DETERMINATION OF BENZO[A]PYRENE LEVELS AND ESTABLISHMENT OF LIMIT OF DETECTION IN SMOKED AND OIL FRIED *Lates niloticus*

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A Thesis Submitted to the Graduate School in Partial Fulfillment for the Requirements of the Award of Masters of Science Degree in Biochemistry of Egerton University

EGERTON UNIVERSITY

MAY, 2012

DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and has not been presented in any other university for a degree award.

Signed……Date……

Mr. Bramuel Muyela

RECOMMENDATION

This thesis has been submitted for examination with our approval as the candidate's supervisors as per the university regulations.

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DEDICATION

This thesis is dedicated to my loving parents Aggrey and Carolyn Muyera and my brothers Clifford and Douglas.

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I wish to express my sincere gratitude to individuals and institutions that made this MSc. research work possible. I wish to thank the Department of Food Science and Technology, Egerton University and the Kenya Bureau of Standards for providing me with the necessary laboratory facilities and equipment to do this work. The Ministry of Fisheries Development, Western region and Kisumu for their assistance in collection of samples. I extend my sincere gratitude to my supervisors Prof Anakalo A. Shitandi and Prof Raphael M. Ngure for their continued support and guidance in planning, conducting and finalizing this study. I extend my heartfelt and sincere thanks to Dr. Michael N. I. Lokuruka for making it possible for me to acquire the necessary resources to do this work. Most of all, I would like to express my gratitude to my Parents, fiancé Thecla, brothers and friends for their relentless support and encouragement throughout the entire course of this study. To God I say thank you.

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds included in the European Union and US Environmental Protection Agency (USEPA) priority pollutant list 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. PAHs have been the subject of much concern in the recent years due to their toxic potential. They are known as highly stable contaminants of foodstuffs and are found frequently in the environment, water and fish. Maximum residue limit (MRL) has been set by the European Commission at 5µg/kg in fish. However, despite the wide use of smoking and deep frying of fish in western Kenya there is limited information on the BaP residues in fish. This study investigated the effects of firewood smoking and oil frying on the BaP levels in Nile perch (*Lates niloticus*) sold in western Kenya. Sampling was done three times in ten randomly selected markets. 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 18.79µg/kg in smoked fish and 4.17 to 11.26µg/kg in oil fried fish. These levels were further compared with the regulatory limits. All smoked fish samples were found to be over the acceptable limit/ MRL of 5µg/kg set by the European Commission while only 20% of the oil fried samples were within the acceptable limit. The limit of detection (LOD) was established at 0.011µg/kg and defined as the smallest amount of BaP that was reliably detected from the background of the HPLC equipment. At the same time these findings indicate that firewood smoking resulted in higher levels of BaP contamination as compared to oil frying. The study also concluded that consumers of these products are exposed to levels that are above the MRL and hence a health risk.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BaP	Benzo[A]pyrene
EC	European Commission
CAC	Codex Alimentarius Commission
HPLC	High performance liquid chromatography
IPCS	International Programme on Chemical Safety
LOD	Limit of detection
LSD	Least significant difference
MCLs	Maximum contamination levels
MDL	Minimum detection limit
MRLs	Maximum residual levels
PAHs	Polycyclic aromatic hydrocarbons
USEPA	United States Environmental Protection Agency

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds included in the European Union and United States Environmental Protection Agency (USEPA) priority pollutant list because of their mutagenic and carcinogenic properties (Ramalhosa *et al.*, 2009). Benzo[A]pyrene is a member of the polycyclic aromatic hydrocarbons (PAHs). It is the only polycyclic aromatic hydrocarbon for which there is sufficient toxicological evidence to allow the setting of a guideline and is recognized to be carcinogenic (Moret *et al*., 2005; Yusty and Davina, 2005).

Except smokers and occupationally vulnerable populations, most individuals are exposed to PAHs predominantly from dietary sources (Bordajandi *et al.*, 2008). In the marine environment, PAHs are bio-available to marine species via the food chain, as water borne compounds and contaminated sediments. As lipophilic compounds they can easily cross lipid membranes and have the potential to bio-accumulate in aquatic organisms. Although for most people, fish and sea food represents only a small part of the total diet, the contribution of this food group to the daily intake of PAHs in some individuals may be comparatively important (Domingo *et al.*, 2007).

Hundreds of individual PAHs may be produced during the incomplete pyrolysis or combustion of organic matter linked to both natural events such as forest fires and volcanic emissions and anthropogenic activities such as industrial processes, fuel combustion, incineration of both wood and urban waste and domestic heating (IPCS, 1998; Codex Alimentarius Commission, 2004).

The formation of deoxyribonucleic acid-adducts by electrophilic metabolites is generally regarded as one of the earliest steps in PAH carcinogenesis. Adduct formation follows the prior conversion of the lipophilic parent PAH to nucleophilic reactive metabolites by xenobiotic metabolizing enzymes, notably cytochrome P_{450} dependent mono-oxygenases. Genetic differences between humans with regard to enzymatic formation as well as enzymatic degradation of these active metabolites has been shown to correlate with adduct formation (Luch and Glatt, 2004).

Traces of PAHs have been detected in many foods (Bartoszek, 2002; EFSA, 2007) including vegetable oils, fruits, sea food (Gianguzza and Santino, 2006), grilled and roasted

meat, smoked fish, tea and coffee (Simko, 2002). In particular, benzo[A]pyrene has been found in these samples at concentration levels between 0.1 and 100mg/kg and hence pose a health risk to consumers (Rey-Salgueiro *et al*., 2008). Contamination of foodstuffs by PAHs can occur at source such as by atmospheric deposition on crops (Culotta *et al*., 2002), or results from preservation of food by drying and cooking procedures and particularly during smoking and intense thermal processing (Chen and Chen, 2001; Orecchio and Papuzza, 2008). Contamination of PAHs by intense thermal processing occur due to generation by direct pyrolysis of food nutrients and also due to direct deposition of PAHs from smoke produced through incomplete combustion of different thermal agents. There has been considerable evidence that meat processing by frying, boiling, barbecuing, roasting among others, produces traces of compounds with mutagenic or carcinogenic potential (Oosterveld *et al*., 2003).

Smoking has been used for centuries as a means for food preservation and is still used widely for this purpose. In the developing world up to 70% of the total fish catch is preserved by smoking (Ward, 1995). The process of smoking requires the penetration of food products by smoke resulting from thermal destruction of wood (Maga, 1998). This increases the shelf life of food products because of the combined effects of heat and reduced water activity during the smoking process. These effects cause microbial destruction, thereby minimizing spoilage (Hintlian and Hothckiss, 1986).

In Kenya, smoked fish is a common and cheap source of protein in the diet component of most households (Bille and Shemkai, 2006). Artisanal fish processors (AFPs) prepare dried and smoked fish mostly for local market, while 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[A]pyrene in smoked fish is 5µg/kg wet weight as set by Commission regulation, for the European union market (EU, 2005). However, there is no maximum residual level set by Kenya Bureau of Standards. This study investigated the presence of benzo[A]pyrene in smoked and oil fried fish sold in western Kenya. It was therefore aimed at establishing the safety of smoked and oil fried fish with regard to benzo[A]pyrene. It also established a data base on the quantity of benzo[A]pyrene in smoked and oil fried fish.

1.2 Statement of the Problem

Polycyclic aromatic hydrocarbons are widespread environmental pollutants. Benzo[A]pyrene is an example of these polycyclic aromatic hydrocarbons and is known to be

both mutagenic and carcinogenic and is therefore of great public health concern. In Kenya, consumers are not aware of the presence of these residues in food products. There is no published data on the residual levels of benzo[A]pyrene on food products and furthermore no safety assessment of food items is done in Kenya with regard to benzo[A]pyrene to ensure consumer protection. As a result no food safety policy has been designed to establish the systems for control and interventions to ensure compliance with maximum residual levels set for these residues.

1.3 Objectives

1.3.1 Main objective

To determine the presence and levels of benzo[A]pyrene in smoked and oil fried *Lates niloticus* sold in western Kenya.

1.3.2 Specific objectives

1. To determine the amounts of benzo[A]pyrene residues in smoked and oil fried fish and whether consumers are exposed to levels above the MRL
2. To establish a limit of detection for benzo[A]pyrene residues in smoked and oil fried fish using the HPLC technique.

1.4 Justification

Safety of food is a growing concern worldwide. PAHs residues if present in food above the MRL pose a serious threat to the public health. Smoked fish is a common source of protein in western Kenya. This food product has been associated with PAHs especially benzo[A]pyrene. In Kenya there is no national program for routine monitoring of benzo[A]pyrene in foods, furthermore there are no standards set for these residues in smoked and oil fried fish. Determination of the residual levels of benzo[A]pyrene in smoked and oil fried fish will help to develop strategies that reduce the actual and potential risk to public health and establish systems for controls and interventions to ensure compliance with the recommended levels of benzo[A]pyrene in smoked and oil fried fish. These findings will further aid in safety assessment of food items with regard to benzo[A]pyrene needed to ensure consumer protection.

1.5 Expected Outputs

1. A basis for putting in place a national programme for routine monitoring of PAHs and development of strategies for the control of these contaminants.
2. A Master of Science degree in Biochemistry.
3. Publication in refereed scientific journals

CHAPTER TWO

LITERATURE REVIEW

2.1 PAH in Foods

Polycyclic aromatic hydrocarbons are persistent chemicals with two or more fused aromatic rings. PAHs are ubiquitous environmental pollutants that have been identified world wide in various matrices, such as water, soil or dust particles. PAHs have also been found as contaminants in various foods, such as dairy products, vegetables, fruits, oils and roasted or smoked meat (Simko, 2002; Camargo and Toledo, 2003; Lin and Zhu, 2005).

The movement of PAHs in the environment depends on their properties. PAHs easily evaporate and disperse into the air and are deposited in soil and thus accumulate in sediments and biota because of their lipophilic properties. For the most part, PAHs enter the environment and then the food chain, through the release from forest fires, residential wood burning or from geochemical processes, such as volcanism or petroleum seepage (IPCS, 1998). Moreover, they can enter water courses through industrial discharges and waste water treatment plants and can be found in soils as a result of their escape from storage containers close to hazardous waste sites (FAO/WHO, 2005).

Concentration of PAHs in home prepared meat dishes depends on many factors, such as the method of thermal treatment, the type of heat source, cooking time and fat content in the meat (Chen and Lin, 1997; Kazerouni *et al*., 2001; EFSA, 2008). In a study by Kazerouni *et al* (2001) highest quantities of PAHs were formed in meat and meat products barbecued on a grill over an open charcoal of wood chips flame. Investigations on carcinogenic compounds from heterocyclic amines group have shown that it is possible to decrease their concentrations in food by applying synthetic and natural antioxidants and vegetables containing antioxidants as food additives (Vitaglione and Fogliano, 2004). However, there are also reports about the enhancing effect of some synthetic and natural antioxidants or spices on heterocyclic amines formation (Johansson and Jagerstad, 1996; Vitaglione and Fogliano, 2004).

PAHs can also contaminate foods during heating and drying processes that allow combustion products to come into direct contact with food. The International Agency for Research on Cancer (IARC, 2004) and the Scientific Committee on Food Regulation 2006/1881/EC (EC, 2006) concluded that a number of PAHs are genotoxic carcinogens. Benzo[A]anthracene, benzo[A]pyrene (BaP) and dibenzo[A,H]anthracene were classified by

IARC as probably carcinogenic to humans and classified as group 2A while 5-methylchrysene, benzo[B]fluoranthene, benzo[K]fluoranthene, dibenzo[A,I]pyrene and indeno[1,2,3-CD]pyrene are possibly carcinogenic to humans and thus classified as group 2B. However, those PAHs that have not been demonstrated to be carcinogenic, such as chrysene classified as group 3 may act as synergists (Hossain *et al*., 2009).

Polycyclic aromatic hydrocarbons have been determined and quantified in some seafood consumed around lakes in Egypt, such as *Oreochromis aureus* (Nile tilapia), *Portuns pelagicus* (crabs), *Venerupis decussate* (bivalves), *Strombus tricornis* (clams) and *Munes spp* (gastropods) (Barakat, 2004). The presence of PAHs in edible oils and fats has also been reported by Pandey *et al*., (2004). Repeated frying of vegetarian and non-vegetarian food in edible oil is a common practice round the globe. Studies have shown that repeated frying of edible oils enhance the generation of variety of carcinogenic PAHs (Khanna, 2000).

In wines, vinegars and distillates production processes, the aging in wood usually oak casks is a well established practice. Aged products are typically distinguishable for their increased sensorial complexity because of the transfer, from wood to beverages, of phenolic and aromatic substances such as the PAHs (Towey and Waterhouse, 1996) and their phenolic evolution, including wine color stabilization, the latter promoted by wood mediated oxygen diffusion (Matejıcek *et al*., 2005). These features are highly appreciated by consumers, and wood aged products are often regarded as quality products. The addition of wood chips as an alternative to the aging of alcoholic beverages in oak barrels has also been proposed (Perez-Coello *et al*., 2000).

In order to protect public health, the EU legislation has fixed maximum levels for benzo[A]pyrene in certain foods containing fats and oils, and in smoked and dried foods since smoking and drying processes might cause high levels of contamination. According to the Scientific Committee on Food, benzo[A]pyrene can be used as a marker for the occurrence and effect of carcinogenic PAH in food, but Commission Recommendation (EC) No. 2005/108/EC required analysis of multiple PAHs including 15 EU priority PAHs to get further information on levels of contamination from different PAHs in food (Rey-Salgueiro *et al*., 2008).

2.2 Exposure to PAHs

PAHs as products of incomplete combustion or pyrolysis of organic material are widespread in the environment. Food can be contaminated by PAHs that are present in the air, water or soil, as well as those that are formed during food processing or certain home

cooking practices including grilling, roasting and smoking. In food they may be formed as a result of thermal degradation of some food components such as triglycerides, fatty acids, amino acids and steroids such as cholesterol (Chen and Chen, 2001; Sharma *et al*., 2003)

PAHs can enter the body as components of the diet where they may diffuse into the membranes of sorptive tissues including the gastrointestinal epithelium. Several studies have documented that PAHs are frequently found in cooked food such as fish and meat, with concentrations ranging from 0.17 to 78mg/kg (Chen and Lin 1997; Kazerouni *et al*., 2001), and in milk and dairy products at levels of 1.5–7.5mg/kg (Cavret *et al*., 2005). BaP has been the most studied PAH because it is the most carcinogenic of all PAHs (IARC, 1983). These cyclic compounds are formed during incomplete combustion or pyrolysis of organic matter (Dreij *et al*., 2005).

The general population is exposed to PAHs primarily through the air and by food-intake, with high levels in leafy plants, smoked fish and meat, where especially frying or charcoal broiling increases the total PAHs content (Barakat, 2004). Certain life-style factors, such as cigarette smoking and some occupations such as gas and coke production may greatly increase exposure to PAHs (IPCS, 1998).

2.3 PAHs Metabolism

Once absorbed in humans and animals, PAHs are distributed by the blood route to several tissues, especially to lipophilic tissues, due to their non polar character. Nevertheless, PAHs are metabolised to a complex mixture of quinines, phenols, dihydrodiols, triols and tetrols in the biological system. Pyrene and benzo[A]pyrene (BaP) are two of the best characterised PAHs and may be bio-transformed in humans and animals to numerous phase 1 metabolites including 1-OH pyrene (1-OH-Pyr) and 3-OH benzo[A]pyrene (3-OH-BaP). These OH-metabolites may be biotransformed to phase 2 metabolites by conjugation with glutathione, glucuronide or sulphate (EC, 2002).

The aim of the metabolism is to increase their polarity to obtain hydrophilic substances in order to expedite their excretion (Grova *et al*., 2002). Though this mechanism produces detoxification, some PAHs are metabolized to active mutagen or carcinogen substances, which are capable of attacking cellular deoxyribonucleic acid (DNA) (Grova *et al*., 2002; Grova *et al*., 2006). Moreover, some assays with animals have shown that some PAHs metabolites are suspected to be endocrine disruptors acting like hormones. Their effects occur mainly in infants of mothers exposed during pregnancy and lactation (Gozgit *et al*., 2004).

Though bile, faeces and urine are the principal elimination routes of PAHs and PAH metabolites, their transfer to milk can occur (Grova *et al*., 2002; Grova *et al*., 2006; Lutz *et al*., 2006; Lapole *et al*., 2007). In this way Grova *et al*. (2006) and Lapole *et al*. (2007) found that the oral exposure of lactating goats to PAHs, resulted in a constant level of native PAH forms and in a significant increase in monohydroxylated metabolites in milk. The results were similar to those obtained by Lutz *et al*. (2006) when they determined the transfer kinetics of soil-bound PAHs to milk in lactating cows. Metabolites are usually not included in the classical monitoring schemes of PAHs in milk samples (Kishikawaa *et al*., 2003; Aguinaga *et al*., 2007; Zanieri *et al*., 2007). Therefore, in the recent years some works have been reported not only for determining native PAHs in milk samples, but also for determining their metabolites (Bulder *et al*., 2006; Grova *et al*., 2006; Lutz *et al*., 2006; Lapole *et al*., 2007).

Dietary fat contains a hydrophobic domain that is maintained during lipolysis and the micellar dispersion of the lipolytic products (Verkade and Tso, 2001), and it has been postulated that many drugs, natural products, and xenobiotics may be bound to dietary fats (Patton *et al*., 1984). The majority of the hydrophobic compounds that enter the gastrointestinal tract are dissolved in lipids, and because of the very low solubility of these compounds in the aqueous environment of the gut lumen, it has been proposed that dietary lipid assimilation might affect the uptake of extremely hydrophobic xenobiotics such as PAHs (Dulfer *et al*.,1998). Most mechanistic models of dietary uptake of hydrophobic organic contaminants (HOCs) in vertebrates assume that only the mobilized (freely dissolved) fraction is available for absorption in the digestive tract (Kelly *et al*., 2004). Hence, it was assumed that hydrophobic organic contaminants such as PAHs would exhibit low dietary uptake efficiency due to their limited mobilization from food under physiological conditions (Gobas *et al*., 1988).

Animal testing is the standard for determining dietary uptake efficiency of nutrients or xenobiotics, however, such tests are expensive, time-consuming and subject to ethical considerations. Hence, researchers have developed in vitro models that simulate human physiological conditions to estimate the uptake of xenobiotics from solid matrices such as food or soil. For example, physiologically based extraction tests (PBETs) use synthetic gastric and intestinal digestive fluids to extract the matrix of interest (Versantvoort *et al*., 2005). Van de Wiele *et al*., (2004) established the simulator of the human intestinal microbial ecosystem (SHIME) reactor which simulates transit through stomach, small intestine, and colon and includes the addition of microbial degradation during gastrointestinal digestion.

However, neither PBET nor SHIME include exposure of gastrointestinal fluids to a gastrointestinal membrane to estimate the sorbed fraction of the contaminants. As the intestinal membrane is the ultimate barrier to uptake, it is important to include this in any model of gastrointestinal absorption and transport (Luba *et al*., 2008).

The Caco-2 cell model is the best characterized among intestinal cell culture models, transport properties of pharmaceuticals (Artursson and Borchardt, 1997), metals (Duizer et al., 1999) and more recently xenobiotics such as polychlorinated biphenyls (PCBs) and several PAHs have been studied (Buesen *et al*., 2003). In extended culture, caco-2 cells differentiate into mature enterocytes with the development of microvilli and the formation of tight junctions that separate the apical and basolateral domains, as well as the expression of lipids and proteins specific for normal intestinal absorptive cells (Jodoin *et al*., 2002) such as microvilli hydrolases, including disaccharidases, peptidases and alkaline phosphatase (Matsumoto *et al*., 1990). Transport systems for sugars, amino acids, bile acids and dipeptides are also present (Artursson and Borchardt, 1997). Like the human intestinal epithelium in vivo, caco-2 cells can metabolize a variety of HOCs via cytochrome P_{450} mono-oxygenase enzymes (CYPs). The caco-2 cell line possesses several phase-I CYP 1A1, CYP 1A2, CYP 1B1, CYP 3A4 and 3A5, many of which are inducible, as well as phase-II enzymes (Ebert *et al*., 2005). Because the intestinal mucosa has a high capacity for metabolism of xenobiotics, even when compared to liver, this enzyme expression may lead to the production of carcinogenic metabolites at the site and increase the risk of cancer (Boulenc *et al*., 1992).

2.4 Carcinogenesis of PAHs

Lifestyle and eating habits are among the main factors responsible for cancer in people. Epidemiological studies show that about 30% of malignant diseases are caused by dietary factors, and that the introduction of some changes into the diet may considerably reduce cancer risk (Dybing *et al*., 2008). Frequent consumption of red meats such as beef, pork and lamb (Sandhu *et al*., 2001) and meat cooked at high temperatures (Sinha *et al*., 2005) is believed to be one of the factors that increase the risk of colon and rectal cancer. Studies on chemical composition of thermally treated and smoked food of high protein content show the presence of various organic substances that are mutagenic, including nitrosoamines, heterocyclic amines and PAHs (Jagerstad and Skog, 2005).

The genotoxic and carcinogenic PAHs can undergo at least three major metabolic pathways to form highly reactive intermediates (Xue and Warschawsky, 2005). The bay-

region dihydrodiol epoxide pathway (Figure 1) is regarded as the quantitatively most important pathway leading to stable, pro-mutagenic DNA-adducts. The pathway is catalysed by cytochromes P_{450}, particularly CYP1A1 and CYP1A2 but also CYP1B and certain CYP2 and CYP3 isozymes, and epoxide hydrolase leading to electrophilic bay-region diol-epoxides (Weyand *et al*., 2002). A one-electron oxidation pathway may play a relevant role for certain PAHs, resulting in the formation of unstable DNA-adducts eventually leading to apurinic sites. The third major pathway is oxidation of PAH dihydrodiols by dihydrodiol dehydrogenases to PAH-derived ortho-quinones. The ortho-quinones can form stable DNA adducts, unstable DNA-adducts resulting in apurinic sites and lead to the formation of DNA-damaging reactive oxygen species. In addition other pathways including sulfate conjugation of hydroxylated meso-methylated PAHs have been described and may, under certain conditions play a relevant role in metabolic activation of PAHs. Induction of certain drug metabolizing enzymes by a number of PAHs occurs via binding and activation of the aryl-hydrocarbon receptor (Diane *et al*., 2009).

The usual sites of adduction to nucleic acids are the extra-nuclear amino groups of guanine and adenine. The major adduct is formed on the N2 position of guanine. Diol-epoxides are thought to react frequently with the N7 position of guanine, but these adducts are labile and are normally spontaneously released. Frame shift, deletion and base substitution mutations can also occur. In addition to base-pair substitutions, PAHs may form bulky DNA-adducts, resulting in frame-shift mutations, deletions, S-phase arrest, strand breakage and a variety of chromosomal aberrations (Xue and Warschawsky, 2005). Some PAHs also exhibit tumour promotion potential, which can affect the carcinogenic potency of PAHs in mixtures (FAO/WHO, 2006).

2.5 Smoking of Fish

Fish is an extremely perishable food and this has consequently resulted in colossal losses due to post harvest spoilage (Eyo, 1983). A number of physical and chemical changes occur in fish after harvest which results into quality deterioration. The main causes of spoilage include autolysis and bacterial decomposition (Adebowale *et al*., 2008). Following the death of fish, certain endogenous biochemical changes take place within the fish making it conducive for the proliferation of spoilage bacteria. Few hours after death, stiffening of the fish muscle occur whereby fish loses its flexibility through rigor mortis (Regenstein and Regenstein, 1991). Oxidative rancidity is another factor which contributes to spoilage in fatty fishes. Of all the preventive measures to arrest spoilage in fish, at domestic and local levels,

smoking remains the cheapest. It is also the most preferred of all the treatment methods in Kenya (Eyo, 1983; Adebowale *et al.*, 2008). In addition to its preservative effect, smoking also impacts flavours on fish (Tull, 1997).

Smoking achieves preservation by four different means. The hot smoke melts down the fat in the fish which drips away, it dries the fish, deposits compounds on the fish that inhibit the growth of micro-organisms which cause decomposition and also kills micro-organisms on the surface of the fish (Andrzej and Zdzislaw, 2004). The preferred woods for fish smoking are the slow burning mainly obtained from the slow growing indigenous hardwoods (Pandey *et al*., 2004).

Traditional smoking involves treating of pre-salted, whole, eviscerated or filleted fish with wood smoke. The smoke is produced by smoldering wood and shavings or sawdust in the oven, directly below the hanging fish or fillets, laid out on mesh trays. The flow rate and distribution of smoke depends upon the natural draft as affected by the construction of the kiln and by the weather conditions. In modern automatic smoke houses, the smoke develops in an external generator under controlled conditions of temperature and air access, while the circulation is forced and controlled by mechanical equipment. The temperature of the smoke is in the range 12–25ºC during cold-smoking and 25–45ºC in warm-smoking. In hot-smoking, the process may be carried out in different stages, during which the temperature of the smoke ranges from about 40–100ºC and that in the centre of the product temperatures may reach up to 85ºC (Abila, 2003). The rate of deposition of different components depends upon the temperature, humidity, flow rate and density of the smoke, the water solubility and volatility of the particular compounds, as well as on the properties of the surface of the fish (Andrzej and Zdzislaw, 2004).

2.6. PAH in Wood Smoke

Wood smoke contains a large number of PAH (Obiedzinski and Borys, 1977). At least 61 PAHs with a wide range of molecular mass, from indene, 116 Da, to dibenzopyrenes, 302 Da are identified (Jagerstad and Skog, 2005). Of these, 15 PAH (Table 1) have been established to be mutagenic/genotoxic in somatic cells in experimental animals (European Scientific Committee on Food, 2002). The PAHs may be regarded as potentially genotoxic and carcinogenic to humans, their carcinogenicity depends on their structure (Bartoszek, 2002). The light PAHs, of molecular mass below 216 Da are regarded as non carcinogenic. BaP with a molecular weight of 252 Da is highly mutagenic and carcinogenic as such it has been accepted as a marker of carcinogenic PAHs in wood smoke, smoked products and

environmental samples (Andrzej and Zdzislaw, 2004). It has been found that the ratio of the concentration of the carcinogenic PAHs to that of BaP in smoked products is generally not higher than 5 (Bartoszek, 2002). If the carcinogenicity of individual heavy PAHs is also taken into consideration, the carcinogenic potency of total PAHs contained in a food product is about 10 times higher than would result from the content of BaP alone (European Scientific Committee on Food, 2002). However, BaP is often accompanied by benzo[E]pyrene, known to be significantly less carcinogenic (Howard and Fazio, 1980). The separation of these two isomers is a difficult analytical task and may not be achieved in all BaP assays. According to regulations in different countries regarding drinking water and foods, the determination of several other PAHs is also required (Andrzej and Zdzislaw, 2004).

The composition of the smoke and the conditions of processing affect the sensory quality, shelf life, and wholesomeness of the product. Potential health hazards associated with smoked foods may be caused by carcinogenic components of wood smoke mainly PAH, derivatives of PAH, such as nitro-PAH or oxygenated PAH, and to a lesser extent also N-nitroso compounds and heterocyclic aromatic amines. The contents of N-nitroso compounds in hot smoked fish generally does not exceed several μg/kg of product, that is lower than in many other foods (Pandey *et al*., 2004). According to Simko (2002), smoked-cured poultry products may contain N-nitrosothiazolidine carboxylic acid in amounts even as high as 1 mg/kg wet weight.

Table 1: Polycyclic aromatic hydrocarbons regarded as potentially genotoxic and carcinogenic to humans

Benzo[A]anthracene	Benzo[A]pyrene	Dibenzo[AH]pyrene
Benzo[B]fluoranthene	Chrysene	Dibenzo[AI]pyrene
Benzo[J]fluoranthene	Cyclopenta[CD]pyrene	Dibenzo[AL]pyrene
Benzo[K]fluoranthene	Dibenzo[AH]anthracene	Indeno[1,2,3-CD]pyrene
Benzo[GHIi]perylene	Dibenzo[AE]pyrene	5-Methylchryzene

(Source; European Scientific Committee on Food, 2002)

2.7 Deep-frying of Fish

The purpose of deep-fat frying a product is to seal the food by immersing it into hot oil so that all the flavors and juices stay inside the crust (Moreira *et al*., 1999). As explained by Mellema (2003), when a food product is added to hot oil in deep fat frying, the surface temperature of the food rises very rapidly and the water at the surface starts boiling. As

boiling starts, convection in the oil will be increased by the turbulent water vapor. Explosive evaporation will lead to the formation of large pores. Water inside the food will be heated and the product will be cooked. Deep-fat frying can be accomplished under three different pressure conditions namely atmospheric pressure, low pressure and high pressure. Under atmospheric or open frying, the temperature of the oil is usually between 160^{o}C and 200^{o}C (Moreira *et al*., 1999)

2.8 Maximum Residue Limits

The average daily intake is the intake of an agricultural or veterinary chemical, which, during the consumer's entire lifetime, appears to be without appreciable risk to the health of the consumer. This is on the basis of all the known facts at the time of the evaluation of the chemical. It is expressed in milligrams of the chemical per kilogram of body weight (FAO/WHO, 2010). The MRL as set by the Codex Alimentarius Commission (CAC) is the maximum level of a chemical that may be in a food in accepted or allowed limits above which can result in serious health concern (CAC, 1997). However, incorporating the MRL into food legislation means that the residues of a chemical are minimized since they must not exceed the MRL, irrespective of whether the dietary exposure assessment indicates that higher residues would not represent a risk to public health and safety. Regulatory levels have been established for drug residues in foods in the form of MRLs (Lee *et al.,* 2000). Sampling and testing protocols are based on standards set by CAC.

Several organisations have proposed maximum values for PAHs in edible vegetable oils. For instance, the German Society for Fat Science proposed a value of 5µg/kg as the limit value for total heavy PAHs with more than four benzene rings fused in their structure and a value of 25µg/kg for the sum of both light and heavy PAHs (Speer *et al*., 1990). Spain has set a maximum level in olive residue oil of 2µg/kg for benzo[A]anthracene, benzo[B]fluoranthene, benzo[K]fluoranthene, benzo[A]pyrene, benzo[E]pyrene, dibenzo[A,H]anthracene, benzo[G,H,I]perylene and indeno[1,2,3-C,D]pyrene and the sum of them should not be above 5µg/kg (Spanish Official Bulletin, 2001). The presence of BaP in baby foods results in health risk to the infant, since they are more sensitive than adults to these contaminants. In 2005 MRL of 1.0µg/kg for BaP in foods for infants and young children, including cereal-based food and milk formulae, was set (Commission Regulation, 2006).

2.9 HPLC Technique for Benzo[A]pyrene Analysis

The most common analytical procedure used in the determination of PAHs in food is high performance liquid chromatography (HPLC) with fluorescence detection. This technique has been successfully used in several food matrices (Camargo and Toledo, 2003; Yusty and Davina, 2005). The isolation of a PAH fraction by HPLC has been introduced improving clean-up procedures. Untreated silica has been successfully used as stationary phase, because of its capacity to retain fat and other interferents allowing the polyaromatic fraction to elute in a few millilitres of the appropriate mobile phase (Moret and Conte, 1998). Recently, more specific sorbents have been introduced, based on a more selective mechanism due to the interaction of the sorbent with the π electrons of PAHs; which has been called donor–acceptor complex chromatography (DACC) (Brouwer et al., 1994). PAHs are retained on the basis of a strong π –π interaction when a non- π -electron containing solvent is used as a mobile phase, eluting other components of the oil such as neutral lipids and tocopherols. Then, the PAH fraction is eluted with a proper solvent that neutralized the π –π interaction (Barranco, 2003)

HPLC utilizes a column that holds chromatographic packing material as a stationary phase, a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed and the solvent(s) used. In this method, high pressure rather than gravity forces can propel mobile phase through the column packed with a stationary phase of much smaller particle sizes. This allows for a quicker and more precise analysis on columns of shorter length, when compared to plain column chromatography (Keith and John, 2000).

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CHAPTER THREE

MATERIAL AND METHODS

3.1 Sampling and Sample Handling

Sampling was done according to Commission Regulation (EC) No. 333/2007 (EC, 2007). Under these regulations ten fish dealers were identified from ten randomly selected fish markets; Asat (0° 5'S, 34° 45'E), Luanda Kotieno (0° 9'S, 34° 26'E), Usenge (0° 14'N, 34° 16'E), Uyoga (0° 20'S, 34° 12'E) and Kamagogo (0° 31′S, 34° 27′E) in Nyanza province (0° 30′S, 34° 40′E) and Marenga (0° 3'N, 33° 59'E), Bukama (0° 4'N, 33° 59'E), Osieko (0° 4'N, 34° 0'E), Busoma (0° 5'N, 34° 22'E) and Sialala (0° 4'N, 34° 26'E) in Western province (0° 30′ N, 34° 35′E). From the selected smoked and oil fried fish dealers, nine samples of 200g each of smoked and oil fried samples were bought and placed in clean, sterilized containers. The containers offered adequate protection from contamination and damage in transit. Four kilograms of freshly landed *Lates niloticus* control samples were bought from Dominion aquaculture farm in Yala, Kenya. 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. All the samples were transported to the laboratory at the Department of Dairy and Food Science and Technology of Egerton University for analysis. The sampling was done three times to ensure the samples were homogenous and representative of the situation in practice. On arrival at the laboratory, the fresh samples were kept in the cold room controlled at between 0 and 5^{o}C. Analytical determinations of benzo[A]pyrene levels were replicated three times using HPLC method. The experimental design was a complete randomized design. All reagents used were of analytical grade sourced from Sigma Ltd through their agent Kobian Scientific.

3.2 Sample Preparation and Analytical Standards of Benzo[A]pyrene

The samples were dissolved in acetone and hexane for 15 minutes before analysis. These solvents dissolve both polar and non-polar compounds and hence were essential in removing impurities. 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 100µg/l of benzo[A]pyrene 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 appropriate dilutions of the stock solution.

3.3 Benzo[A]pyrene Extraction

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

3.4 Post- Extraction Cleanup

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

3.5 Analysis by HPLC

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 Millenium 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 30µl. The signal due to

benzo[A]pyrene was identified by comparison of sample chromatograms with the chromatogram of the benzo[A]pyrene standard.

3.6 BaP Quantification

The external standard plot method was used. Duplicate injections of 0.2µl-12.8µl benzo[A]pyrene standard solutions were used to construct linear regression lines which are peak area ratios versus benzo[A]pyrene concentration. Detection limit, defined as the concentration corresponding to peak height of three times the baseline noise level, was calculated for benzo[A]pyrene following Miller and Miller (1993) guidelines. Conversion of these values to µg of benzo[A]pyrene per kg of sample was done by division of the results obtained by the mass of the sample analysed.

3.7 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) with three different concentrations of the benzo[A]pyrene standards ranging from 0.1 to 5µg/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 benzo[A]pyrene 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 benzo[A]pyrene performed during the recovery tests.

3.8 Data Analysis

The data from the three sampling times were pooled and the mean values for benzo[A]pyrene levels in smoked, oil fried and fresh fish were analyzed for each of the treatments. The means of benzo[A]pyrene levels of the selected smoked, oil-fried and fresh fish analyzed for each of the treatments at each sampling occasion were subjected to analysis of variance. 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).

CHAPTER FOUR

RESULTS

4.1 Recovery Studies and Detection Limit

Precision, detection limit, recovery and retention time for BaP are presented in Table 2. The average recovery for benzo[A]pyrene was 95% with a standard deviation of 1.3% and a coefficient of variation of 4.23. The limit of detection for benzo[A]pyrene was established at 0.011µg/kg and was defined as the smallest amount of analyte that was reliably detected from the background of the HPLC equipment. This value showed enough sensitivity for the detection of the compound in the samples analyzed.

Table 2: Detection limits and mean recovery levels of BaP in spiked fish sample

PAH	*R*[a] (%)±STDEV	CV (%)	Detection Limit (µg/kg)	Retention Time (min)
BaP	95±1.3	4.23	0.011	27.01

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

4.2 Benzo[A]pyrene Standard and Sample Chromatographs

Identification of benzo[A]pyrene was achieved on the basis of comparison of HPLC retention times of the benzo[A]pyrene standard and those of spiked or unspiked, smoked and oil-fried fish samples. Benzo[A]pyrene standard and sample chromatographs obtained from HPLC are shown in Figure 1, 2 and 3. Figure 1 shows a BaP standard chromatogram with a retention time of 27.01 minutes and an instrument response of 100mv which gave a peak area of 214,654. No other peaks were observed in this chromatograph since it represented a chromatogram of BaP standard in pure solution. Figure 2 and 3 represent 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 was not detected. BaP content was below the detection limit of 0.011µg/kg. Instrument response (mV) is directly proportional to concentration of an analyte. Figure 2 which represents BaP concentration in smoked fish has the highest instrument response of (280mV) and hence the largest peak area for BaP.

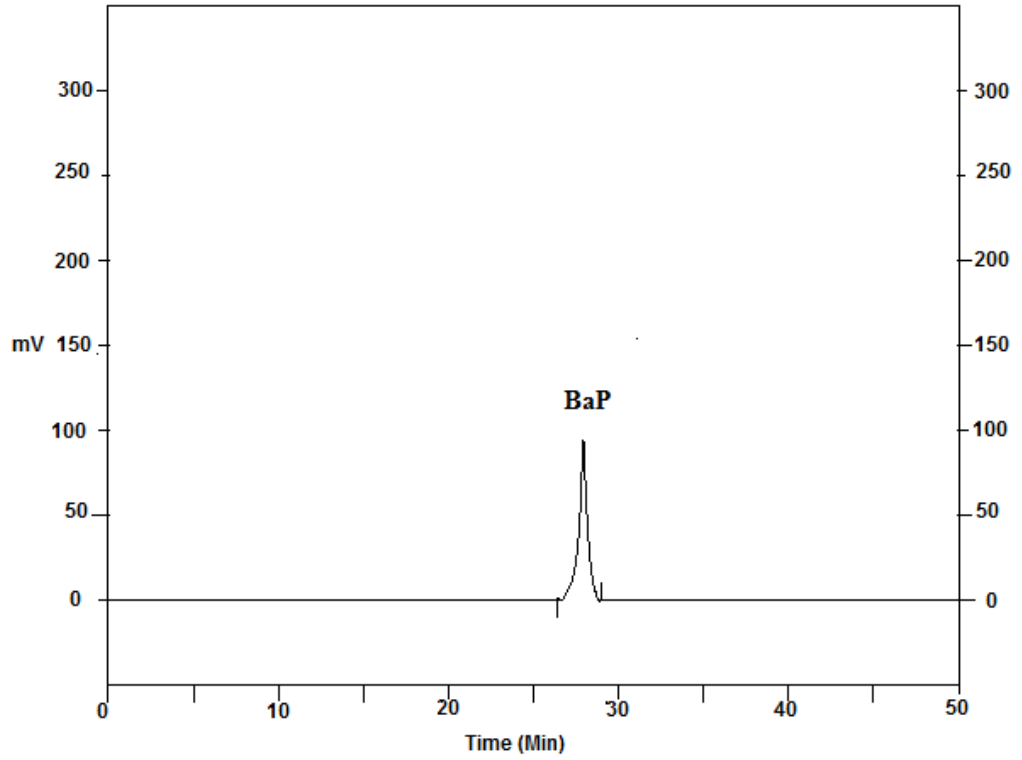

Figure 1: BaP standard chromatogram

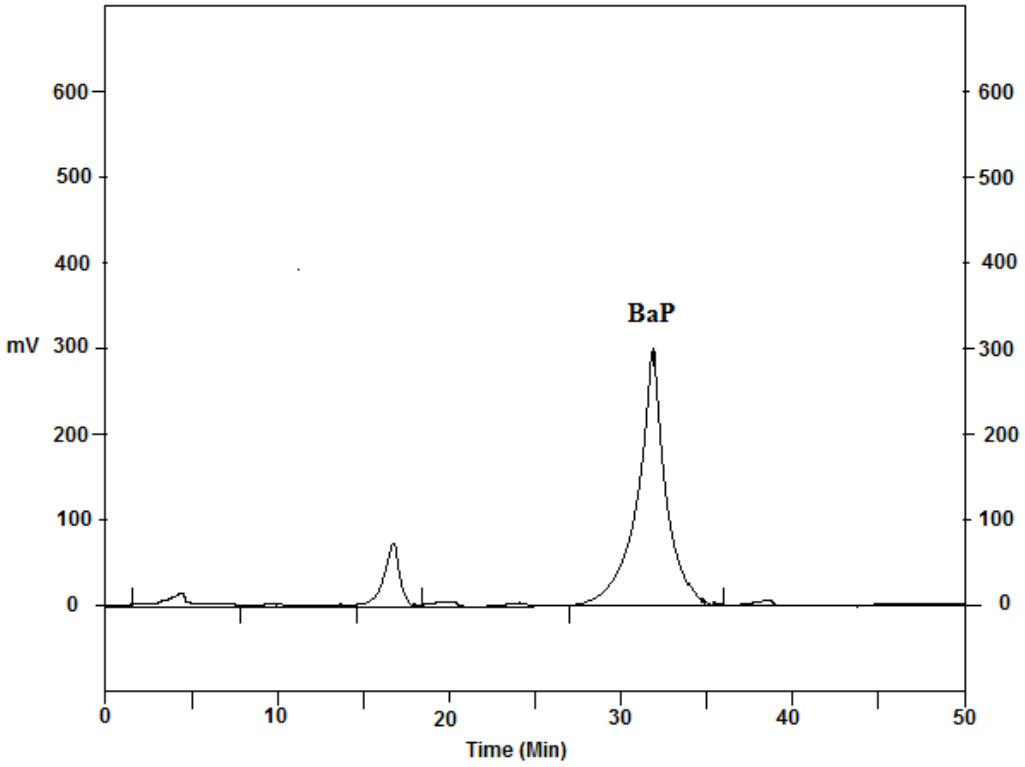

Figure 2: BaP fraction isolated from smoked *Lates niloticus*

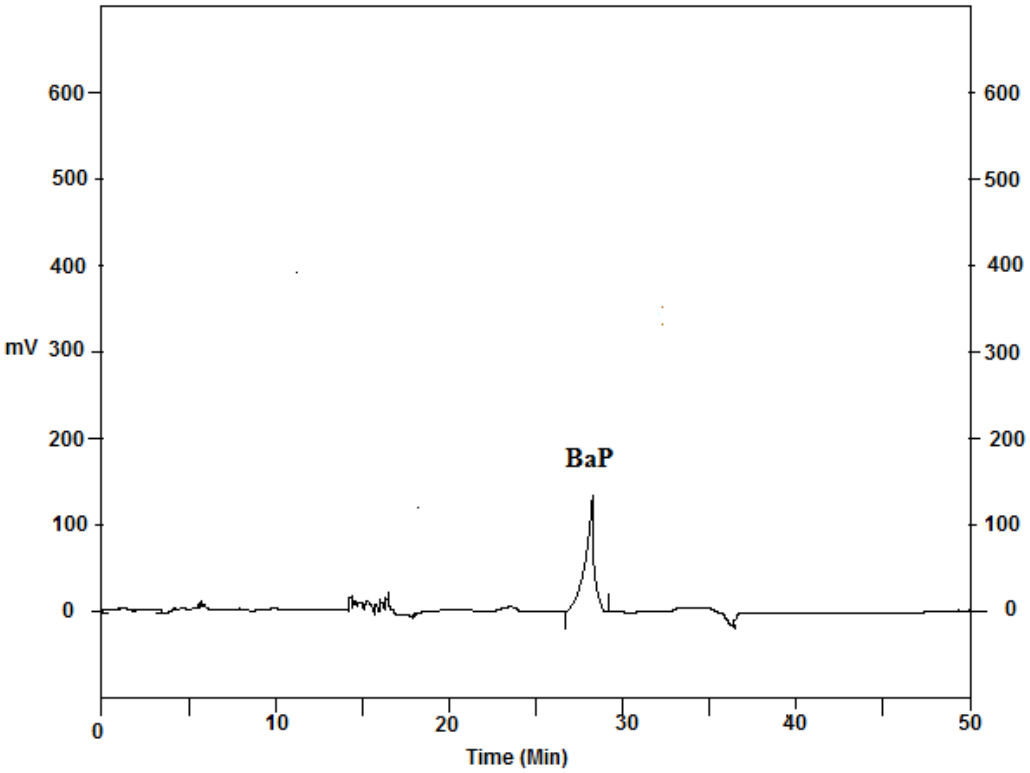

Figure 3: BaP fraction isolated from oil fried *Lates niloticus*

4.3 External Standard Plot

The calibration curve, linear equation, coefficient of regression (R^2) and limit of detection obtained using the signal to noise ratio as S/N=3 for benzo[A]pyrene are presented in Figure 4. The response was highly linear (R^2 = 1.00, y =1,338,247.83x)

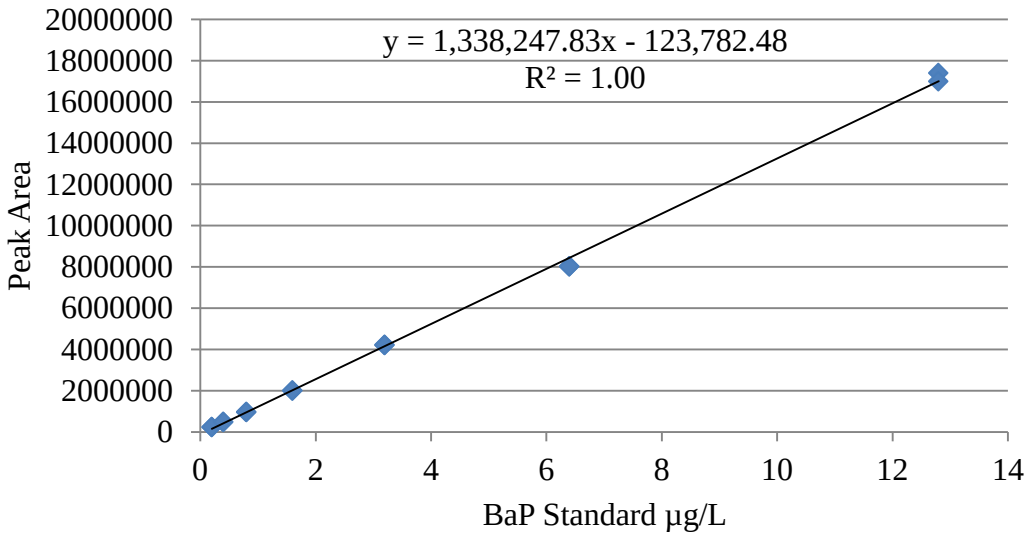

Figure 4: External standard plot

4.4 Benzo[A]pyrene Levels in Smoked and Oil fried Fish at Different Occasions of Sampling

The means and standard deviation for BaP levels in smoked and oil fried fish are presented in Table 3 and Table 4, respectively.

Table 3: 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}

n=9: Mean values in the same row with the same superscript are not significantly different ($p>0.05$)

Table 4: Mean BaP concentration in oil fried fish at different sampling occasion

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]

n=9: Mean values in the same row with the same superscript are not significantly different ($p>0.05$)

4.5 Benzo[A]pyrene Levels in Smoked and Oil Fried *Lates niloticus*

Mean BaP levels in smoked and oil fried fish are presented in Table 5. Benzo[A]pyrene concentration in smoked *Lates niloticus* ranged from 7.46 to 18.79µg/kg among the sampled markets Table 5. 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 (Appendix 1).

The difference in BaP concentration in oil fried fish among the sampled markets is not statistically significant ($p>0.05$) (Appendix 1). The levels of BaP in oil fried fish samples ranged from 4.17 to 11.26µg/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 5. The difference between smoked and oil fried groups was significant ($p<0.05$)

Table 5: Mean BaP concentration (µg/kg) in smoked and oil fried *Lates niloticus* samples

Sampled Markets	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	17.71 ± 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	12.69 ± 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

n=9: Mean values with the same superscript are not significantly different ($p>0.05$)

4.6 Frequency Distribution (%) of BaP Concentration in Smoked and Oil Fried *Lates niloticus*

All smoked fish samples (90) examined in this study were found to be over the acceptable limit (5µg/kg) specified by the European Commission (E.C, 2005), but when they were evaluated according to the FAO/WHO limits (10µg/kg), 72 samples (80%) analyzed were over the acceptable limit. Twenty percent (18 samples) of the oil fried fish samples were found to be within the acceptable limit set by the European Commission while only 10% (9 samples) were found to be above the limit set by the FAO/WHO (Table 6). This suggests that smoking of fish results in higher BaP levels in the food product as compared to oil frying.

Table 6: Frequency distribution (%) of BaP concentration in analyzed samples

BaP (µg/kg)	Smoked fish samples (n=90)	Oil fried fish samples (n=90)
<5	0	20
5-10	20	70
>10	80	10

CHAPTER FIVE

DISCUSSION

The clean up procedure based on solid phase extraction and adsorption column chromatography was used to isolate the benzo[A]pyrene fraction from the smoked and oil fried fish samples bought from the ten sampled markets. The adsorption chromatography on silica gel enabled the purification of the benzo[A]pyrene fraction from fat and residues of interfering components from the final fish extracts. This clean up step was particularly important in this work because samples of fish prepared by either smoking or oil frying might have contained other components such as proteins, amino acids, organosulphur compounds and phenolic compounds. The application of the clean-up procedure enabled the isolation of benzo[A]pyrene concentrates from every sample of smoked and oil-fried fish where a quantitative determination of benzo[A]pyrene by HPLC-FLD method was possible.

Recovery studies are usually conducted to verify the accuracy and precision of an analytical procedure. This study recorded an average recovery of 95% which is much higher than the 66.5% recovery for benzo[A]pyrene analysis in meat samples reported by Janoszka *et al* (2004). Moreover, Wu *et al* (1997) also reported a lower recovery (75%). The range of recovery in this study was 93.6-96.2%. These values fulfill the performance criteria for methods of analysis for benzo[A]pyrene, according to which the recovery for this compound should be in the range of 50-120% for food samples (E C, 2007; EFSA, 2008).

Modern chromatographic equipment are fitted with computers which gather data from the detector, process the data into a chromatogram (detector response versus time), integrate areas under peaks or measure peak heights and present the peak area/height in a printout or screen display. These integrators offer several modifiable parameters which must be set by the analyst in order to consistently integrate the peak area or measure peak height for standards, controls, fortified controls, and treated samples. Integration parameters cannot be changed within a batch or sequence. In this study, the integrator on and off times of the HPLC equipment was carefully set at between 0 and 40 minutes so as to eliminate integrator peaks which are not of interest 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.

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 within fatty species, of lipids rich in n-3 fatty acid residues. The wood smoke used in smoking of fish may contain, depending predominantly on the temperature of generation, a large variety of PAHs, including the most carcinogenic ones.

The variation in the levels of BaP observed among sampled smoked fish from the selected markets could be related to differences in processing (Duration of smoking), differences in the type of wood used for smoking or even differences in construction of smoking kilns. According to Kazerouni *et al* (2001) the concentrations of BaP in home prepared meat dishes 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. Various studies have reported the levels of BaP in smoked fish, according to Stolyhwo and Sikorski (2005), most of the PAHs in smoked foods come from the wood smoke and that smoked fish contain much more PAH than the raw material from about 0.05 to about 60µg of BaP/kg of product, this is dependent on the properties of the fish, method and parameters of smoking, composition of the smoke and exposure of the edible parts to the smoke. This was satisfactorily confirmed in this study.

Fish and marine invertebrates may naturally contain small amounts of different PAH absorbed from the environment (Stolyhwo and Sikorski, 2005). In this study, BaP in raw fish samples was below the detection limit of 0.011µg/kg and therefore not detected. This is similar to a study by Rainio *et al*., (1986) who reported that the edible parts of raw fish from unpolluted waters generally do not contain detectable amounts of BaP.

. 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 as compared to the smoked fish samples could be as a result of diffusion of BaP from fish fraction in oil fraction, which could reduce the level of benzo[A]pyrene in fish. According to a study conducted by Stumpe-Viksna *et al*. (2007), separate detection of BaP concentration in oil and fish fraction of canned fish in oil as well as undivided sample, a decrease by about 73 up to 95 percent of the initial BaP amount in fish was found. The study further suggested that if a person consumes only the fish fraction of canned fish in oil, he becomes less contaminated by 56±8 percent than if he consumes the same amount of fish and oil together.

Research has shown that BaP concentration of charcoal fire cooked meat samples was much higher than gas fire cooked meat (Kazerouni *et al.*, 2001; Anderson *et al*., 2002).

However, Rivera *et al*. (1996) detected BaP concentrations of 4 to 19µg/kg in charcoal grilled meat. The levels of BaP in smoked foods, including turkey, pork, chicken, beef and fish products were found to be between 0.15 and 8.93µg/kg by Gomaa *et al*. (1993). This study however, found much higher values. According to Dennis *et al*. (1984), the concentration of BaP detected by HPLC-FL in grilled sausage was 0.4µg/kg, in grilled pork chops 8.2µg/kg, smoked herring 8.5µg/kg, smoked ham 0.2µg/kg and in hard grilled sausage 191µg/kg. Binnemann (1979) found BaP concentrations of 0.6 to 100µg/kg in sausages and special products while Terzi *et al*. (2008) found mean levels of BaP to be 24µg/kg for charcoal fire cooked meat samples and 5.7µg/kg gas fire cooked meat samples.

Essentially BaP formation is largely determined by cooking method and the 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, and so significant levels of PAH will be produced during cooking directly on the meat as well as via the charcoal or wood fuel (Lijinsky, 1991; Wu *et al*., 1997; Phillips, 1999; Kazerouni *et al*., 2001). PAH production by cooking over charcoal or wood fuel is a function both of the fat concentration in the meat and proximity of the food to the heat source and can be reduced by cooking for longer periods at lower temperatures (Phillips, 1999)

CHAPTER SIX

CONCLUSSION AND RECOMMENDATIONS

6.1 Conclusion

The levels of BaP in smoked and oil fried fish determined in this study indicate that the BaP levels are generally above the maximum residue limit recommended by different international and European regulations and hence pose a health risk to consumers. However oil frying results in lesser accumulation of this toxic residue as compared to smoking. This study also validated HPLC technique as an efficient and reliable tool for determination of BaP in fish below the recommended MRLs.

6.2 Recommendations

The following recommendations were drawn from the study

1. The Kenya Bureau of Standards and the Ministry of Fisheries Development should adopt the findings of this study in determination of benzo[A]pyrene in smoked and oil fried fish and subsequently develop a Kenyan standard for benzo[A]pyrene.
2. There is need to investigate methods that would significantly reduce contamination of these products with carcinogenic PAHs.
3. Further research on determination of individual carcinogenic hydrocarbons to guarantee unequivocal separation and identification of these contaminants in fish.

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APPENDICES

Appendix 1: ANOVA for BaP concentration in smoked fish samples among the different sampled markets in western Kenya

Matrix	Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Smoked fish	Between Groups	42.2	9	7.0	8.9	0.000
	Within Groups	16.6	27	0.8		
	Total	58.8	36			

Appendix 2: ANOVA for BaP concentrations in oil fried fish samples among the different sampled markets in western Kenya

Matrix	Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Oil fried fish	Between Groups	18.559	9	3.093	2.490	0.056
	Within Groups	26.086	27	1.242		
	Total	44.644	36			