1.9.2014. Furthermore the maximum level for Sum of Benzo (a) pyrene, Benz(a)anthracene, Benzo(b)fluoranthene and Chrysene is 30.0 µg/kg as from 1.9.2012 until 31.8.2014 12.0 µg/Kg as from 1.9.2014.

The newest classification on carcinogenicity of PAHs by the International Agency for Research on Cancer paper, it has been established that Benzo[a]pyrene is a definite carcinogenic (group 1), whereas Benzo[a]anthracene, Chrysene, Benzo[b]fluoranthene, and are classified as possible human carcinogens (group 2B).

According to this major four PAHs levels in smoked fish, all thesamples experimented consists at least twodefinite carcinogenic and possible human carcinogenic PAHs. Highest amount of carcinogens were detected in *C.catla*species.

The lowest sum of Benzo (a) pyrene, Benz(a)anthracene, Benzo(b)fluoranthene and Chrysene obtained for smoked fish samples are 55.86 and highest sum is 2706.60 µg/Kg. These concentrations are higher than the standard levels for the sum of Benzo(a)pyrene, Benzo(a)anthracene, Benzo(b)fluoranthene and Chrysene (12.0 µg/Kg) according to commission regulations (EU) 2014.

An ANOVA analysis conducted at 95% confidence interval shown a statistically significant difference ($P < 0.05$) in toxic PAH levels between smoked fish samples of three species.

In the above table 4, sample 1 to sample 4 represents *C. catla*, sample 5 to sample 7 *L.rohita* and sample 8 to sample 10 represents *Oreochromisniloticus* smoked fish species.

4. Conclusion

Polycyclic Aromatic Hydrocarbon contamination occurs in higher levels in smoked fishdue to the traditional smoking method whichused by Sri Lankan fisher community. The different smoked fish species from different areas had different PAH levels accumulate from the smoking process. As this can cause adverse health effects on consumers much improvements on traditional smoking is needed.

The water activity, moisture content and fat content records obtained from all the species were below the critical levels of contamination. Therefore these smoked fish obtained from traditional methods can be recommended as in good organoleptic quality though further research is required.

Furthermore therewas nosignificant difference in moisture content values of three species (L.rohita, C.catla and *Oreochromisniloticus)*. ($P>0.05$)

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