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Influence of Cambodian traditional smoking practices on the concentration of Polycyclic Aromatic Hydrocarbons (PAHs) in smoked fish processed in Tonlé Sap area, Cambodia

Dissertation thesis

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Supervisor:

Prof. Ing. Jan Banout PhD.

Co-supervisor:

Ing. Adéla Fraňková, Ph.D.

Author:

Ing. Tereza Slámová

Declaration:

"I Tereza Slámová hereby declare that this thesis entitled Influence of Cambodian traditional smoking practices on the concentration of Polycyclic Aromatic Hydrocarbons (PAHs) in smoked fish processed in the Tonlé Sap area, Cambodia is my own work and all the sources have been quoted and acknowledged by means of complete references."

In Prague ……

(signature of student)

Ing. Tereza Slámová

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Abstract:

The main aim of this work is to investigate in detail the traditional practice of smoke-cured fish from Tonlé Sap lake area, Cambodia, and to monitor the concentration of selected polycyclic aromatic hydrocarbons (PAHs) in the final product. Levels of BaP, ΣPAH4 and ΣPAH12 in 18 species of smoked fish commonly consumed in Cambodia were determined by modified QuEChERS–EMR Lipid–DLLME method and analysed by gas chromatography–mass spectrometry (GC/MS). This method was sucessfully developed and applied with a recovery rate of 50 – 120% and RSD values of <16.7%. Overall results highly exceed the limits set by the European Commission (EU) No 1881/2006. The highest ΣPAH4 and ΣPAH12 concentration were detected in *Paralaubuca typus,* 2701.45 $\mu g.kg^{-1}$ and 16818.19 $\mu g.kg^{-1}$ and the lowest measured in *Paralaubuca barroni*, 76.33 $\mu g.kg^{-1}$ and 536.95 $\mu g.kg^{-1}$, respectively. Among fish species, the highest ΣPAH4 and ΣPAH12 were detected in *Paralaubuca typus* (1644.19 $\mu g.kg^{-1}$ and 9171.56 $\mu g.kg^{-1}$, respectively). Results showed significant increase of ΣPAH12 mean values between smoking times T1 (3 – 16 hours) and T2 (1 - 4 days) and when fuel wood was used. The total fat content was measured, and the correlation between fat content and PAHs contamination was analysed, but was not proven. Altogether, the extremely high concentrations of PAHs measured in this study are attributable to a combination of factors, such as the type of fuel used and the length of the process, but other factors, such as the use of inappropriate fire-starting techniques, the use of a direct heat source, the distance from the heat source, the lack of temperature regulation systems and the size and physical state of the smoked fish cannot be excluded, although not supported by statistics in the present study. Such a burden can lead to an elevated risk of the development of diseases related to PAHs exposure. However, by following good manufacturing practices. PAHs contamination can be controlled and decreased.

Key words: smoke-cured, food preservation, PAHs, fat content, modified QuEChERS, Cambodia, fish

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List of abbreviations used in the thesis

°C	degrees Celsius
µA	microampere
µl	micro liter
µm	micro meter
Ace	acenapthylene
acetyl-CoA	acetyl coenzym A
ALA	alpha-linolenic acid
Ant	antracene
ASE	accelerated solvent extraction
B[a]a	Benz[a]anthracene
BaP	Benzo[a]pyrene
C18	octadecyl
C2H2	acetylene
C2H4	ethylene
C2H6	ethane
C6H6	benzene
cm	centimeter
CO	carbon oxide
CO2	carbon dioxide
CVD	cardiovascular disease
DHA	docosahexaenoic acid
DLLME	Dispersive Liquid-Liquid Microextraction
DNA	deoxyribonuleic Acid
DoF	Department of Fisheries
DP	daily production
EC	European Commission
EFSA	European Food Safety Authority
EMR-lipid	Enhanced Matrix Removal
EPA	eicosapentaenoic acid
EU	European Union
FA	fatty acids
FAO	Food and Agriculture Organisation
FID	fluorometric detection
Flu	fluorene
GC	gas chromatography
GPC	gel-permeation chromatography
h	hours
H2O	water
HMW	high molecular weight
HPLC	high-performance liquid chromatography
HPLC-FLD	high-performance liquid chromatography with fluorometric detection
IARC	International Agency for Research on Cancer
kg	Kilograms
LC	long-chain
LLE	liquid-liquid extraction
LMW	low molecular weight
LOD	Limits of Detection
LOQ	Limits of Quantifiaction
LSF	liquid smoke flavourings

MeCN	acetonitril
MeNap1	methylnaphtalene
MgSO4	magnesium sulphate
ML	Maximal limits
mm	millimeter
MS	mass spectrometry
MUFA	Monosaturated fatty acids
NaCl	sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
Nap	naphtalene
NCD	Noncommunicable Diseases
ng	nanogram
P	producent
PAH	Polycyclic Aromatic Hydrocarbons
PE	polyethylene
PET	polyethylene terephthalate
PLE	pressurized liquid extraction
PM	particulate matter
PSA	primary secondary amine
PUFA	polyunsaturated fatty acids
QuEChERS	Quick Easy Cheap Effective Rugged Safe method
RSD	relative standard deviation
SCF	Scientific Committee on Food
SD	standard deviation
SFA	saturated fatty acids
SIM	single ion mode
SPE	solid phase extraction
SPV	sulfophosphovanillan
t	tonnes
T	time
TAG	Triacylglycerides
UFA	unsaturated fatty acids
US EPA	United States Environmental Protection Agency
WHO	World Health Organisation
Z-Sep	zirconium dioxide-based

1 Introduction

Cambodia, a country in South East Asia, has one of the largest fresh-water lakes with a fascinating water system, bringing every rainy season valuable fingerlings to the Tonlé Sap lake area. With an average consumption of 42 kg/year/person in Cambodia, fish is one of the major sources of protein intake (Ahmed et al. 1999; Hortle 2007; FAO 2020). They represent up to 37% of the total and 76% of the animal protein intake, respectively (Vilain & Baran 2016; FAO 2020). Of 16.5 million people (WHO 2020), 66% are economically active in agriculture (World Bank 2018) and particularly in fisheries and fisheries-related activities (Vilain & Baran 2016). Fisheries in the Tonlé Sap area are further considered a major source of income, supporting the national economy and contributing to the country's food security (Belton & Thilsted 2014; Vilain et al. 2016). With its high water content, fish is a highly perishable material due to restricted access to electricity for cooling in most of the rural areas. Fast and basic processing and subsequent preservation is crucial to ensure a continuous supply of protein throughout the year. Smoking is one of the oldest food preservation techniques known for more than 9,000 years, and is still widely used in the food industry (Simko 2002; Essumang et al. 2013; Kartalovic et al. 2015). Smoked fish products are favoured for their longer shelf life compared to fresh unprocessed fish, and their lightweight and organoleptic properties. However, the smoked products can also be a source of contaminants formed during the process itself. The most known xenobiotics formed during smoking are polycyclic aromatic hydrocarbons (PAHs) (Bansal & Kim 2015). This large group of organic compounds is characterized by its structure composed of two or more aromatic rings, lipophilicity, and relatively high stability (Pensado et al. 2005; Bansal & Kim 2015; Silva et al. 2017). Despite their great abundance in the environment, the main exposition route to human organisms is via food (Xia et al. 2010; Gomes et al. 2013; Rengarajan et al. 2015). Even though current commercial smoking is performed by modern controlled methods which effectively eliminate the incidence of PAHs in final products, traditional ways of smoking in the smokehouses (kilns) are still popular and widespread in households and with small-scale producers. A typical examples would be traditional Khmer smoke-cured fish. However, smoking under uncontrolled technological conditions and non-existent legislative measures, leads to enormous PAHs content in smoked foods (Šimko 2005). Consequently, those products can be associated with potential health hazards (Stołyhwo & Sikorski 2005; Alomirah 2011). From

preliminary studies conducted in the Tonlé Sap area (Slámová et al. 2017) we know that the ML (maximal limits) of the studied samples highly exceed the limits imposed by European Commission (EC) No 1881/2006 (EFSA 2008)(2 $\mu g.kg^{-1}$ and 12 $\mu g.kg^{-1}$)(Table 1), for both benzo[a]pyrene (BaP) and the sum of four PAHs (ΣPAH4) (2 – 60 times and 2 – 50 times, respectively). One of the main challenges in the determination of PAHs in smoked fish is their high fat content (e.g., lipids) and the extraction of PAHs from this complex matrix is usually laborious and not effective enough (Muller & Holst 2001; Lund et al. 2009). The fat residues can also contribute to the deterioration of the chromatographic system or suppress the analytes signal. Finally, PAHs are known to be associated with fat content due to their lipophilic nature (Basak et al. 2010). Considering that smoked fish are a regular part of the diet for the Cambodian population, we assume that these products can play a significant role in the total burden of Cambodians with PAHs and subsequent bioaccumulation in the food chain. Nevertheless, there is currently a lack of studies about the traditional smoking process and PAHs occurrence in fish products in Cambodia. Therefore, the aim of this study is to investigate in detail the traditional practices of fish smoke curing in the Tonlé Sap area and their influence on the final content of selected PAHs in smoked fish, and to develop an effective sample preparation procedure for the determination of PAHs in smoked fatty fish products.

2 Literature review

2.1 Tonlé Sap area, Cambodia

Tonlé Sap lake, located in a tropical climate in Cambodia, is known as a unique flood pulse and the largest body of freshwater in Southeast Asia. This lake covers an area of 2,500 km^2 during the dry season and expands up to 16,000 km^2 during the rainy season, providing the benefits for life and the environmental ecosystem. The water in the lake exchanges periodically with the waters of the Mekong River. The Mekong River is considered the biggest fishery in the world producing 2.1 million tonnes of fish per year. Cambodia contributes 33% of this amount (Baran et al. 2014). During the rainy season from May to October, the water flows from the swollen Mekong River into the Tonlé Sap lake via the Tonlé Sap river, while the flow is reversed during the dry season from December to April. Due to this natural phenomenon, the water quality and amount of fish in the Tonlé Sap lake change from season to season (Irvine et al. 2011; Ung et al. 2019). The Tonlé Sap lake, the largest body of fresh water in Southeast Asia, plays an important role in the lives and environment of Cambodians. The wetlands of the Tonlé Sap area in Cambodia are part of the Mekong watershed, with one of the most productive fisheries in Southeast Asia. Fisheries are the main industry and source of household income in this region, especially for poor villagers. Approximately 85% of the total fish catch in Cambodia comes from inland fisheries. In addition, wetlands provide two-thirds of the people's dietary protein (70%) (Van Zalinge et al. 2000; Kanchanaroek et al. 2013). With an annual catch estimated at between 289,000 and 431,000 t, the lake is the fourth most productive captive fishery in the world, serving 1.5 million people (van Zalingen et al. 2003; Berdik 2014). The reversal and subsequent southward draining once the rains recede is the key driver of the interaction between hydrology and fish which supports food security and livelihoods (Bonheur & Lane 2002). The wetlands support Cambodian fisheries and the fisheries of other countries by acting as an important fish nursery ground. In addition, the Tonlé Sap wetlands are considered a biodiversity hotspot of international significance (Hortle 2004). Cambodia’s three most important cities, Phnom Penh, Battambang, and Siem Reap, are all built around the lake, just as were the historic capitals of the Angkor period. The lake’s biodiversity, in terms of both variety and abundance of species, as well as the extraordinarily complex and diverse interactions of physical, biological, and human systems, make it a key element in the ecology of the lower Mekong River system and the economy, culture, and

identity of the Cambodian people. The Tonlé Sap area consists of six of Cambodia's provinces divided over 25 subdistricts and contains over 1000 villages. Namely provinces Battambang, Pursat, Kampong Thom, Kampong Chhnang, and Siem Reap, Kampong Cham. Kampong Chhnang province has among the highest per capita consumption of fresh fish, and, more particularly smoked fish processing in Cambodia. The total consumption of fresh fish and processed fish in the province is almost 120 kg per year (Ahmed et al. 1999). Furthermore, the area near Kampong Chhnang city, along with the Tonlé Sap river is famous for the high concentration of smoked fish producers.

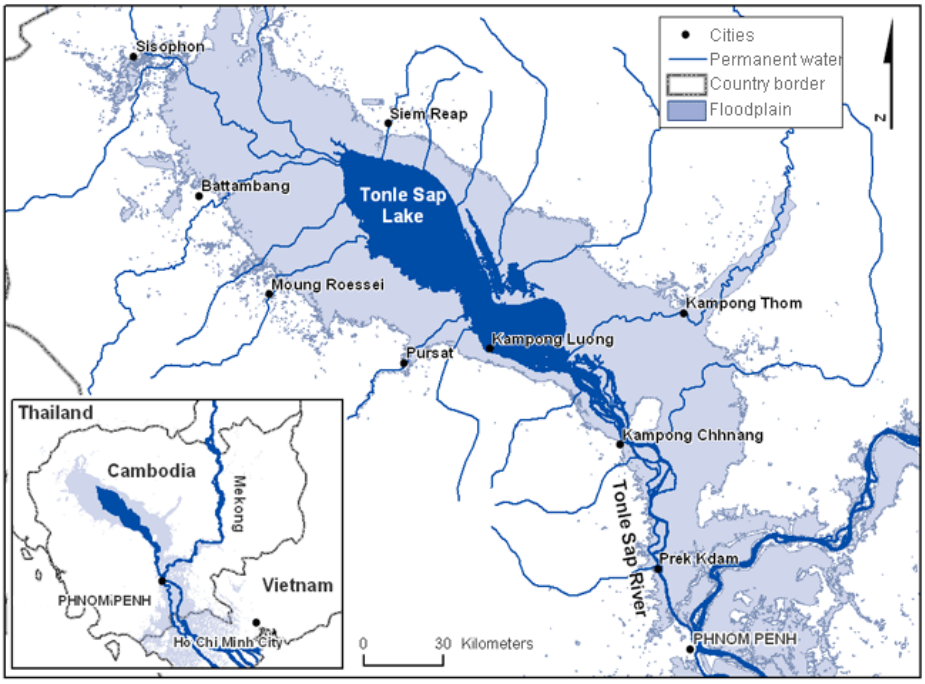

Figure 1 Cambodia, Tonlé Sap area, source: (Mkummu 2006)

2.2 Preservation by smoke

Smoking is one of the oldest food preserving technologies, having been in use for approximately 10,000 years. It is believed that the man hung his catch over the fire to protect it from dogs and so s the preserving effect of smoke was probably discovered (Simko 2002; Šimko 2005). The first evidence of smoking as a technological process dates back 9,000 years to Poland, where the oldest smokehouse was discovered by archaeologists in a Stone Age colony located in Zwierzymec, near Krakow (Ledesma et al. 2017). Other evidence of the adoption of the smoking process for the preservation of meat and fish may be observed in other countries and cultures all over the world (Ledesma et al. 2014). Smoking was widely used mainly for the special organoleptic properties of smoked products and because of its

useful inactivation effect on enzymes and microorganisms (Simko 2002). Only the introduction of cooling systems such as refrigeration in the 1900s reduced smoking, in particularly as a preservation method. The introduction of cooling systems reduced the need for smoking technology, mainly due to the reduction of the delivery time from the production site to the market (McGee 2007). Nowadays smoking is still widely used, and not only in fish processing. According to Stołyhwo & Sikorski (2005), about 15% and 40 – 60% of the total quantity of fish and meat, respectively, for human consumption in Europe is offered on the market in the form of either cold- or hot-smoked products. Since the beginning of traditional, uncontrolled biomass burning, smoking techniques have been increasingly improved and various and specific procedures for treating meat and fish products, related to different regions and cultures, have been developed (Šimko 2005; Ledesma et al. 2016). Currently, we can consider that the technology is mainly used and widely demanded on the market for the enrichment of foods with specific taste, odour, and appearance (Šimko 2005; Ledesma et al. 2016). On the other hand, the role of the preservative effects is in decline, thanks to the latest trends in alternative preservation procedures. Originally, smoke impaired an offflavours and helped to preserve both the fish and its particular flavour. This was achieved by the burning of wood biomass with a qonssequent release of smoke containing compounds with antimicrobial and antioxidant properties. Most smoking technology combines other preservation techniques such as salting or drying. Traditionally, the product is of high salt and low moisture content (Rahman 2007; Zachara et al. 2017). Today's pretreatment techniques are kept at a low level of salt content to extend the shelf life of the fish products up to a few days or weeks (McGee 2007).

Figure 2 Salting as pretreatment of smoked fish products. (Source:FAO (2020))

2.2.1 Smoke generation

Smoke-curing is a fish or meat preservation method carried out through a combination of drying and deposition of naturally produced chemicals such as phenols, aldehydes, acetic acids, and a range of polycyclic aromatic hydrocarbons (PAHs) from smoke by attaching to a food surface with their subsequent migration into a food bulk (Simko 2002). Traditionally, smoke generation is a result of thermal degradation of wood or charcoal, followed by the oxidation of some of the products of pyrolysis under limited oxygen supply (Stumpe-Viksna et al. 2008; Malarut & Vangnai 2018). The generation of wood smoke during curing is a typical example of incomplete combustion (Phillips 1999; Stołyhwo & Sikorski 2005). In general, smoke is a polydisperse mixture of liquid and solid components with diameters of 0.08 - 0.15 µm in the gaseous phase of air, carbon oxide, carbon dioxide, water vapour, methane, and other gases (Šimko 2005; Stołyhwo & Sikorski 2005). The smoke has a variable composition which depends on various conditions, such as the procedure and temperature of smoke generation, the origin and composition of the wood, the water content in the wood, etc. (Simko 2002; Stołyhwo & Sikorski 2005; Duedahl-Olesen et al. 2010; Gomes et al. 2013).

The composition of the smoke and the conditions of treatment affects the sensory quality, shelf life and wholesomeness of the product. The suitability of smoke for treating fish and meat depends primarily on the contents of phenols, since they are mainly responsible for imparting desirable sensory properties to the products and are valuable as antioxidants (Stołyhwo & Sikorski 2005). The most commonly used woods for smoke generation are those termed hardwood (beech, hickory, and oak), but softwoods (pine and fir) are also used. The colour components and sweet scented aroma are mostly provided by the structural materials of wood cells; cellulose and hemicellulose (linear polysaccharides), which when burnt effectively, caramelize and produce carbonyls (McGee 2007; Garcia-Perez 2008; Rowell 2012). Moreover, lignin, a bonding glue of wood cells, a highly complex arrangement of interlocked phenolic molecules, produces a number of distinctive aromatic products when burnt as smoky, spicy, and pungent, antimicrobial compounds such as guaiacol, syringol, PAHs and phenols (Hui et al. 2001; Klemm et al. 2005; McGee 2007; Garcia-Perez 2008). According to Šimko (2005) the wood smoke of different trees may impart a distinct flavour to smoke-cured fish due to their specific ratio of components. The smoke produced at 650 – 700 °C is the richest in components such as phenols and it is able to impart desirable organoleptic

properties to treated products. The thermal degradation of hemicelluloses, cellulose, and lignin of wood proceeds at 180 – 300, 260 – 350, and 300 – 500 °C, respectively (Stołyhwo & Sikorski 2005).

2.2.2 Polycyclic Aromatic Hydrocarbons (PAHs) in smoke

Among its hundreds of components, wood smoke also contains at least 100 polycyclic aromatic hydrocarbons (PAHs) and their alkylated derivatives (Stołyhwo & Sikorski 2005). Although the exact mechanism of the formation of PAHs in grilled/smoked foods is not precisely known, it is generally considered that at least three possible mechanisms exist. The first mechanism is the pyrolysis of organic matter such as fats, protein and carbohydrates at temperatures above 200 °C. PAH formation is favoured at a temperature range of burning biomass between 500 – 900 °C (Knize et al. 1999; Stołyhwo & Sikorski 2005; Alomirah et al. 2011). The second mechanism is by the direct contact of lipid drippings with intense heat directly over the flame. This condition can generate volatile PAHs that, in turn, will adhere to the surface of the food as the smoke rises (Lijinsky 1991; European Commission (EC) 2002; Farhadian et al. 2010; Alomirah et al. 2011). The third mechanism is the incomplete combustion of charcoal which can generate PAHs that are brought onto the surface of the food (Conde et al. 2005; Djinovic et al. 2008; Rey-Salgueiro et al. 2008; Wretling et al. 2010; Lorenzo et al. 2010; Alomirah et al. 2011; Gomes et al. 2013; Hitzel et al. 2013; Pöhlmann et al. 2013; Škaljac et al. 2014; Ledesma et al. 2016).

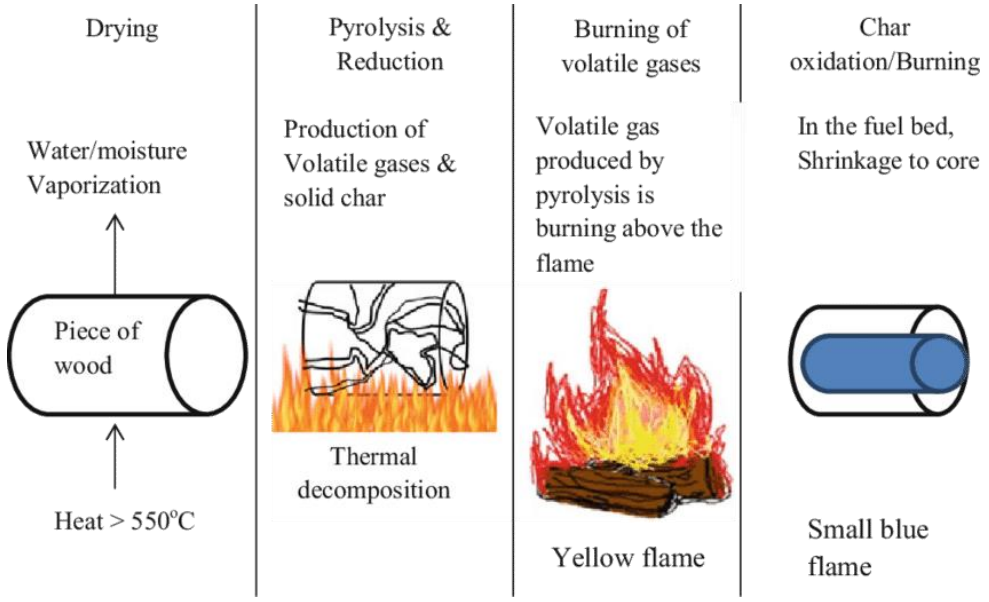

Figure 3 Four stages of combustion of wood. (Source: Chandel & Sukumaran 2017)

PAHs also play an important role in soot formation, but they can appear adsorbed on the soot surface as well as at the gas phase due to their different volatility and molecular weight. After generation, smoke is driven by aerosols into a kiln while its temperature is going down and is accompained by the partial condensation of smoke components (especially compounds with a high boiling point) in pipes, walls and smoke chambers. PAHs and other compounds can also be coveyed by smoke to the fish or meat products being smoked, which eventually become contaminated. The rate of deposition of different components of smoke 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 (Chen & Lin 1997; Šimko 2005; Lund et al. 2009). PAHs produced from wood smoke are known to originate from the thermal pyrolysis (depolymerisation) of lignin and the subsequent condensation of the lignin components in lignocelluloses at temperatures above 350 °C (Garcia-Perez 2008; Nakamura et al. 2008). For instance, according to Sun et al. (2019) and PPRIS (2020), lignocelluloses compositions of hardwoods are cellulose (40 – 50%), hemicellulosis (25 – 35%) and lignin (20 – 25%) and for softwoods: cellulose (45 – 50%), hemicelluloses (25 – 35%) and lignin (23 – 35%) (Sun & Cheng 2002). Nakamura et al. (2008) and Garcia-Perez (2008) found that softwood produces higher PAHs than hardwood when it is burnt at temperatures above 400 °C, explaining this as a result of high lignin content. Therefore, hardwoods rather than softwoods have been recommended for the smoking of products. Similarly, dry woods generate more PAHs because of their higher smoke generation temperature (Guillén et al. 2000; EFSA 2008).

2.2.3 Smoking technology

Smoking technology usually involves multiple steps, such as pre-treatment, smoking and cooling. This is because smoke-curing only affects the surface of the foods. Therefore, other preservation principles were introduced such as salting and drying (McGee 2007). Nowadays, they are almost inseparable. Smoking is traditionally done in a chamber filled with heated smoke. Classification of the smoking technology may be done according to the temperature of smoke, placement of foodstuff and location of fire, or the structure of the smokehouse (Figure 5). Most often, the technology is classified into two. One cooks the product (hot smoking), and the other does not (cold smoking) (Rahman 2007; Ledesma et al. 2016).

Cold-smoking technology

During cold smoking, fish or meat products are hung from shelves or rods in a separate room from the source of heat. Smoke is then led to the chamber where the product is placed and the preservation take place there. When burning finishes, the fire is not always poked and sometimes the smoke is allowed to cool. For cold smoking, smoke temperatures between 15 and 35 °C is used. This low smoke temperature is obtained by regulation of air (Vazquez Troche et al. 2000). The texture and microflora of the final product treated by cold-smoking are relatively unaffected. Therefore this technique is mainly used for the aromatization of uncooked sausages, raw hams and fermented thermally untreated salami (Šimko 2005; Stołyhwo & Sikorski 2005; McGee 2006; Rahman 2007).

Hot-smoking technology

During hot-smoking technology, the chamber is preheated by the burning of wood. Sawdust may then be introduced into the chamber and the fire is stoked with the aim of producing a large amount of smoke (Ledesma et al. 2014). Temperatures of 130 °C for smoke and 80 °C for the fish or meat are needed in hot-smoking (Ahmad 2003; Ledesma et al. 2014), although some authors specify lower temperatures, between 55 and 80 °C for the product (McGee 2007). Food is then placed directly inside the chamber, where the product is heated and dried. This will give it a more or less firm, dry texture, depending on the temperature and time involved, and can kill microbes not just on the surface but also inside the meat, (McGee 2007). Hot-smoking technology is mainly used for the aromatization and thermal treatment of hams, salami, sausages, etc. (Šimko 2005).

Additionally, smoke vapours are deposited onto the surface of the meat as much as seven times faster in hot smoking; however, cold-smoked meats tend to accumulate higher concentrations of sweet-spicy phenolic components and so may have a finer flavour. Cold-smoked meats also tend to accumulate more possible carcinogens. The humidity of the air also makes a difference; smoke vapours are deposited most efficiently onto moist surfaces, so “wet” smoking has a stronger effect in a shorter time.

Nowadays, the modern controlled method occurs in several steps: pretreatment, smoking itself, and cooling. First, pretreatment such as salting (soaking in brine or injection) take place for a few hours or days. The muscle tissue of meat or fish absorb a samll percentage of salt

(not enough to decrease water activity and subsequent microbial growth), and the protein (mainly myosin) from the muscle tissue is extruded to the surface. This process leads to the development of the pellicle at later stage in the process. The pellicle is the “golden” layer on top of the smoked fish from the sticky dissolved myosin (see Figure 4). The colour is formed by browning reaction (Maillard reaction) between aldehydes in the smoke and amino acids from myosin (muscle) in combination with dark resin from the smoke (McGee 2007). Secondly, smoking is done either hot or cold, depending on the product and desirable organoleptic attributes of the final product. Finally cooling achieves the uniform taste and best quality product from the food safety point of view. Smoking is still widely used in fish processing, and it involves using either modern controlled methods or traditional uncontrolled kilns.

Figure 4 Example of "Golden" smoked Osteochilus schlegeli, Tonlé Sap area, Cambodia.

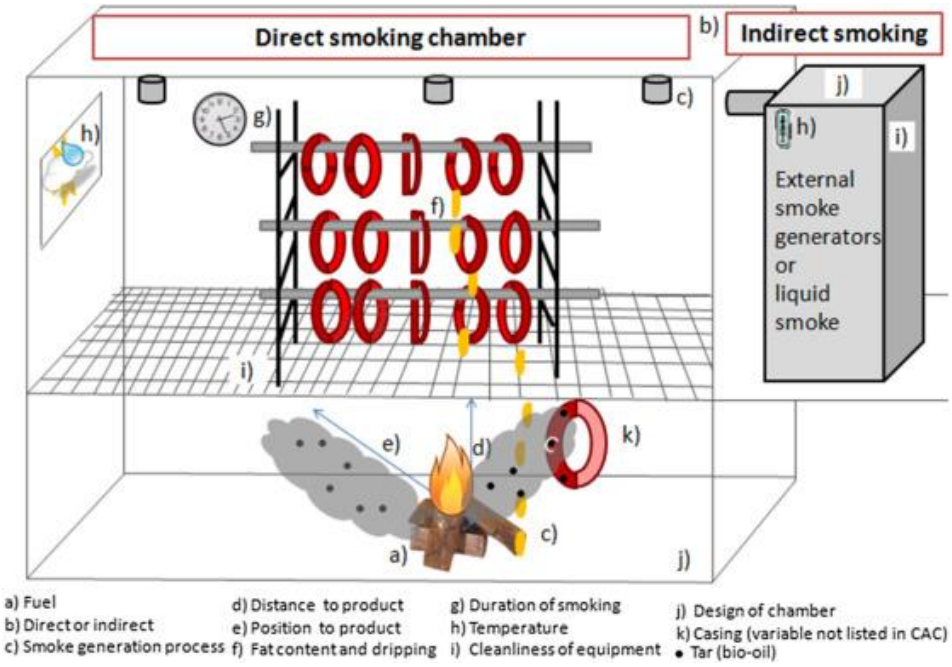

Figure 5 Smoking chamber: Representation of CAC/RCP 68/2009 variables to control polycyclic aromatic hydrocarbons (PAHs) contamination of meat products in direct and indirect smoking processes. (Source: Ledesma et al. 2016)

2.3 **Traditional fish smoking practices in Cambodia**

Cambodia has very few income generating possibilities beyond its natural resources and it is economically almost completely dependent on agriculture, forestry, and fisheries (FAO 2020). Fish and fisheries in Cambodia are essential in providing food security to the people (Clayton et al. 2003). More than 66% of the population in Cambodia is strongly dependent on agriculture, of which freshwater aquaculture is one of the most important sources of food production (Hortle 2007; FAO 2020). Yearly production of fish is about 514,000 t life weight and about 470,000 t is emarked for consumption. Although there is a marked taste preference for fresh inland species of fish by the Cambodian population, large quantities of freshwater fish, and to a lesser extent marine species, are processed for human and animal consumption (Doulman 1993).

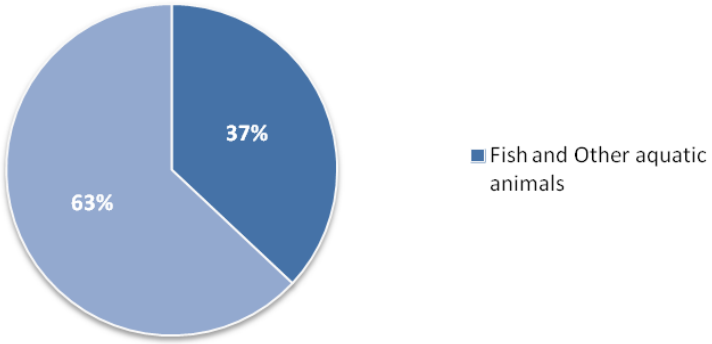

Figure 6 Contribution of fish, fish products, and other aquatic animals to total protein intake in Cambodia. (Source: Vilain et al. 2016)

Fish processing provides a continuous source of protein throughout the year (Tickner 1996). Recent estimates show that proteins obtained from fresh fish and fish products make up to 37% of the total protein intake (see Figure 6) and 76% of the animal protein intake (see Figure 7) (Vilain et al. 2016).

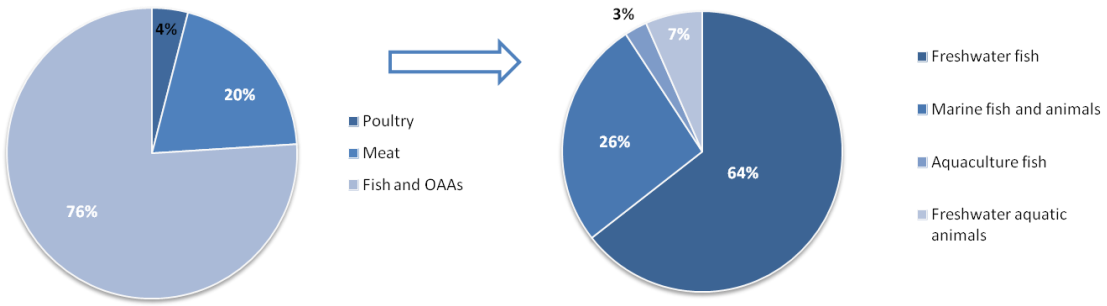

Figure 7 Total animal protein intake and breakdown of fish sub-group contribution to total animal protein intake. (Source: Baran et al. 2016)

Even in relatively less important fishing areas seasonally available fish and other aquatic animals are an essential part of the diet, contributing up to 42 kg per person per year (FAO 2020). Since fish are being caught in a very short peak period, it is necessary to process the fish quickly and in a basic way (Eong & Hariono 2003; FAO 2020). Fresh fish contains up to 80% water by mass and is considered highly perishable material, which results in an extremely short shelf life when left unprocessed (Bala & Mondol 2006). Processing involves a range of basic but effective preservation techniques mainly focusing on decreasing water activity, and the process of spoilage. These techniques include sun-drying, salt-drying, and smoking

(Doulman 1993; Ahmed et al. 1999; Hortle et al. 2004). Even though commercial smoking is currently performed by modern controlled methods that effectively eliminate the incidence of PAHs in the final products, traditional methods of smoking in smokehouses (kilns) are still popular and very common in households or small-scale production. However, smoking under uncontrolled technological conditions and non-existent legislative measures leads to enormous PAH contents in smoked foods (Šimko 2005). Additionally, although the access to electricity of the Cambodian population has increased in the past few years from 44% to almost 90%, based on data from the World Bank (2018). Access to electricity is not evenly distributed across the population and electircity is not used for the purposes of food conservation. Therefore, smoking, as one of the oldest preservation methods, is still widely used in the country (Stołyhwo & Sikorski 2005).

Available literature describes traditional smoking of fish in Cambodia as a series of several processes occuring at the same time. Traditional smoking involves treating pre-salted, sun-dried, whole, eviscerated, or filleted fish with wood or charcoal smoke. The smoke is usually produced by smouldering wood and shavings or sawdust in the oven, directly below the hanging fish or fillets, laid out on mesh trays. Generally, the traditional fish processing establishments are classified as small, medium, and large scale, according to the number of workers and production (Eong & Hariono 2003). The production of fish products is highly affected by seasonality and therefore, traditional ways of processing fish are well adapted to the irregularity of the seasonal fish catch (Vilain & Baran 2016). As a consequence, during the peak season, thousands of people travel to the Tonlé Sap area, the Mekong basin, and other waterways to trade rice for fish, to fish on their own, or to buy small-sized/low value fish to produce fish products (Nam et al. 2009). Due to the seasonality of the fish capture, and the limited use of ice or electricity for conservation, a number of techniques, in fish processing have developed. Therefore, the consumption of processed fish is expected to be high in Cambodia (FAO 2020).

Cambodians are considered one of the highest per capita consumers of freshwater fish globally (Nam et al. 2009; Vilain et al. 2016). Eong and Hariono (2003) reported that about 60% of the total fish were consumed fresh, 18% were fermented, 13% salt-dried, 5% smoked, 2% fish sauce and 2% other derived products (e.g. prahoc – fermented fish paste) (see Figure 8).

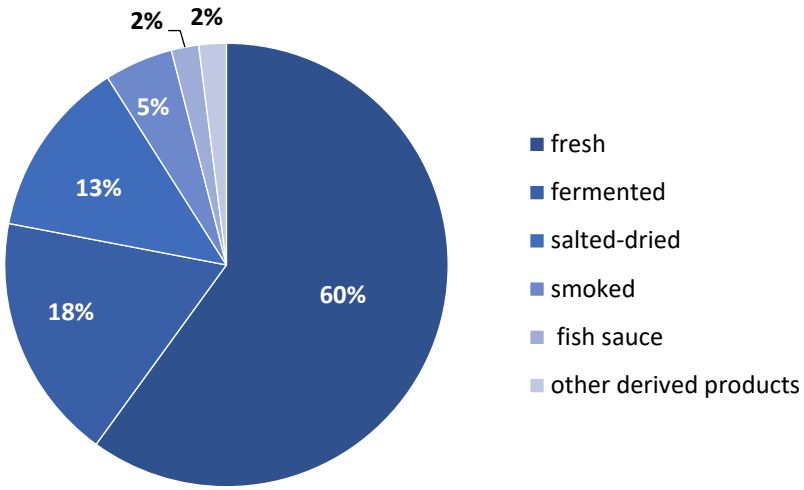

Figure 8 Reported processing techniques and consumation preferences in Cambodia. (Source: (Eong & Hariono 2003))

The wholesomeness of smoked fish products using the traditional kiln depends on the type of wood used for the smoking process, the temperature used, the duration of smoking, the type of kiln used, the proximity of the fish to the fire, the type of fish being smoked and the fat content of the fish. In the case of traditional Cambodian smoke curing, it is possible to regulate all these parameters, except for the smoking temperature, to give a quality smoke-cured fish product. Given the lack of temperature regulating systems in the traditional kilns and little knowledge of the control of smoking temperature to meet quality standards, it is difficult to effectively control smoking temperature, hence the release of toxins like PAHs in the fish product (Phillips 1999; EFSA 2008).

The technology used for the traditional smoking of fish in Cambodia may be considered as "wild", described by Šimko (2005) as: "smoking under uncontrolled technological conditions and non-existent legislative measures, what is typical especially for households and developing countries and leads to enormous PAHs contents in smoked foods"(Afolabi et al. 1983; Alonge 1987, 1988).

Although the final product has low quality, it is a way of handling a large amount of fish during the peak period. In general, two groups of traditional smoking fish processors exist: small-scale and medium-scale. Small-scale fish processing is an activity in households who produce for family consumption. These are people living near the river, fishing lots, lakes, and people who live in upland areas (Nam et al. 2009). Medium-scale is usually fish processing done by households, which work by using family labour, relatives, and some hired labourers during the

peak period (Eong & Hariono 2003; Nam et al. 2009). Their location is usually near the fishing lots, fishing villages, and landing places. The fishing calendar is divided into two seasons: open (October-May) and closed (June-September). The small-scale fishing has an open access during the whole year, with imposed restrictions mainly on fishing efforts, for example, type, number, size, and mesh size of gear, whereas middle and large-scale fishers are allowed to fish only in the open season and require a license issued by the Department of Fisheries (DoF) (Hortle 2007; Vilain & Baran 2016).

Besides traditional smoked fish ("trey Cha-ar") Cambodians also generally produce: dry salted fish, "Pho-ork" (fermented fish), or fish sauce. Since there is a market for sun-dried fish for animal feed, its production has also expanded in the last few years and it is exported to Vietnam (Nam et al. 2009).

Smoked fish is a popular product that is mostly made by the women involved in small or medium-scale fish processing. Main smoking activities are from March to April (Khim et al. 2003). As a primary source of fuel, wood for smoking fish is provided by the flooded forests that surround the Tonlé Sap Lake. In practice, the family-scale fishermen sell their catch to middlemen who collect fish at the fishing ground, in case the market is too far from their house, and some of them sell their catch directly to consumers at their place or bring it to the landing place and sell it to wholesalers (Hortle 2007).

The fish used must be fresh. Any size and weight of fish can be used, but each batch to be smoked must be of a similar type and size. The fish must be well prepared by cutting and cleaning. Before smoking, the fish is usually cleaned and the head and guts are often removed because of aesthetic, sapidity, and contamination reasons (Kawarazuka 2010; Vilain & Baran 2016). Once the fish are gutted, the family threads them in dozens to skewers and then smoked them over an open fire – a task requiring all family members. The fish are skewered using small bamboo sticks about 20 cm long and 5 mm in diameter. The prepared skewers are placed over a low fire on a bamboo frame to dry out in the fish smokehouse or outside to sun-dry. They are placed in vertical rows and a 1 cm gap is left between each skewer to allow for the smoke to circulate (see Figure 9). On average 100 - 150 skewers of fish are placed in the smokehouse together. The fish are smoked for 5 to 6 hours. Once the fish are smoked, it must be dried again by smoking or sun-dried 2 to 3 times a week (Hortle 2007). During the high season one family can smoke about 200 to 300 fish per day, in total weight around 30 kilograms (Ou 2011).

Figure 9 Sun-drying pre-treatment and placement of skewers in the smokehouse, Tonlé Sap area, Cambodia.

2.4 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous pollutants, eco-toxicants that are harmful to human health, with some known to be carcinogenic (Phillips 1999; Vazquez Troche et al. 2000; Kishikawa et al. 2003; Janoszka et al. 2004; Šimko 2005; Stołyhwo & Sikorski 2005; Lage Yusty & Cortizo Daviña 2005; Okuda et al. 2006; Tfouni et al. 2007; Xia et al. 2010; Alomirah et al. 2011; Essumang et al. 2012; Purcaro et al. 2013; Ledesma et al. 2016; Urban & Lesueur 2017; Malarut & Vangnai 2018). PAHs are a class of organic compounds consisting of 2 to 7 fused aromatic rings in a linear, angular, or clustered arrangement (see Figure 10 (Xia et al. 2010; Alomirah et al. 2011). Generally, they are divided into two groups according to their molecular weight, light/low (LMW PAHs) and heavy/high (HMW PAHs) (Alomirah et al. 2011; Nguyen et al. 2020). Low molecular weight PAHs (containing 2 - 3 aromatic rings) are generally considered more volatile than the high molecular weight PAHs (containing more than 3 aromatic rings) (Xia et al. 2010; Alomirah et al. 2011).

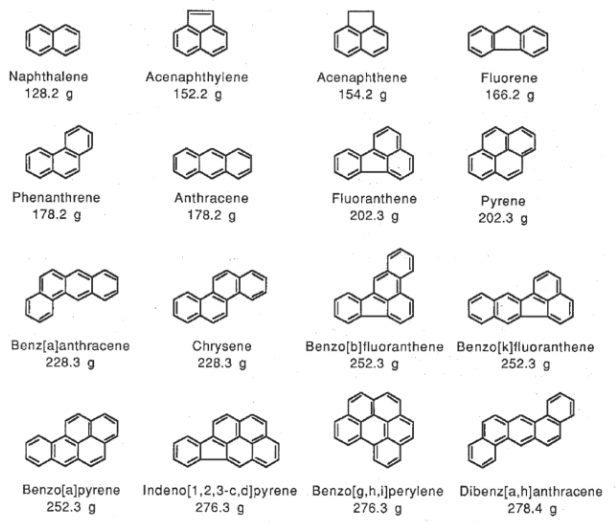

Figure 10 The Polycyclic Aromatic Hydrocarbons with their molecular weight. (Source:Henner et al. 1997)

The origin of PAHs is defined either as anthropogenic such as the exhaust of motor vehicles, petroleum refineries, heating in power plants, combustion of refuse, deposition from sewage, oil/gasoline spills, tobacco smoke, and coke production (Christensen & Bzdusek 2005; Moon et al. 2006; Alomirah et al. 2011; Nguyen et al. 2020), or as incomplete combustion of charcoal or thermal decomposition (pyrolysis) of wood (Conde et al. 2005; Djinovic et al. 2008; Rey-Salgueiro et al. 2008; Lorenzo et al. 2010; Wretling et al. 2010; Gomes et al. 2013; Hitzel et al. 2013; Pöhlmann et al. 2013; Škaljac et al. 2014; Ledesma et al. 2016). The formation of PAHs is alsi known to occur through pyrolysis of fat at temperatures above 200 °C (EFSA 2008), and it is highly stimulated at temperatures over 400 °C (Garcia-Perez 2008; Nakamura et al. 2008; Rose et al. 2015). However, the formation of PAHs during biomass combustion should also be taken into account (Ledesma et al. 2016). PAHs can also be found as tertiary tar products formed during biomass pyrolysis (Basu 2010). Pyrolysis products can be classified as solids (mostly char or carbon), liquids (tar, heavier hydrocarbons, and water) and gases (carbon dioxide - CO_2, water - H_2O, CO – carbon oxide, C_2H_2 - acetylene, C_2H_4 - ethylene, C_2H_6 - ethane, C_6H_6 - benzene, etc.).

Despite their high abundance in the environment, the main route of exposure to human organisms is via food (Phillips 1999; Xia et al. 2010; Alomirah et al. 2011; Gomes et al. 2013; Rengarajan et al. 2015). Foodstuffs usually represent the major source of exposure for non-smokers, although a few instances of direct exposure due to combustion processes have been found (Alomirah et al. 2011; Rose et al. 2015). Their presence in food is usually a consequence of the ubiquitous nature of these compounds in the environment or formation during a cooking process (Rose et al. 2015). PAHs are lipophilic in nature and usually accumulate in the fatty tissues of organisms and consequently in the food chain (Pensado et al. 2005; Bansal & Kim 2015). Chen and Lin (1997) also concluded that smoking time increased contamination by PAHs. Based on the study of Rose et al. (2015), PAHs can be formed during cooking and the amounts produced and occurring in the cooked food depends upon the food type, fuel used, and cooking method. Among various food categories, meat, meat products, and fish are generally most prone to elevated concentrations of PAHs (EFSA 2008; Plaza-Bolaños et al. 2010; Xia et al. 2010; Rose et al. 2015; Singh et al. 2019). Although the fish is not always declared as having the highest concentration, even though it is among the most contaminated,

it often has the highest Benzo[a]pyrene (BaP) compared to other products, probably because of the higherincidence of high molecular weight PAHs.

2.4.1 PAHs presence in smoked fish products

Grimmer and Böhnke found about 100 PAHs and their alkylated derivatives in smoked fish aa early as 1975. Thus, it is a very rich mixture of compounds similar in chemical character, and difficult to analyse, especially if also accompanied by other nonpolar components. Fish, too, contains naturally occurring hydrocarbons, such as squalene $C_{30}H_{50}$, which is abundant, e.g., in some fish oils. These naturally occurring hydrocarbons present in some fish oils behave the same as the analyzed PAHs during the analytical procedures and thus complicate further steps of analysis (Chen & Lin 1997; Reinik et al. 2007; Lund et al. 2009). The normal amount of benzo[a]pyrene in smoked fish is between 0.1 and 1 $\mu g.kg^{-1}$ (Gómez-Guillén et al. 2009). Residual PAHs concentrations in smoked foods are highly variable and result from the use of different smoking methods. Traditional direct smoking, in which the smoke is generated in the same chamber where the product is processed, exposes the foodstuff to higher PAH content than indirect smoking which uses a separate chamber for smoke generation (Akpambang et al. 2011). The highest concentration of PAHs in smoked products is immediately after of smoking is finished, and then it decreases due to light decomposition and interaction with the present compounds (Dennis et al. 1984; Šimko 2005). However, PAHs also penetrate into smoked product bulk, where they are protected from light and oxygen, and after some time, the concentration stabilises at a certain constant level (Šimko 1991). Commission Regulation (EC) No 1881/2006 sets the maximum levels for certain contaminants in foodstuffs, see Table 1 (EFSA 2008). According to this regulation, since 1st September 2014, the limits of benzo[a]pyrene for muscle meat of smoked fish and smoked fishery products, smoked sprats and canned smoked sprats, and bivalve molluscs (smoked) were defined as the following: 2.0 $\mu g.kg^{-1}$; 5.0 $\mu g.kg^{-1}$; 6.00 $\mu g.kg^{-1}$ of benzo[a]pyrene and 12.0 $\mu g.kg^{-1}$; 30.0 $\mu g.kg^{-1}$; 35.0 $\mu g.kg^{-1}$ sum of benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene and chrysene (ΣPAH4), respectively (see Table 1). European Commission Regulation was selected for its complex, detailed and regularly updated evidence base set of data, for comparaison of PAH conent in smoke-cured fish. Fat-binding PAHs are capable of accumulating in the food chain (McLachlan 1997; Roeder et al. 1998) and therefore the amount of PAHs per gram of fish consumed is essential data to help advise on the long-term implications for human health.

Table 1 Limits given by European Commission regulation No. 1881/2006 for selected foodstuffs. (Source: EFSA 2008)

		Maximum levels *(µg.kg^{-1})*	
	Foodstuff	**BaP**	**ΣPAH4**
6.1.4	Smoked meat and smoked meat products	**5.0** until 31.8.2014 **2,0** as from 1.9.2014	**30.0** as from 1.9.2012 until 31.8.2014 **12.0** as from 1.9.2014
6.1.5	Muscle meat of smoked fish and smoked fishery products, excluding fishery products listed in points 6.1.6 and 6.1.7. The maximum level for smoked crustaceans applies to muscle meat from appendages and abdomen. In the case of smoked crabs and crab-like crustaceans (*Brachyura* and *Anomura*) it applies to muscle meat from appendages.	**5.0** until 31.8.2014 **2.0** as from 1.9.2014	**30.0** as from 1.9.2012 until 31.8.2014 **12.0** as from 1.9.2014
6.1.6	Smoked sprats and canned smoked sprats (*sprattus sprattus*); bivalve molluscs (fresh, chilled, or frozen); heat-treated meat and heat treated meat products sold to the final consumer	**5.0**	**30.0**
6.1.7	Bivalve molluscs (smoked)	**6.0**	**35.0**

2.4.2 Health risks related to dietary exposure to PAHs

PAHs are of significant concern primarily because of their ubiquitous presence in the environment and well-recognized carcinogenicity, teratogenicity, and mutagenicity (Tobiszewski & Namieśnik 2012). According to the Scientific Committee on Food (2002), 15 PAHs ‘‘show clear evidence of mutagenicity/genotoxicity in somatic cells in experimental animals in vivo. They may be regarded as potentially genotoxic and carcinogenic to humans’’; their carcinogenicity depends on their structure (Alomirah et al. 2011; Essumang et al. 2012; Rose et al. 2015; Ledesma et al. 2016). Carcinogenic risks are estimated as the incremental probability of an individual to develop cancer over a lifetime as a result of exposure to a potential carcinogen (IELCR, or just carcinogenic risk) (Essumang 2010). Based on the SCF “Opinion on the risk to human health from PAH in food”, the European Commission first established levels of benzo[a]pyrene (BaP) in a number of food types including smoked meat and fish in 2005 (EC 2005), accepting BaP as a marker of carcinogenic PAHs (Stołyhwo &

Sikorski 2005). In 2008, EFSA (European Food Safety Authority) published an opinion on PAHs (EFSA 2008) and concluded that BaP alone was not a suitable general marker for PAHs in food but identified a group of 4 PAHs (PAH4), and a group of 8 PAHs (PAH8) as better indicators based on data relating to occurrence and toxicity. Based on the EFSA opinion, in 2011, the European Commission extended the scope of the regulation to include other types of food and to add limits for PAH4 (EC 2011). According to the latest classification on the carcinogenicity of PAHs by IARC monograph, it has been established that benzo[a]pyrene is defined as carcinogenic (group 1), dibenzo[a,h]anthracene is probably carcinogenic (group 2A). In contrast, naphthalene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[j]fluoranthene and indenol[1,2,3-c,d]pyrene are classified as possible human carcinogens (group 2B), (Essumang et al. 2012). Wood smoke has also been classified by the IARC (2012) monograph as certainly carcinogenic (group 1).

Potential health hazards associated with smoked foods may be caused by the carcinogenic components of wood smoke – mainly PAHs and derivatives of PAHs. Carcinogenic, mutagenic and bio-accumulative effects of PAHs as well as their occurrence and toxicity have been reported by several institutions concerned with public health, food security and safety such as: Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO), the International Agency for Research on Cancer (IARC), the European Scientific Committee on Food (SCF), the European Food Safety Authority (EFSA) and the US Environmental Protection Agency (EPA) (European Commission (EC) 2002; EFSA 2008). Recently discussed consequences on health after PAH exposure were reported as growth retardation, low birth weight, small head circumference, low IQ, damaged DNA in unborn children and disruption of endocrine systems, such as estrogens, thyroid and steroids (Shen et al. 2013). Skin changes (thickening, darkening, and pimples) and reproductive-related effects such as early menopause due to destruction of ova; have also been connected to exposure to PAHs (Essumang et al. 2012). It is due to the binding to cellular macromolecules in mammalian cells, including DNA, where PAHs undergo metabolic activation to diol and epoxides, thereby causing errors in DNA replication and mutations, that the carcinogenic process is initiated (Lijinsky 1991; Phillips 1999; Alomirah et al. 2011).

According to WHO (the World Health Organisation) Noncommunicable Diseases (NCD) Country Profiles from 2018 (WHO 2018), the incidence of cancers in the Cambodian

population increased from 2014 to 2018 by 1 percent to 14% (see Figure 11). Although PAHs are not the only causes of cancer in Cambodia, NCD Country Profiles also highlight ambient air pollution (Exceedance of WHO guidelines level for annual PM2.5 concentration) and household air pollution (population with primary reliance on polluting fuels and technologies) which was 82% in 2016, where PAHs played a significant role (WHO 2018).

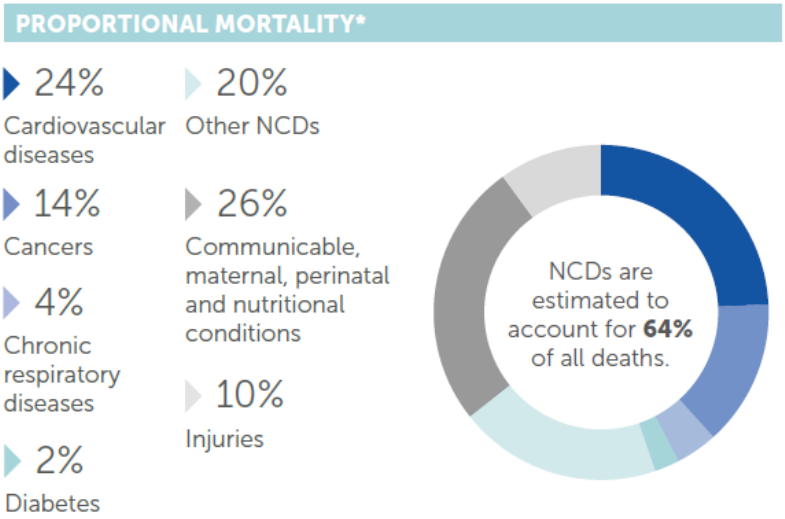

Figure 11 Proportional mortality from Noncommunicable Diseases for Cambodia, data 2018. (Source: WHO 2018)

The PAHs content in foods has been commonly considered as not affected by environmental factors and additional operations, e.g., cooking or even packaging. However, photodegradation of PAHs by UV light is possible, and the formation of oxidative products (such as aromatic alcohols, ketones, quinones, and ethers) has already been proven (Simko 2002; Chen & Chen 2005; Nguyen et al. 2020). However, in spite of the decreased BaP content, the total toxicity of the PAHs might be even elevated due to the presence of oxidized PAHs compounds. In an attempt to reduce PAH levels in charcoal grilled meat, two treatments, preheating (steam and microwave) and wrapping (aluminium foil and banana leaf) have been investigated. Using these pretreatments before charcoal grilling resulted in reduced levels of carcinogenic PAHs in grilled meat samples (Farhadian et al. 2010; Alomirah et al. 2011). Also, as Lijinsky (1991) stated, simple processing practices are known to result in a significantly reduced contamination of foods by PAHs as well as by other undesirable contaminants. This may include selecting preferentially lean meat and fish, avoiding contact of foods with flames for barbecuing, using less fat for grilling, and, in general, cooking at lower temperatures for a

longer time. Broiling (using a heat source above the product) instead of grilling can significantly reduce PAH levels. Variation in PAH levels in foods, apart from the analytical discrepancy, is mainly due to the type and fat content of the food, cooking process (fried, grilled, roasted, boiled and smoked), temperature and duration of cooking, type of fuel used (electricity, gas, wood, and charcoal) and proximity and direct contact with heat source (Farhadian et al. 2010; Akpambang et al. 2011; Alomirah et al. 2011; Rose et al. 2015).

2.4.3 PAH Determination

Part of the thesis consists of the optimisation of methodology of analysis of PAHs for gas chromatography–mass spectrometry (GC-MS). Therefore in the following chapter a short introduction and description of the analytical method is presented with a focus on used sample extraction, clean-up and pre-concentration. In the chapter 4 Materials and methods references and detailed description of method are provided.

At present, chromatographic techniques, mainly gas chromatography (GC) and high-performance liquid chromatography (HPLC), are the dominant effective analytical tools capable of separating individual isomers of PAH fraction to be isolated from both smoked fish meat and liquid smoke flavourings (LSF) matrix (Chen et al. 1997; Guillén et al. 2000; Simko 2002; Jira 2004; Tamakawa 2004; Šimko 2005).

The most common approach for the determination of PAHs in fatty foods involves the saponification of lipids by methanolic or ethanolic KOH or NaOH solution, followed by the isolation of the PAHs by liquid-liquid extraction (LLE) with cyclohexane, hexane, dichloromethane or its mixtures. The obtained extracts are then cleaned up using gel - permeation chromatography (GPC), solid phase extraction (SPE), or adsorption chromatography with the use of silica or Florisil sorbents. For the detection and quantification of PAHs, gas chromatography with mass spectrometry (GC-MS) and high-performance liquid chromatography with fluorometric detection (HPLC-FLD) are usually used (Rose et al. 2007; Purcaro et al. 2013; Silva et al. 2017; Slámová et al. 2017; Urban & Lesueur 2017; Zachara et al. 2017). Quantification of polycyclic aromatic hydrocarbons (PAHs) in smoked fish products often requires multiple clean-up steps to remove fats and other compounds that may interfere with the chemical analysis (Lund et al. 2009).

With subsequent detection and quantification by mass spectrometry (MS) or fluorescence detector (FLD), respectively, it is possible to determine individual PAHs in smoked foods at concentrations of the order of 0.1 $\mu g.kg^{-1}$ or even 0.01 $\mu g.kg^{-1}$, because of the very low contents of individual PAHs in foods, of the order of 1 $\mu g.kg^{-1}$, and the requirement to determine BaP, with a reproducibility not lower than 48% of the value tolerated in the products (EFSA 2008). The efficiency of extraction of PAHs depends upon the polarity of the solvent, on the nature of the matrix, and on the preparation of the sample (Stołyhwo & Sikorski 2005; Ghasemzadeh-Mohammadi et al. 2012; Purcaro et al. 2013). It has been shown by Grimmer and Böhnke (1975) that alkaline hydrolysis of samples previously extracted with boiling methanol increased (about 3-fold) the total recovery of PAHs from meat (Simko 2002; Stumpe-Viksna et al. 2008; Essumang et al. 2012). On the other hand, prolonged alkaline hydrolysis may lead to some loss of BaP due to degradation (Takatsuki et al. 1985). There is a significant correlation between the fish lipid content and the total PAH levels (Chen & Chen 2005; Akpambang et al. 2011; Rose et al. 2015). At present, there is still no official procedure accepted by all concerned organisations which would solve the difficulties associated with the quantitative isolation of PAHs from the food material, clean-up of the extract without significant loss of the analytes, and separation of all individual PAHs contained in the purified extract, including the detection of the separated components, unequivocal identification of the PAHs, and quantification of the identified compounds.

Depending on the final determination method, the low levels of PAHs sometimes require the application of an extract pre-concentration step, such as, e.g., evaporation in a stream of nitrogen and dissolution of the residues in a small volume of solvent that will then be injected into the chromatographic system. However, in the case of lighter PAHs, the stream of gas can lead to the loss of analytes.

QuEChERS method

The QuEChERS (quick, easy, cheap, effective, rugged, safe) method is another concept that can be applied to the determination of PAHs in fatty food samples. It is characterized by quick extraction and purification times, as well as low solvent consumption. In the clean-up step, mainly PSA (primary secondary amine), C18 (octadecyl), and Z-Sep (zirconium dioxide-based) sorbents are used for the fat removal, but also an implementation of freezing out has been reported in the literature (Rejczak & Tuzimski 2015; Sadowska-Rociek et al. 2016; Kim et al.

2019). However, these modifications might be insufficient to achieve adequate sample clean-up, and additionally these sorbents can exhibit non-selective interactions with analytes resulting in the loss of analysed compounds.

EMR-Lipid

Recently, a new material “enhanced matrix removal” (EMR-Lipid), has been proposed for fat removal from fat-rich food products. The structure of EMR-Lipid is a proprietary secret, and it does not function as a conventional sorbent, but dissolves to saturation in a sample extract solution. Its mechanism is said to involve both size exclusion and hydrophobic interactions. Long-chain hydrocarbons associated with lipids fit within the EMR-Lipid structure, where they are trapped. The EMR-Lipid complex is either precipitated out of the solution or remains in the aqueous phase during the final salting-out step (Lucas & Zhao 2015; Han et al. 2016). The manufacturer claims that EMR-Lipid selectively removes lipids from QuEChERS extracts without loss of analytes (Huang et al. 2019).

Dispersive Liquid–Liquid Microextraction (DLLME)

An alternative to evaporation by nitrogen is the direct transfer of analytes from the extract into a small volume of another non-miscible solvent. This approach is used in the dispersive liquid-liquid microextraction (DLLME) method that is based on the system of three solvents: aqueous sample, dispersive solvent, and extraction solvent. The mixture of an extraction solvent (e.g., chloroform) and a dispersive solvent (a water-organic miscible solvent e.g., acetonitrile) is rapidly injected into an aqueous sample, forming a cloudy solution. After centrifugation, the analytes are pre-concentrated into the phase of extraction solvent (Viñas et al. 2014; Kamankesh et al. 2015). Until now, DLLME has demonstrated promising results in extract preconcentration without any loss of analytes, also in the case of the determination of PAHs in food samples (Sadowska-Rociek et al. 2016; Petrarca & Godoy 2018).

2.5 Lipids

Lipids are one of the macronutrients which, together with carbohydrates and proteins, are essential to human nutrition. For this reason, lipid analyses are performed routinely in many different research areas. Generally, lipids cnsists of the following groups: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides (carbanion-based) and/or prenol lipids and sterol lipids (carbocation-based) (Sargent et al. 2002; Fahy et

al. 2009). All the aforementioned classes, except cholesterol, contain fatty acids, esterified to alcohol groups (glycerides), and amino groups (sphingolipids). According to Gurr et al. (2016), lipids are defined based on their solubility properties, not primarily on their chemical structure. It also denotes a chemically heterogeneous group of substances having in common that they are insoluble in water, and soluble in organic solvents such as chloroform, ether or benzene (Gurr et al. 2016). It also contains long-chain hydrocarbon groups in their molecules and are present in or derived from living organisms. Another classification is based on the physical properties at room temperature, fats being solid and oils liquid, their polarity (polar and neutral lipids) and their essentiality for humans (essential and non-essential) (Sargent et al. 2002). Designation of fatty acids is based on their chain length, degree of unsaturation (number of double bonds), and position of the double bonds.

As is evident, the issue of lipids is complex, and would be sufficient for the entire thesis or a book, therefore for the purpose of this literature review we will focus only on fats, triacylglycerols.

2.5.1 Fatty acids, Fats, triacylglycerols (TAG)

Fatty acids (FA) are the basis of lipid molecules and have a crucial role in all living organisms, being one of the main constituents of cellular membranes. The creation of fatty acids is made by so-called fatty acid synthesis from acetyl-CoA and NADPH through the action of enzymes called fatty acid synthases (see Figure 12). This process occurs in the cytoplasm of the cell. Most of the acetyl-CoA which is converted into fatty acids is derived from carbohydrates via the glycolytic pathway. Fatty acids are accumulated as energy reserves and transported and metabolised for different final purposes.

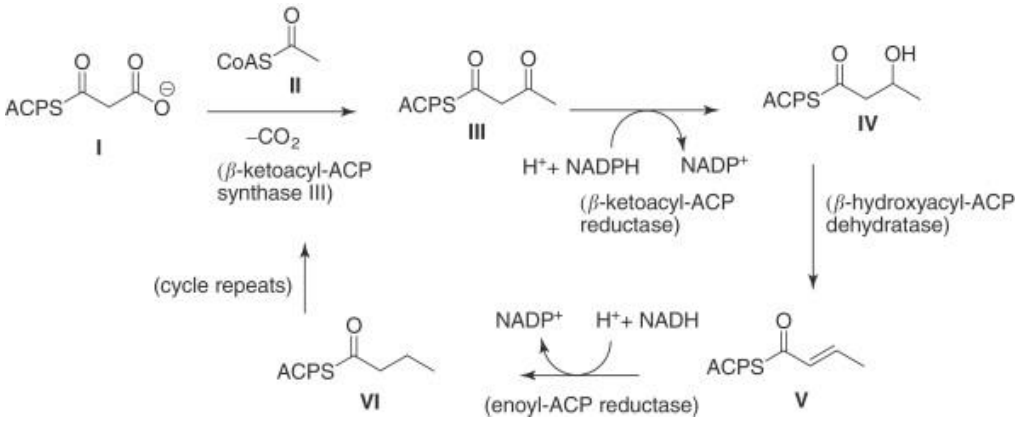

Figure 12 Schema of fatty acid synthesis. (Source: Casida 2010)

The terms “fats” and “lipids” are often used interchangeably. Fats are generally substances clearly fatty in nature, greasy in texture and immiscible with water. Natural fats and oils are composed predominantly of esters of the three-carbon alcohol - glycerol with fatty acids, often referred to as “acyl lipids” (Gurr et al. 2016). Fats and oils are also called triacylglycerols (TAG) because they consist of three fatty acids joined to glycerol, a trihydroxy alcohol (see Figure 13). If all three OH groups of glycerol are joined by three same fatty acids (FA), this results in simple TAG (McMurry 2004; Gurr et al. 2016). However, natural oils and fats are typically built of so-called mixed TAGs, which consist of two to three different FAs.

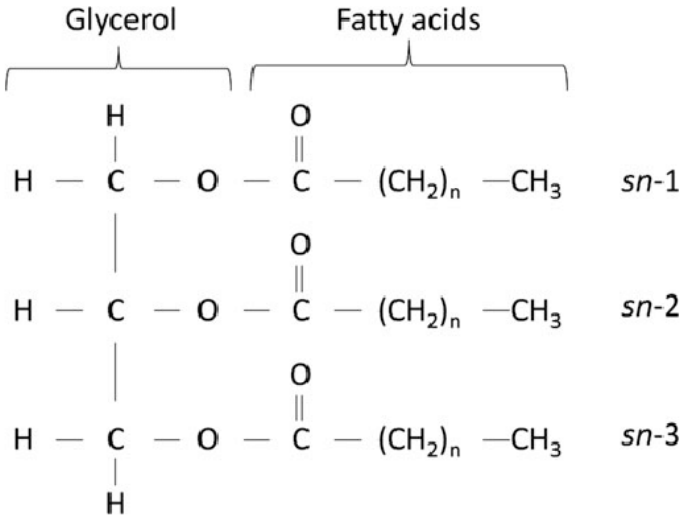

Figure 13 Structure of triacylglycerols. (Source: Mills et al. 2017)

TAGs, which are solid at room temperature (25 °C), are called fats, on the other hand, oils are TAGs that are liquid at room temperature. Due to this fact, TAGs gained from animal sources we usually call “fats” and TAGs from plant sources are commonly called “oils”. Pure fats and oils can be described as tasteless, odourless, and colourless. However, animal fats and vegetable oils are known more for their specific sensory characteristics, caused by foreign lipid soluble substances, such as colourants and volatile compounds (Gurr et al. 2016). Furthermore, fats are categorized according to a number of double or triple bonds between carbons in the aliphatic chain. Saturated fats (SFA) do not contain double or triple bonds. Unsaturated fats (UFA) contain one or more double bonds (Sargent et al. 2002; Fahy et al. 2009; Gurr et al. 2016). Configuration of carbons in double bonding by *cis*-*trans* isomerism is the subsequent sorting of unsaturated fats. Generally, *Cis*-FA’s are commonly found in nature, compared to *trans*-FA’s. Unsaturated FAs are further divided into monounsaturated (MUFAs)

and polyunsaturated fatty acids (PUFAs). Essential fatty acids, which cannot be produced in the human body, include linoleic acid (C18:2 n-6) and alpha-linolenic acid (C18:3 n-3) and are required for normal growth and development as well as the physiological function of body systems (Moghadasian & Shahidi 2017). Fats and oils, which are nutritionally a major source of energy, are present naturally in many foods such as dairy products, meats, poultry, fish, and nuts.

Saturated FAs (SFA)

Saturated fatty acids are not essential and contain only single carbon–carbon bonds in the aliphatic chain, while all other available bonds are taken up by hydrogen atoms. SFAs are the simplest of all FAs. They are chemically low reactive, and their melting point increases with chain length. Typical for SFAs are chains of 12-24 carbons long, but there are several biochemically important FAs with shorter chains, for instance, butyric (C4:0) and caproic (C6:0) acids well known as FAs found in milk. Saturated FAs are predominantly found in butter, margarine, coconut, and palm oils, as well as foods of animal origin, and their excessive intake can raise cholesterol levels (Moghadasian & Shahidi 2017).

Unsaturated FAs (UFA)

Unsaturated FAs are specific by one or more double bonds between carbon atoms, signifying that the number of bonded hydrogen atoms is not maximum. When a FA has only one double bond, it is called monounsaturated (MUFA) and when there are two or more double bonds, it is called polyunsaturated (PUFA) (Moghadasian & Shahidi 2017).

There are up to one hundred naturally occurring MUFA, but most of them are very rare compounds. The most nutritionally important MUFA is oleic acid. MUFA sources are olive and canola oil, avocados, peanuts, nuts, and seeds. They have certain health benefits over saturated fatty acids, and it is currently recommended that MUFA and PUFA sources are consumed more frequently than foods rich in saturated fat or trans-fat but within the restriction of 20 – 35% of total calories from fat (Moghadasian & Shahidi 2017). Because MUFAs are present in relatively high amounts in the traditional Mediterranean diet, mainly due to the high intake of olives and olive oil, and because regions consuming this diet generally have lower rates of CVD (Cardiovascular diseases), it has been speculated that MUFAs are cardioprotective. Recent studies have reported a significant negative association between

regular consumption of Mediterranean diet and cancer and other chronic disease risk factors (Moghadasian & Shahidi 2017). However, this effect is most likely caused by other associated factors such as high consumption of fruit, replacement of animal-based fats, etc.

The major classes of PUFAs are the omega-3 (n-3) and omega-6 (n-6) fatty acids. Omega-3 fatty acids are a type of PUFA containing more than two double bonds. They differ from other fatty acids because of the location of the first double bond in the aliphatic chain. The omega-3 fatty acids that are most important nutritionally are alpha-linolenic acid (ALA), which is essential, as well as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The human body can synthesize EPA and DHA in minimal amounts from ALA. Dietary sources of omega-3 FAs are flaxseed oil, canola oil, soybean oil, walnuts, and seafoods, particularly fatty fish. The most common omega-6 essential fatty acid is linoleic acid, which is found in many vegetable oils, cereals, snack foods, and baked goods. Omega-6 FAs are recommended for lower intake than omega-3 FAs (Council 1988; Moghadasian & Shahidi 2017) but the ratio should be within the ideal range between 4:1 and 1:4 (Simopoulos 2002), or 3:1 to 1:1 as presented by another author (Kim et al. 2007). However, more important is the absolute amount of omega-3 and omega-6 FAs.

2.5.1 Fat content in fish

Fish is one of the best sources of animal protein due to the composition of fish protein compared to those of other animals. Fish contains more favourable amino acid composition and significant amounts of free amino acids, as well as all essential amino acids the human body needs (Vladau et al. 2008). The vitamin and mineral content in freshwater fish meat is very favourable (Özyurt et al. 2009). Fish and shellfish also provide an almost unlimited variety of fatty acids with beneficial effects on human health (Ackman 2000; Guler et al. 2008). The potential health benefits related to fish consumption are due to the presence of proteins, unsaturated essential fatty acids, minerals, and vitamins (Guler et al. 2008). Fish lipids are well known to be rich in long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA), especially eicosapentaenoic acid (EPA or 20:5n-3) and docosahexaenoic acid (DHA or 22:6n-3) (Alasalvar et al. 2002; Regulska-Ilow et al. 2013). Over the past decades, evidence for the health benefits of long chain (LC) omega-3 (n-3) polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3), has been increasing. These health benefits are mainly related to maintaining normal brain function,

vision, and cardiac function (EFSA 2008). However, essential EPA and DHA are originally synthesised by microalgae, not by fish. When fish consume phytoplankton that consume microalgae, they accumulate the omega-3s in their tissues. Long chain omega-3 PUFA cannot be synthesised by humans and must be obtained from the diet (Alasalvar et al. 2002; Guler et al. 2008). However, the consumption of these products is inadequate in most Western countries (EFSA 2008; Yesiltas et al. 2021). Moreover, LC n-3 PUFAs are highly prone to oxidation (Frankel et al. 2012). The lipid content, type, and amount of fatty acids in fish tissue differs depending on the condition of fish such as size or age, season or reproductive status, and what the fish is fed with (Ackman 1989; Murray & Burt 2001; Alasalvar et al. 2002; Guler et al. 2008; Ćirković et al. 2011). The fatty acid composition is further influenced by temperature. Therefore, seasonal changes affect the FA profile of fish (Guler et al. 2008). Generally, the composition of saturated and unsaturated fatty acids in fish is 15 - 36% saturated FAs (Ackman 1989; Buchtová et al. 2007; Zakęś et al. 2010) and 58 - 85% unsaturated FAs (Domaizon et al. 2000; Caballero et al. 2002). Taking all species into account, the fat content of fish can vary much more widely than the moisture, protein, or mineral content. While the ratio of the highest to the lowest value of protein or water content encountered is not more than three to one, the balance between the highest and lowest fat values is more than 300 to one (Murray & Burt 2001). Generally, fresh fish contains 0.1 - 22% of fat in wet weight according to Abraha et al. (2018), another study from Emre et al. (2018) reported that fat content as 4.57 – 21.29% depending on the season. In addition, freshwater fish are often lower in cholesterol than marine fish, although levels depend on the species. Therefore, freshwater fish in the diet is more favourable for human health (Moreira et al. 2001; Luzia et al. 2003; Ćirković et al. 2011).

According to a study by Kaya (2008), during the smoking process, the temperature and wood smoke components negatively affect the fatty acid composition, especially EPA, DHA, and some essential amino acids. On the other hand, a study by Rahimabadi and Faralizadeh (2016) shows the opposite results, with the amounts of EPA and DHA increasing after smoking. However, this increasing trend of essential fatty acids is in conflict with other studies from Beltrfin and Universitaria (1991) and Swastawati (2004). Thermal treatment reduces the water and fat content of fish meat. During the smoking process, fats and water drip from the fish resulting in the physical loss of lipids, protein, and micronutrients (Kiczorowska et al. 2019).

However, the fat loss phenomenon was more intensive in boiled and solar dried fish than in smoked samples, although fat may be excluded with moisture evaporation through extended heat treatment (Abraha et al. 2018).

2.5.2 Fat content determination

In this thesis determination of fat content by modified microquantity colorimetric sulfophosphovanillan method (SPV) has been used. Therefore in the following chapter a short introduction and description of analytical methods are presented. In the chapter 4 Materials and methods references and detailed description of method used are provided.

Lipids are an essential group of compounds that aid several biological functions such as energy storage, cell membrane structure, and signalling (Wang 2004; Wymann & Schneiter 2008). For this reason, lipid analyses are performed routinely in many different research areas. There are different extraction methods for isolating lipids from tissues using various solvents or mixtures of solvents. One of the most widely used methods for the extraction of lipids is that proposed by Soxhlet (Horwitz & Albert 2006). This method is simple and efficient, but a disadvantage is its time duration and use of large amounts of solvent, which is usually petroleum ether. Other methods were proposed by Hara & Radin (1978), as well as other contemporary extraction methods such as pressurized liquid extraction (PLE) and accelerated solvent extraction (ASE) (Schäfer 1998; Dodds et al. 2009). Some researchers have prepared *in situ* direct methylation of FAs without lipid extraction and purification steps (Carrapiso & García 2000; Meier et al. 2006; Polak et al. 2008; Ichihara & Fukubayashi 2010).

Standard lipid analyses require large numbers of samples. However, with improved technological and analytical capabilities, “microquantity” approaches that require only micrograms of the sample and microlitres of solvents for estimating lipid content, have been developed. These micro-methods would reduce this need.

Microquantity colorimetric sulfophosphovanillan (SPV) method

One example is the SPV reaction (microquantity colorimetric sulfophosphovanillan method). It is performed in two steps, an initial reaction of the lipids with concentrated sulphuric acid at high temperature followed by a second reaction of the derived products with vanillin in the presence of phosphoric acid. Consensus understanding is that a positive SPV reaction requires the presence of double bonds or free hydroxyl groups within the lipid analytes (Knight et al.

1972; Johnson et al. 1977). The chemical reactions are complex and are thought to involve the formation of relatively stable (up to several hours) (Johnson et al. 1977) carbonium ion (or carbo cation) chromogens (alkenyl cations) in the initial reaction followed by generation of a pink chromophore upon the addition of vanillin to the reaction (Frings et al. 1972; Knight et al. 1972; Johnson et al. 1977; Inouye & Lotufo 2006; Yi & Jean 2011; Anschau et al. 2017).

2.6 Fish species in Tonlé Sap area, Cambodia

In Cambodia, there can be found more than 500 fish species from the 1,200 indigenous species of the Mekong Basin (Rainboth 1996; Roos et al. 2007) and fish from floodplains and rivers is a basic in the daily diet for millions of people (Roos et al. 2007). Cambodia also features 468 marine species and 26 species that can live in both environments. Fish plays a fundamental role in providing livelihood, income, and food security for large population groups in the densely populated Mekong river basin (Van Zalinge et al. 2000). The following fish species were found as commonly processed by traditional Cambodian smoke-curing. These fish species are smoked by small-scale producers in provinces around Tonlé Sap lake. Small fish species are generally less preferred than large species and therefore have a low market value and are more accessible to the poor. Khim et al. (2003) reported that most full-time fishers are very poor (85.37%) based on wealth ranking and poor (34.18%). Small, low-valued fish species are therefore likely to be the main or only animal food in the diet of the Cambodian population (Roos et al. 2007) and source of raw material for smoking.

Family Belonidae

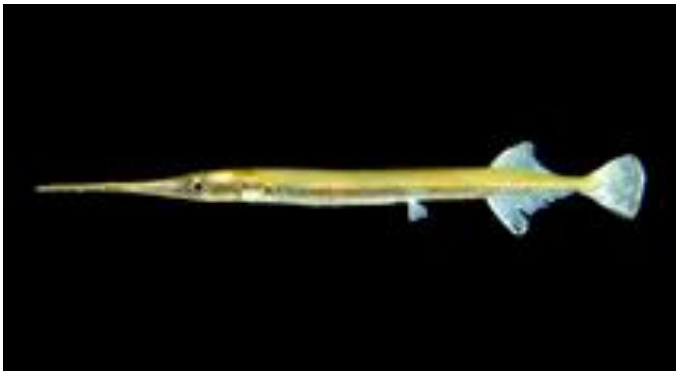

Figure 14 *Xenentodon cancila*. (Source: Froese & Pauly 2000)

Scientific name: *Xenentodon cancila* (Hamilton 1822)

Family: Belonidae

Local name/English name: Trey phtoung/Freshwater garfish

Xenentodon cancila is a species of needlefish found in freshwater and brackish habitats in South and Southeast Asia particularly in Sri Lanka and India eastward to the Mekong. It is found most commonly at the surface in sluggish or standing waters. Moreover, it is considered a popular aquarium fish (Talwar & Jhingran 1991). In common with other needlefish, this species has an elongated body with long, beak-like jaws filled with teeth. The dorsal and anal fins are positioned far back along the body close to the tail (Talwar & Jhingran 1991). The body is silvery-green, darker above and lighter below with a dark band running horizontally along the flank. This needle fish feeds on small fishes and insects (Rainboth 1996). Slight sexual dimorphism exists for this species, the male fish often have an anal and dorsal fins with a black edge (Talwar & Jhingran 1991).

Family Clariidae

Figure 15 *Clarias batrachus*. (Source: Froese & Pauly 2000)

Scientific name: Clarias *spp.* (Günther 1864)

Family: Clariidae

Local/English names: Trey andaing toun/ Walking catfish

Clarias *spp.* is usually found in rivers, lowland streams, ponds, swamps, rice paddies, and pools after floods or standing waters. It can reach a length up to 120 cm. All species of Clarias genus are freshwater fishes and are a type of air-breathing catfish. This means that the fish can breathe either under water, taking oxygen from water, or from air. This gained advantage can be useful during the dry season when the water resources are dried out and the fish needs to get back to the water. For these purposes, Clarias developed air-breathing organs next to regular gills. This special organ is only used when Clarias does not have enough water for breathing (Frimodt 1995). The ability to breathe air can be from several hours to a few days as long as the fish's skin is moist. This is considered an advantage for sellers who sell these fish alive and thus prolong the freshness of the "goods" over already killed fish species (Rainboth 1996). *Clarias batrachus* is also capable of moving on land by wriggling from side to side on its erect pectoral fins, helping them to move towards the source of water during the dry season (Rainboth 1996). Other typical characteristics of this family are a slender body with long dorsal and anal fins that gives them a similar appearance to eels. A broad mouth with four pairs of sensory barbels situated on a depressed bony head. Clarias *spp*. occurs in water resources from Africa to East, Southeast, and South Asia, and it has been introduced to other parts of the world. In total, 4 species of the genus Clarias are likely to occur in Cambodia (Rainboth 1996). The broadhead catfish (*Clarias macrocephalus)* is very often mistaken with a female of walking catfish (*Clarias batrachus*), although the broadhead catfish is considered better in

taste and more nutritious. The introduction of the species outside its habitat was found to be invasive in its ecological impact.

Family Cyprinidae

Figure 16 *Henicorhynchus siamensis*. (Source:Froese & Pauly 2000)

Scientific name: *Henicorhynchus siamensis* (Sauvage 1881)

Family: Cyprinidae

Local name/English name: Trey riel (tob)/Siamese mud carp

The siamese mud carp is a freshwater fish that can be found in the Mekong and Chao Pryah basins. The Cambodian name for Siamese mud carp is the same as thet of a local currency (riel), which shows the importance of this fish for the annual fishery on the Tonlé Sap lake. It naturally occurs in large and small rivers where it lives in mid-water to bottom in great shoals. During the rainy season, the Siamese mud carp migrates out to floodplains and comes back when water returns to the river. Main characteristics of the Henicorhynchus genus are 8 branched dorsal fin rays and a thin lower lip tightly attached to the lower jaw. The siamese mud carp typically has a plain silvery body, a miniscule, maxillary barbel, and a dorsal fin with a dark distal margin. Siamese mud carp feed on algae, periphyton, and phytoplankton and can grow up to 20 cm length. This fish is used for prahok production.

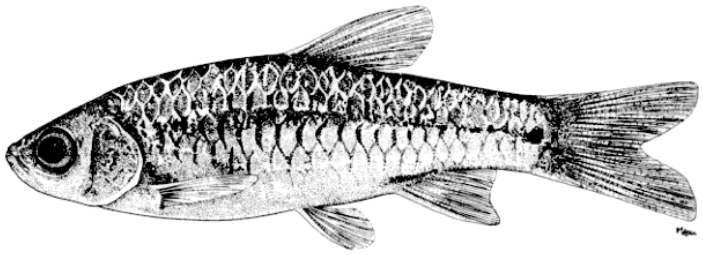

Figure 17 *Rasbora hobelmani*. (Source: Froese & Pauly 2000)

Scientific name: *Rasbora hobelmani* (Kottelat 1984)

Family: Cyprinidae

Local name/English name: Trey changwa/Kottelat rasbora

A freshwater fish *Rasbora hobelmani* can be found from mid-water to the surface of small streams and ponds in the area from Myanmar to Cambodia. It grows up to 6 cm and has a dark lateral stripe which ends in a dark spot. It feeds on exogenous insects. The fish is not often seen on markets but may be sold to the aquarium trade and it is often used for smoked fish production (Rainboth 1996).

Figure 18 *Hypsibarbus malcolmi*. (Source:Froese & Pauly 2000)

Scientific name: *Hypsibarbus malcolmi* (Smith 1945)

Family: Cyprinidae

Local name/English name: Trey chhpin/Goldfin tinfoil barb

It occurs in mid-water to the bottom depths in large and medium-sized rivers. It is found in large rivers in the dry season, it moves to medium-sized rivers in the wet season. Usually found over the coarse substrate. Its gut is usually full of fine matter with occasional insect exoskeletons They reproduce at the end of the rainy season. In February to March the level decreases and the young of the year appear with a length of about 2 cm. It is marketed fresh or to the aquarium trade (Rainboth 1996).

Figure 19 *Labeo chrysophekadion*. (Source: Froese & Pauly 2000)

Scientific name: *Labeo chrysophekadion* (Bleeker 1849)

Family: Cyprinidae

Local name/English name: Trey kaek/Black sharkminnow

This species occurs in rivers, streams, canals, and inundated floodplains and sometimes in impoundments. Like other planktivorous and detritivores carps, it begins spawning after the first thunderstorms of the rainy season. It spawns upstream from shallow sandbars that line long river bends. The eggs are spawned in shallow water and hatch just as water levels begin to rise following the onset of seasonal rains. They immediately move into inundated grasses along the bank and continue to follow the leading edge of the advancing water as floodwaters spread over the land. Adults also migrate out into seasonally flooded areas where they feed on algae, periphyton, phytoplankton and detritus. They return to rivers from October to December (Rainboth 1996). In Laos and Thailand, they migrate upstream at the onset of the rainy season. In Cambodia, they undertake upstream migration between October and March and downstream migration from March to August (Sokheng et al. 1999). A desirable fish that is marketed fresh, dried, and salted (Rainboth 1996).

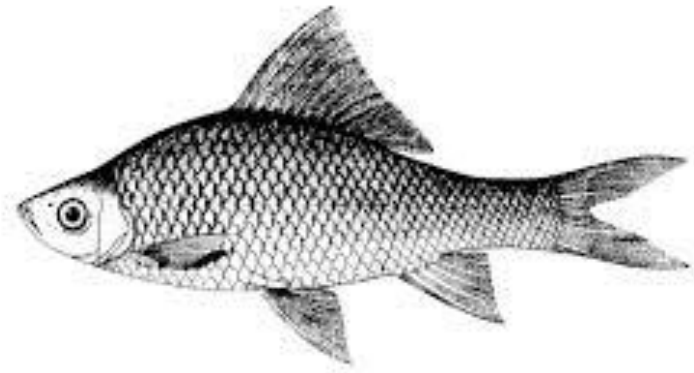

Figure 20 *Osteochilus schlegelii*. (Source: Froese & Pauly 2000)

Scientific name: *Osteochilus schlegelii* (Bleeker 1851)

Family: Cyprinidae

Local name/English name: Trey lolok sor/Giant sharkminnow

The species of *Osteochilus schlegelii* usually occurs from midwater to the bottom depth in large and medium sized rivers. Found in Great lake (Tonlé Sap) but does not flourish in impoundments. Seasonal movements are similar to those of *O.microcephalus.* It occurs regularly in fisheries of the middle and lower Mekong. It is used to make prahok (Rainboth 1996).

Figure 21 *Paralaubuca barroni***.** (Source: Froese & Pauly 2000)

Scientific name: *Paralaubuca barroni* (Fowler 1934)

Family: Cyprinidae

Local name/English name: Trey slak russey

It is found in large rivers in slow flowing to standing water (Kottelat 1998) and at shallow and medium depths (Rainboth 1996) in continental Southeast Asia. Due to its resemblance to *P.typus* it is little known. *Paralaubuca barroni* feeds on zooplankton and occasionally insects. It is probably used to make prahok (Rainboth 1996).

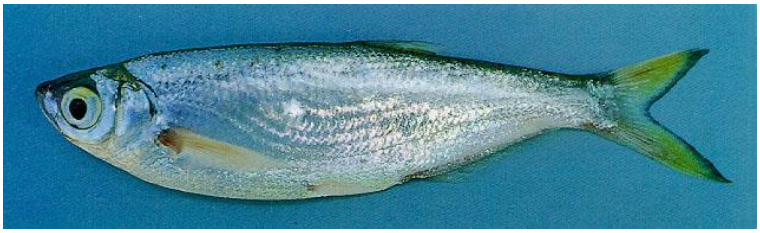

Figure 22 *Paralaubuca typus.* (Source: Froese & Pauly 2000)

Scientific name: *Paralaubuca typus* (Bleeker 1864)

Family: Cyprinidae

Local name/English name: Trey slak russey

The *Paralaubuca typus* species occurs naturally in the shallow depths of large rivers (Rainboth 1996). Found in slow flowing large rivers (Kottelat 2001). It is a school-forming species, often harvested in large numbers throughout its range. Feeds on zooplankton and occasionally on insects. It moves into flooded forests when the water level is high and returns to the mainstream after the water level considerably declines (Rainboth 1996). Spawning occurs at the onset of the flood season (May to July) and the eggs and larvae are swept downstream and out onto the flooded areas (Sokheng et al. 1999). Sometimes it is marketed fresh, but more often used to make prahok (Rainboth 1996).

Figure 23 *Puntioplites proctozystron*. (Source: Froese & Pauly 2000)

Scientific name: *Puntioplites proctozystron* (Bleeker 1865)

Family: Cyprinidae

Local name/English name: Trey chrakaing

Puntioplites proctozystron is a species with a brownish body colour and reticulated dark scale edges (Rainboth 1996). It is commonly found in standing and slowly moving water in streams, canals, ditches, and reservoirs (Kottelat 2001). This species moves into flooded forests and marshes during high water periods. The occurrence of *Puntioplites proctozystron* covers an area from Malaysia to northern Thailand, including Cambodia and Vietnam. It usually occurs around submerged aquatic or inundated terrestrial vegetation where it consumes some algae but mostly insects and zooplankton. Larger fish are marketed fresh while smaller ones are used to make prahoc along the Tonlé Sap, Cambodia (Rainboth 1996). It generally reaches more than 30 cm in length (Taki 1974).

Family Notopteridae

Figure 24 *Notopterus notopterus.* (Source: Froese & Pauly 2000)

Scientific name: *Notopterus notopterus* (Pallas 1769)

Family: Notopteridae

Local name/English name: Trey slat/ Bronze featherback

N. notopterus naturally occurs in the freshwaters of South and Southeast Asia. During the rainy season, adults stay in the standing waters of lakes, ponds, rivers, and canals. The species can be recognized by the brown colouring of adults and the slightly concave dorsal head profile. The usual length is about 25 cm. It feeds on insects or fish. It colonizes and breeds in seasonally inundated areas during the rainy season. It is most active at night and around twilight. It is usually sold fresh or dried (Rainboth 1996).

Family Siluridae

Figure 25 *Phalacronotus micronemus*. (Source: Froese & Pauly 2000)

Scientific name: *Phalacronotus micronemus* (Bleeker 1846)

Synonym: Kryptopterus micronema, Silurus micronemus

Family: Siluridae

Local/English name: Trey kes

Phalacronotus micronemus is a freshwater fish species native to the Mekong River. It is widespread in water courses, such as rivers, lakes, and streams, from Thailand to Indonesia. It is well adapted to impoundments. Its maximum length is 50 cm. Recognition signs for genus Phalacronotus are: subcutaneous eye, orbital rim continuous with skin covering eye, short mouth that is not extending eye, the dorsal fin may be present, a maxillary barbel that extends past the gill opening, dark spot at caudal-fin base and vomerine teeth in a smoothly curved band. The usual diet of *Phalacronotus micronemus* is shrimp and pelagic fish. It is mostly consumed smoked or as traditional fish paste called prahok (Rainboth 1996).

Figure 26 *Wallago attu*. (Source: Froese & Pauly 2000)

Scientific name: *Wallago attu* (Bloch & Schneider 1801)

Family: Siluridae

Local name/English name: Trey sanday/ Freshwater shark

Wallago attu is a freshwater fish that can naturally occur in brackish water. This fish can be found throughout Cambodia except forhighland streams, mainly in large rivers of the lower Mekong floodplains, or large lakes and tanks. It easily adapts to impoundments. It can reach 200 cm in length but is commonly about 80 cm. Typical for *W. attu* is a broad head with a depressed snout, caudal fin plainly forked, and a large mouth extends back as far as the eye. Its eyes have a free orbital margin. The teeth in its jaw are set in wide bands. This allows it to feed on smaller fish, crustaceans, and molluscs. It is considered an excellent game fish. It is usually caught with gillnets and hooks and then sold on market fresh or in ice exported to Thailand (Rainboth 1996).

Figure 27 *Belodontichthys truncatus*. (Source: Dignall 2021))

Scientific name: *Belodontichthys truncatus* (Kottelat & Ng 1999)

Family: Siluridae

Local/English name: twisted-jaw catfish

The twisted-jaw catfish is freshwater fish that belongs to the endemic species of the Mekong Basin. The fish can reach up to 60 cm length. It has a strongly upturned head with mouth that is in angle of 60 ° above horizontal. The natural habitat of the fish is in deeper parts of large rivers. It usually eats smaller fish close to the water surface. The fish can be caught by hook-and-line as a game fish, or usually by gillnets and cast-nets (Rainboth 1996). Other species of the genus Belodontichthys can be found, comprising *B. dinema* (Bleeker 1851) and *B. truncatus*. The natural occurrence of the first mentioned species is in central and southern Thailand, Malaysia, Sumatra, and Borneo, while the second species occurs in northeast Thailand, Laos, Cambodia, and Vietnam. In Cambodia, *Belodontichthys truncatus* is often

cought, stored in ice, and exported to Thailand (Ng & Kottelat 1998). For local consumption, the fish is marketed fresh, dried, or salted (Rainboth 1996).

Figure 28 *Micronema hexapterus.* (Source: Froese & Pauly (2000))

Scientific name: *Micronema hexapterus* (Bleeker 1851)

Synonymy: *Krptopterus hexapterus, Silurus hexapterus*

Family: Siluridae

Local/English name: Trey kamplieu

Found in rivers, streams, and canals. Feeds mainly on small fishes, along with prawns and insect larvae. It is usually marketed fresh (Rainboth 1996).

Figure 29 *Ompok bimaculatus*. (Source: Froese & Pauly (2000))

Scientific name: *Ompok bimaculatus* (Bloch 1794)

Family: Siluridae

Local/English name: Trey krormorm/Butter catfish

Adults are found in quiet, shallow (0.5 - 1.5 m), often muddy waters, in sandy streams, rivers and tanks (Pethiyagoda 1991). The species also occurs in canals, and inundated fields during

the flood season (Rahman 1989). It is a slow-moving predator that feeds on crustaceans, fish, and occasionally on molluscs (Pethiyagoda 1991).

Figure 30 *Pangasius elongatus*. (Source: Planetcatfish 2021)

Scientific name: *Pangasius elongatus* (Pouyaud, Gustiano & Teugels 2002)

Family: Siluridae

Local/English name: Trey chhwiet

Pangasius is found mainly in the lower courses of major rivers from Thailand to Indonesia. An omnivorous fish, feeding mainly on benthic animals such as molluscs and crustaceans. During the rainy season this Mekong specimen feeds on fruits and various debris (Pouyaud et al. 2002). Pangasius is one of the most economically important fish species across Southeast Asia. It is marketed fresh (Rainboth 1996; Gustiano et al. 2018).

Figure 31 *Phalacronotus bleekeri*. (Source: Froese & Pauly 2000)

Scientific name: *Phalacronotus bleekeri* (Günther 1864)

Synonyms: *Kryptopterus bleekeri, Micronema bleekeri*

Family: Siluridae

Local/English name: Trey kes

Phalacronotus bleekeri occurs in rivers, streams and lakes as well as in impoundments (Rainboth 1996). It is a migratory species (Hill & Hill 1994). This species undertakes lateral migrations from the Mekong River into smaller tributaries and into the floodplains at the beginning of the flood season, returning to the main river channel when the water begins to recede at the onset of the dry season (Sokheng et al. 1999). Migrations are triggered by the first rainfall at the end of the dry season, as well as water level changes. The lunar cycle also affects its movements. It returns to the river from the floodplain and tributaries on, or immediately before, the full moon (Sokheng et al. 1999). It feeds on small fishes, shrimps and aquatic insect larvae (Ukkatawewat 1984).

3 Objective

The main objective of this thesis is to investigate in detail the traditional practices of smoke-curing fish in the Tonlé Sap lake area, Cambodia, and monitoring the concentrations of selected contaminants in the final product, particularly polycyclic aromatic hydrocarbons (PAHs). Determination of PAHs is often linked with challenges in determination in smoked food of animal origin. The main obstacle is its high fat content (e.g. lipids, triglycerides and fatty acids) and the extraction of PAHs from these complex matrices is usually laborious and often not effective enough. Fat residues in analysed extracts can contribute to the deterioration of the chromatographic system (especially GC) as well as suppressing a signal of analytes. Thus another aim of this study is to develop an effective sample preparation procedure with less solvent and time input for the determination of polycyclic aromatic hydrocarbons (PAHs) in smoked fatty products of animal origin, particularly smoked fish.

4 Materials and methods

4.1. Site area description, sampling, and questionnaire survey

4.1.1 Site area description and sampling

The wetlands of the Tonlé Sap area in Cambodia are part of the Mekong watershed, with one of the most productive fisheries in Southeast Asia (Rainboth 1996; Hortle 2004). Fisheries in this location are the main industry and source of household income, especially for poor villagers. Approximately 85% of the total fish catch comes from the inland fisheries in Cambodia. In addition, the wetlands provide two-thirds of people's dietary protein intake (Kanchanaroek 2013). The location of villages where samples of smoked fish were collected is presented in Figure 32. These are Spean Trong, Kandal, Phsar Leur, Preak Trab, Chamkar Reusey village and Lor Eit in the 3 provinces Kampong Cham, Kamong Chhnang and Battambang in the wetlands of the Tonlé Sap lake area in Cambodia. Sampling and questionnaire surveys were carried out among small-scale producers of smoked fish products in Cambodia in villages within the Tonlé Sap area with a history of smoking. Kampong Cham province is situated about 100 km from the capital city Phnom Penh. Kampong Chhnang province is located about 95 km north from the capital Phnom Penh and Battambang province, approximately 300 km northwest direction from the capital city. Kampong Chhnang province is among the highest per capita consumption of fresh fish and more particularly smoked fish processing in Cambodia. The total consumption of fresh fish and processed fish in the province is almost 120 kg per year (Ahmed et al. 1999). In addition, the area near Kampong Chhnang city along the Tonlé Sap river is famous for a high concentration of smoked fish producers. Hence this area was selected for our sample collection. About 84% of the Cambodian population is considered as rural (Hortle 2007). At least 45% of the population work full time in fisheries or fisheries-related activities and are dependent on these wetlands thus improving local people's livelihoods (Nam & Bunthang 2011). In total, 63 samples of smoked fish were collected directly from the smokehouses of 30 producers. Samples were collected during the period from October to December 2018 in the period reported as production period. After collection, the samples were identified, marked, photographed, and placed in clean, properly labelled plastic bags and vacuum sealed. Samples were then transported to the Czech University of Life Sciences Prague, the Czech Republic for further laboratory analyses. All samples were analysed in triplicates.

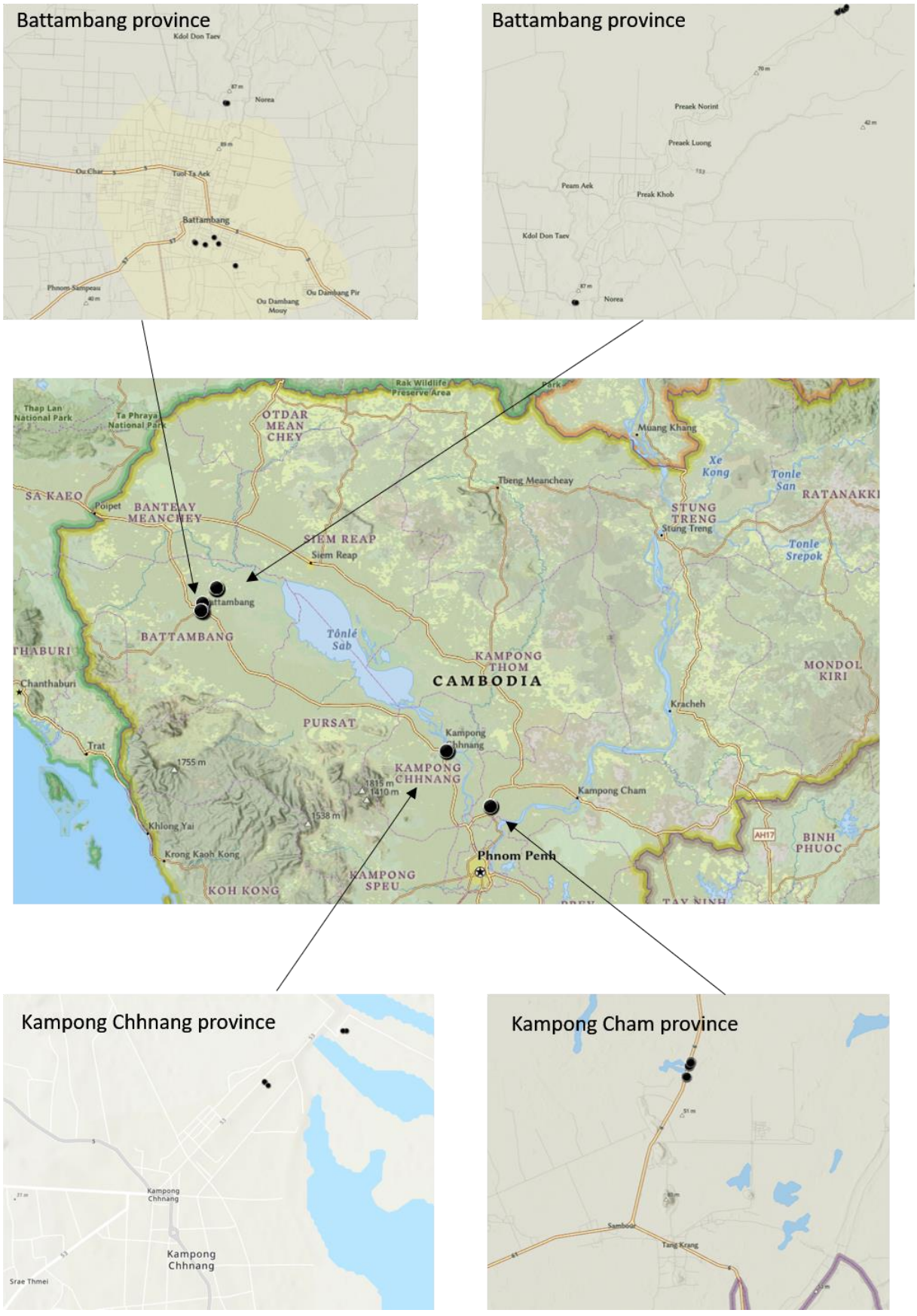

Figure 32 Map of sampling and questionnaire survey area Tonlé Sap, Cambodia.

Each of the samples weighted approximately 100 g. The samples were frozen (-20 °C) until analyses were performed. The fish samples were determined to be species of the five families Belonidae, Clariidae, Cyprinidae, Notopteridae and Siluridae order and Beloniformes, Cypriniformes, Osteoglossiformes and Siluriformes. The number of samples collected, listed according to their family and order is displayed in Table 2.

Table 2 Fish species collected for sampling listed according to Family and Order.

Scientific name	Family	Order	Reference	No. of samples collected
Xenentodon cancila	Belonidae	Beloniformes	*Hamilton, 1822*	1
Clarias *spp.*	Clariidae	Siluriformes	*Linnaeus, 1758*	6
Henicorhynchus siamensis	Cyprinidae	Cypriniformes	*Sauvage, 1881*	12
Hypsibarbus malcolmi	Cyprinidae	Cypriniformes	*Smith, 1945*	1
Labeo chrysophekadion	Cyprinidae	Cypriniformes	*Bleeker, 1849*	3
Osteochilus schlegeli	Cyprinidae	Cypriniformes	*Bleeker, 1851*	1
Paralaubuca barroni	Cyprinidae	Cypriniformes	*Fowler, 1934*	3
Paralaubuca typus	Cyprinidae	Cypriniformes	*Bleeker, 1864*	3
Puntioplites proctozystron	Cyprinidae	Cypriniformes	*Bleeker, 1865*	2
Rasbora hobelmani	Cyprinidae	Cypriniformes	*Kottelat, 1984*	8
Notopterus notopterus	Notopteridae	Osteoglossiformes	*Pallas, 1769*	1
Belodontichthys truncatus	Siluridae	Siluriformes	*Kottelat & Ng, 1999*	2
Micronema hexapterus	Siluridae	Siluriformes	*Bleeker, 1851*	6
Ompok bimaculatus	Siluridae	Siluriformes	*Bloch, 1794*	4
Pangasius elongatus	Siluridae	Siluriformes	*Pouyaud, Gustiano & Teugels, 2002*	1
Phalacronotus bleekeri	Siluridae	Siluriformes	*Günther, 1864*	4
Phalacronotus micronemus	Siluridae	Siluriformes	*Bleeker, 1846*	3
Wallago attu	Siluridae	Siluriformes	*Bloch & Schneider, 1801*	2
Total number of fish samples collected				**63**

4.1.2 Questionnaire survey

To gather supplementary data to evaluate the final PAH concentrations in the fish samples a questionnaire survey and personal interviews were conducted. The questionnaire survey was carried out in the period from October to December 2018. A questionnaire survey was conducted among small-scale producers of smoked fish products in Cambodia in five villages within the Tonlé Sap area in 3 provinces with a history of smoking. Namely, Spean Trong,

Kandal, Phsar Leur, Preak Trab, Chamkar Reusey village and Lor Eit. The number of respondents (31) was equal to producers visited within sample collection in the targeted area Figure 32.

Figure 33 Questionnaire survey conducted by local student from Royal University of Agricultue, Cambodia.

To better understand the whole process of traditional smoking, three groups of questions were prepared - Introductory, technical and marketing and selling practices. Questions in the first part were related to the location of the producer, source of fish, location of the collection of the raw product, fish species used for smoking, pre-treatment used before the smoking procedure, technique used for fish smoking and readiness estimation. Questions in the technical part were focused on parameters affecting the deposition of PAHs in the product such as fuel used for smoking, type of fuel wood, period of smoking, fire-starting techniques and others. The last group consisted of questions regarding the storage, main production period and selling practices and consumption habits (see Appendix I). All the data were collected in local units and names, and all the interviews and questionnaires were conducted in the Khmer language and then translated into English (Figure 33).

4.1.3 Temperature measurements

During the sampling measurements of temperature were taken, although not all producers had production in operation during the period of collection. This was mainly due to a shortage of raw material and/or fuel or the fact that production had already ended or not yet started. Although the general timescale is known it is highly affected by weather conditions since most

of the producers are in the wetlands surrounding Tonlé Sap lake. On sites where production was ongoing temperature data were collected. For that purpose, a one-channel Testo 925 thermometer (Testo s.r.o., Prague, Czech Republic) with a Testo TE type K immersion probe (Testo s.r.o., Prague, Czech Republic) was used (see Figure 34). Temperature was measured in each level of trays in the centre of the product in three repetitions. The first level of tray is usually between 50 - 90cm above the fireplace. However, due to the low amount of collected data the exact numbers are not displayed in this work, just as the percentage of range in Table 4.

Figure 34 Temperature measurements, Tonlé Sap area, Cambodia.

4.2 Modified QuEChERS-EMR Lipid-DLLME method development

Most of the primary products of animal origin used for smoking are high in fat content. This might subsequently cause difficulties in PAH extraction from the complex matrix and due to

their lipophilic nature. Nowadays, there is a need for methods which are fast and reliable, as well as ecological and economical. Therefore, optimisation and validation of the new method was carried out. Development of this method was done in collaboration with Malopolska Centre of Food Monitoring, Faculty of Food Technology, University of Agriculture in Krakow during summer 2018 (June – October 2018). For the purpose of the method development, optimisation and validation smoked mackerel obtained from the Polish retail market was used. This testing material was considered as standard because it is subject to the limits of the European Union we refer to in this study. QuEChERS (quick, easy, cheap, effective, rugged, safe) (see chapter QuEChERS method) multi-class, multi-residue analytical approach was introduced and used as a default method. Two different approaches were compared: 1) classical QuEChERS with PSA and C18 sorbents and 2) procedure with the use of EMR-Lipid (see chapter EMR-Lipid) according to the manufacturer. We have also compared two different methods of extract pre-concentration: under a nitrogen stream and with the use of DLLME

Figure 35 Modified QuEChERS-EMR Lipid-DLLME method in laboratory, faculty of Food Technology, University of Agriculture in Krakow during summer 2018.

method (see chapter Dispersive Liquid–Liquid Microextraction (DLLME)). All samples were analysed using gas chromatography-mass spectrometry. All samples were prepared in triplicates.

4.2.1 Chemicals and reagents

Polycyclic aromatic hydrocarbons suitable for EPA Method 610 (method for PAH analysis) (Kinzer et al. 1984), anthracene-d_{10} (Internal Standard 1; IS1), chrysene-d_{12} (Internal Standard 2; IS2), hexachlorobenzene (Syringe Standard; SS) were obtained from Sigma-Aldrich, Saint Louis, Missouri, USA. Magnesium sulphate anhydrous p.a. and sodium chloride p.a. were

purchased from Krakchemia SA, Krakow, Poland. Acetonitrile, chloroform and hexane, were purchased from Merck KGaA, Darmstadt, Germany. PSA, C_{18}, SPE Bulk Sorbents and EMR-Lipid material derived from Agilent Technologies, Santa Clara, California, USA. Deionised water (18MΩ) was produced by a Milli-Q system (Millipore, Burlington, Massachusetts, USA). Stock, intermediate and working standard solutions of PAHs, chrysene-d_{12}, and anthracene-d_{10} (all at the concentration of 1 $\mu g.mL^{-1}$) were prepared in hexane. Calibration standards of PAHs at the concentrations ranged from 2 to 400 $ng.mL^{-1}$ were prepared by diluting the standard mixture solution to the corresponding hexane volume. All reagents were at least of analytical purity.

4.2.2 Instrumentation

Analyses were carried out on a Varian 4000 GC-MS (Agilent Technologies, Santa Clara, California, USA) system consisting of 3800 gas chromatographs with a DB-5MS column (30 m x 0.25 mm x 0.25 µm; Agilent Technologies, Santa Clara, California, USA) and 4000 Ion Trap MS detector. The GC oven was operated with the following temperature program: initial temperature 50 °C (1 min) – 15 $°C.min^{-1}$ – 300 °C (6.0 min) for PAHs. Helium 5.0 (Linde Group, Munich, Germany) was used as the GC carrier gas at a flow rate of 1.0 $mL.min^{-1}$. The auto sampling injector was CP-1177 Split/Splitless Capillary Injector, with a temperature of 270 °C and with the volume of 1.0 µL for all standards and samples. Each injection was repeated three times. The ion trap mass spectrometer was operated on the internal ionisation mode, scan from m/z 45 to 500 in full scan mode, used for the evaluation of the quality of sample extracts. Quantitative analyses were conducted in the selected ion monitoring mode (SIM mode) and analysed compounds were identified according to their ions and retention times. The trap and the transfer line temperatures were set at 200 and 270 °C, respectively. The emission current of the ionisation filament was set at 15 µA. Acquisition and processing data were performed using Varian Star Workstation software and NIST 2.0 library (National Institute of Standards and Technology, Gaithersburg, Maryland, USA).

4.2.3 Extraction and clean-up of the sample

In the experiment, the samples of smoked mackerel obtained from the local market were used for the preparation of blank and spiked samples. Recovery studies in each case involved three samples being spiked at the level of 100 $\mu g\ kg^{-1}$ with the PAH standard and internal standards

solutions (anthracene-d_{10} and chrysene-d_{12}, both also at the level of 100 µg kg^{-1}) at the preparation phase before using the different proposed schemes. Blank samples and reagent blanks were prepared similarly to the fortified samples. The tested procedures are presented in Figure 37. The experiment was based on the comparison of two different concepts of analyte extraction from the samples and its clean up: 1) classical QuEChERS method, using freezing out, clean-up step with PSA and C_{18} and 2) the protocol involving implementation of EMR-Lipid according to its manufacturer (Lucas & Zhao 2015).

The second part of the research included the selection of the best method of the final extract pre-concentration: 1) by evaporation to dryness under the stream of nitrogen and dissolution of the residues in a small volume of hexane or 2) the use of the DLLME method; the choice of the solvents and its volumes were based on the previous results, according to the procedure developed and optimised recently (Sadowska-Rociek et al. 2015; Surma et al. 2018). To summarize, four different variants combining different analyte extractions and final extract pre-concentration were prepared, analysed, and subsequently evaluated based on analyte recoveries and the quality of obtained chromatograms to develop effective sample preparation procedure. In all tested variants, the recovery values were calculated after the final pre-concentration step, involving all conducted sample preparation stages.

4.3 Determination of PAHs in smoked fish from Tonlé Sap area, Cambodia

Samples were analysed by modified QuEChERS-EMR Lipid-DLLME method (Slámová et al. 2020). PAHs were then analysed by gas chromatography–mass spectrometry (GC/MS). Fat content in fish samples was determined by modified SPV method (Microquantity colorimetric sulfophosphovanillan (SPV) method). All samples were prepared in triplicates.

Figure 36 Preparation of the modified QuEChERS-EMR Lipid-DLLME analysis

4.3.1 Modified QuEChERS-EMR Lipid-DLLME method

Chemicals and reagents

A standard mixture of 16 important polycyclic aromatic hydrocarbons (QTM PAH mix) and a mixture of deuterated internal standards (Semivolatile Internal Standard Mix) were purchased from Sigma Aldrich, CZ. Anhydrous magnesium sulfate (p.a. anhydrous, ReagentPlus®, ≥99.5%) and sodium chloride (p.a. ReagentPlus®, ≥99.5%) were purchased from Sigma-Aldrich, CZ. The material EMR-Lipid was obtained from Agilent Technologies, USA. Acetonitrile, chloroform, and hexane were purchased from VWR Chemicals, CZ. Stock, intermediate and working standard solutions of PAHs and the internal standards were prepared in hexane. Calibration standards of PAHs with concentrations ranging from 2 to 2500 $ng.ml^{-1}$ were prepared by diluting the standard mixture solution to the corresponding hexane volume.

Sample preparation for GC/MS analysis

First, the fish samples were homogenized using a laboratory blender (IKA, DE) and liquid nitrogen. The PAHs were extracted by the modified QuEChERS–EMR Lipid–DLLME method described by Slámová et al. (2020) (see Figure 37). The classical QuEChERS method uses freezing out and clean-up step with PSA and C_{18}. Our modified method uses extraction step as

in the classical QuEChERS method with acetonitrile (MeCN) and water. Further addition of NaCl and MgSO4 is followed by clean-up by new EMR-Lipid material and final pre - concentration step by DLLME. Briefly, 1 g of dried smoked fish was weighed into a 50 ml Falcon tube and spiked with an internal standard solution at a level of 500 µg.kg^{-1}, and then 10 ml of acetonitrile and 5 ml of deionized water were added. The tube was shaken for 2 minutes and then allowed to stand for 10 minutes to properly rehydrate the dried samples. Next, 5 g of magnesium sulfate and 1.5 g of sodium chloride were added, and the tube was shaken for another 2 minutes. The sample was then centrifuged at 4600 rpm and 0 °C for 15 minutes. The supernatant (7 ml) was transferred to 15 ml tubes containing 1 g of the EMR-Lipid sorbent previously activated with 5 ml of deionized water. After the addition of the supernatant, the tube content was vortexed for another 1 minute. This mixture was then centrifuged at 4600 rpm and 0 °C for 15 minutes. Five millilitres of the obtained supernatant were transferred to a 15 ml tube containing 1.6 g of magnesium sulfate and 0.4 g of sodium chloride, and the tube was shaken for 2 minutes. Then, the sample was centrifuged at 4600 rpm and 0 °C for 15 minutes. The upper layer (2 ml) was transferred to a 15 ml tube containing 6 ml deionized water and 200 µl chloroform, the tube was shaken for 2 minutes, and the mixture was allowed to stabilize. The bottom chloroform layer was transferred to a vial and allowed to evaporate until dry. Finally, the evaporated sample was diluted in 500 µl of hexane and analyzed by gas chromatography–mass spectrometry (GC/MS). All samples were prepared in triplicates.

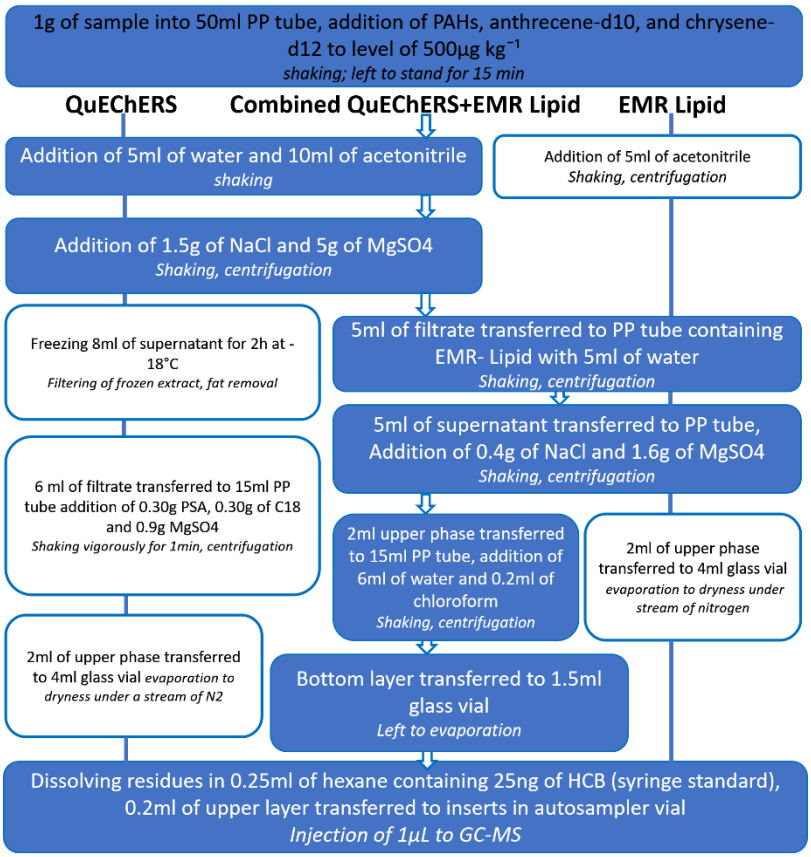

Figure 37 Schema of the sample preparation process by modified QUEChERS-EMR Lipid-DLLME method.

Instrumentation

Analyses were conducted on a GC 7890A instrument coupled to a 5975C MS quadrupole detector (Agilent Technologies, Santa Clara, California, USA). The samples were separated using a VF5-ms column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies, Santa Clara, California, USA) under a constant He flow (1 ml.min^{-1}). The GC oven was operated according to the following temperature program: initial temperature: 50 °C (1 min), 15 °C.min^{-1} to 150 °C, 8 °C.min^{-1} to 310 °C, and 310 °C (10 minutes). The sample (1.0 µl) was injected in splitless mode at 280 °C, the MS instrument was operated in the internal ionization mode, and scans were performed from m/z 45 to 500 in full scan mode to evaluate the quality of the sample extracts. To quantitatively analyze the PAH ion monitoring mode (SIM mode), quantitative ions were used. The temperatures of the transfer line, ion source, and quadrupole were set to 280, 230 and 150 °C, respectively.

PAH identification and quantification

Data acquisition and processing were performed using Agilent software (Agilent MSD ChemStation E.02.01.1177) and the NIST 2.0 library (National Institute of Standards and Technology, Gaithersburg, Maryland, USA). The PAHs were identified by comparing the retention times of the peaks and target ions with those obtained from a standard mixture of PAHs. The quantification was performed by internal standard calibration using the standard solutions of each of the PAHs and corresponding IS (phenantrene-d10, chrysene-d12, perylene-d12). Altogether, 12 PAHs, namely, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene and benzo[ghi]perylene, were identified.

4.3.2 Determination of the fat content in fish by a modified Sulfo-phospho-vanillin (SPV) method

Due to the limited amount of samples, the total fat content was determined by the microquantity colorimetric sulfo-phospho-vanillin method (SPV see Microquantity colorimetric sulfophosphovanillan (SPV) method) described by Anschau et al. (2017) with slight modifications. The determination of fat content was carried out in triplicate according to AOAC procedure (AOAC 1975). Homogenized fish samples (40 – 100 mg) were extracted for 5 minutes in a 4 ml chloroform : methanol mixture (1:1). Then, 1 ml of a 0.9% NaCl solution in water was added, and the tube was vortexed for 30 s. Falcon tubes containing the homogenate were centrifuged for 5 min at 9000 rpm to separate the chloroform layer with fat from the rest of the sample. Aliquots of the chloroform layer (250 µl) were transferred to glass tubes. Meanwhile, a six-point calibration (1 to 40 $mg.ml^{-1}$) was prepared from commercially available fish oil (Moller´s) in acetone, and aliquots (250 µl) were transferred to glass tubes. The tubes with the samples and calibration standards were placed in a dry heat block at 100 °C until the solvent was evaporated (approx. 10 min). After the tubes had cooled, 250 µl of concentrated sulfuric acid was added, and the sample was again heated for 10 min in a dry heat block (100 °C). Finally, 2.25 ml of the phospho-vanillin reagent (300 mg vanillin, 50 ml hot distilled water and 200 ml of 85% o-phosphoric acid) was added to the cooled sample and properly mixed. After 5 minutes, 100 µl of the samples and calibration standards was transferred to a 96-well microtiter plate, and the absorbance was measured on a Biotek reader (SYNERGY H1, USA) at a wavelength of 490 nm. All samples were prepared in triplicate, and three technical replications of the measurements were performed. The results are

expressed as the percentage of fat in dry fish. All chemicals were analytical grade and delivered by VWR (Czech Republic).

4.3.3 Statistical analysis of the data

All samples, in total 63 from 18 fish species (Table 2) were prepared in triplicates for all analysis (PAHs concentration and fat content determination). The data obtained from the laboratory measurements were processed in Microsoft Office 365 Excel. Samples where more than one sample, within one fish species, were collected, were statistically compared. Analysis were done using the STATISTICA 12 software. Analysis of variance (ANOVA) followed by Tukey's HSD test at the $p < 0.05$ significance level was applied to the PAH concentration levels, for samples where more than one sample was collected from one fish species. Correlations were used to assess the relationship between the PAH concentration and total fat content.

5 Results and Discussion

5.1 Questionnaire survey results

5.1.1 Source of fish for traditional smoking

Based on a questionnaire survey, we can briefly describe the traditional production of smoke-cured fish in the Tonlé Sap area. Selected parameters and relevant responses about traditional smoking are shown in Table 4. In this study, the small-scale producers could be divided into three groups according to the reported daily production DP1 (40 – 100 kg), DP2 (100 – 500 kg) and DP3 (500 – 1000 kg), which accounted for 37%, 23% and 33%, respectively. Although the production places and households are often next to the water source, more than 60% of producers buy raw products from local fishermen or on the market. The other source reported was direct fishing. This is most likely influenced by financial means or distance of the producer from the water bodies (Ahmed et al. 1999). This is in accordance with Nam et al. (2009), who described the seasonal movements of the Cambodian population towards the Tonlé Sap area to gather fish for their own production by trading, fishing or buying. All questioned producers, 100%, within all provinces, process fish directly by the smokehouse or in their households, there is no additional transportation (Figure 38). This practice is reasonable due to natural conditions in the area and fast spoilage of the fish meat. It is the same as the technique used for conservation itself.

Figure 38 Household processing, Kampong Chhnang province, Cambodia.

5.1.2 Traditional smokehouse

Traditional smoke curing take place in typical smokehouses along the river or near the water areas. Dimensions of the smokehouse vary according to the production and financial means of the producer. Nam et al. (2009) divide producers according to smokehouses to small-scale

and medium scale. A traditionally smoking kiln has the dimensions 200 – 1500 cm × 90 – 40 cm × 70 – 400 cm, length, width, and height, respectively, except for producers with production over 500 kg.day^{-1}. There we can find ventilated or covered bricks smoke kilns with dimensions up to 10 m in length, 1 m height and 1.5 m width (see Figure 39).

Figure 39 Example of larger, big dimension brick smokehouse, Battambang province, Cambodia.

Based on observation smokehouses are wooden, bamboo or brick constructions with or without walls made of mats, from leaves or bamboo, fibre, or metal sheets. However, the roof, if present, is made from palm leaves or metal sheets. The structure and diameters may result from the financial means available to the family . Bamboo and wooden smokehouses are mostly made from material gathered in the surrounding area in contrast to a brick building for which the family has to buy the material. All of the producers reported use of direct smoking with a direct source of heat. Therefore, the technology used may be classified as the direct and hot smoking method (Ledesma et al. 2016). Traditional smoking kilns consist of a fireplace, various levels of ventilation and a number of trays (1 - 4) according to production, where grouped fish are placed. The first level of trays is placed at a height from 50 up to 100 cm above the fireplace. The next levels of trays are usually placed above with approximately 40 cm between them (Figure 40). Hokkanen et al. (2018) reported significantly lower amounts of PAHs if the heat source is more than 5 meters from the smoked product. The trays are made from bamboo sticks or, rarely, metal rods. There are basically two main dimensions used for smoking of fish, square dimension 25 - 40 × 30 – 50 cm and rectangular 15 – 30 × 80 – 250 cm width and length, respectively.

Figure 40 Example of typical smoking kiln dimensions, Tonle Sap area, Cambodia.

5.1.3 Fuel used for traditional smoking of fish

Fires are generally placed on the ground considered as a fireplace; most of the producers use a net arrangement which produces a better circulation of hot air, and therefore a better quality of smoked products. As a main source of fuel for traditional fish smoking three types of fuel are used; wood and charcoal, and a combination of both (see Figure 41). More than 70% of respondents reported using wood as the primary source of fuel compared to 13% who used charcoal and a combination of both charcoal and wood (10%). These results are consistent with results presented by San et al. (2012) for the majority of the population in Cambodia. In contrast, in the same study charcoal was used in 6%, followed by LPG (liquid petroleum gas) 5.2%. This great difference in the use of wood compared to charcoal may be caused by the ostensibly infinite sources of fuel wood in the surrounding area and the higher charcoal price compared to fuel wood. Based on the questionnaires, more than 70% of

respondents use fuelwood for smoking. Consumption of wood varied between 10 – 300 kg per day and comparable to charcoal 25 – 250 kg.day^{-1}.

Figure 41 Example of typical fuel used for smoking in Tonlé Sap area, Cambodia; left bottom and top – wood; right bottom and top - charcoal.

The wood used is mostly local fuel wood gathered in the surrounding area. It is common to use mixtures of locally available wood in ready-to-buy batches, locally called "*Kreak*". It is described as an unspecified mixture of woods. However, 50% and 20% of respondents reported that the most collected and used woods were from *Barringtonia asiatica,* locally called "*Deam Reang*" and *Havea brasiliensis*, respectively. *Barringtonia asiatica* is known for the favourable properties of the smoke it produces (Figure 42).

Figure 42 Barringtonia asiatica; C – leaves, D - wood. (Source: Sourav 2019)

In comparing to other plant species which produce smoke with higher probability of darker final product which is undesirable. However, the seeds of *Barringtonia asiatica* also contain antinutritional compounds known for their ichthyotoxic properties (Ravikumar et al. 2015). Among other species named by respondents were *Anacardium occidentale* (3%), *Barringtonia acutangula* (7%), *Combertum trifoliatum* (10%), *Mallotus anisopodus* (7%) and *Tamarindus indica* (3%) (Figure 43). Usually, the distribution of fuel used for smoking is determined by province.

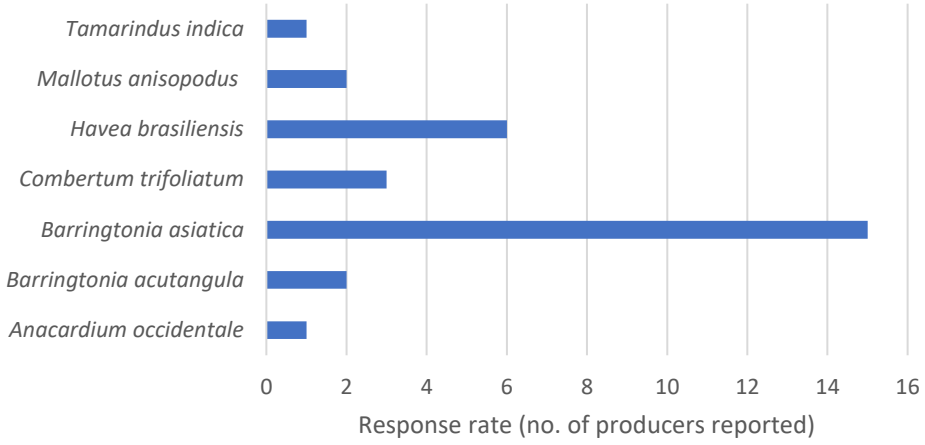

Figure 43 Display of species of commonly used fuelwood for smoking named by producers

5.1.4 Fish used for traditional smoking

Figure 44 Example of a raw fish mixture of species. Kampong Cham province.

The producers identified 20 fish species mainly processed by smoking in the targeted area. The variability of fish species is affected by the season and financial capacity of the producer (Khim et al. 2003). Fish species were determined to be 20 fish species belonging to 5 different families. Fish species used for smoking in the target area and their response rate as were reported by each producer are summarized in Table 3. As it is presented in Table 3 among fish species mainly used for smoking are species belonging to the family Cyprinidae, which is in accordance with Rainboth (1996). Fish species are described in detail in Chapter 2.6 Fish species in Tonlé Sap area, Cambodia.

Table 3 Fish species identified by producers for smoke-curing.

Fish species	Response rate (no. of producers)	Family
Belodontichthys truncatus	5	Siluridae
Henicorhynchus lobatus	1	Cyprinidae
Henicorhynchus siamensis	15	Cyprinidae
Clarias spp.	7	Clariidae
Hypsibarbus pierrei	2	Cyprinidae
Micronema hexapterus	4	Siluridae
Phalacronotus micronemus	9	Siluridae
Labeo chrysophekadion	1	Cyprinidae
Notopterus notopterus	2	Notopteridae
Ompok bimaculatus	4	Siluridae
Osteochilus lini	1	Cyprinidae
Pangasius elongatus	1	Siluridae
Parachela oxygastoides	1	Cyprinidae
Paralaubuca barroni	3	Cyprinidae
Puntioplites proctozysron	3	Cyprinidae
Systomus orphoides	1	Cyprinidae
Rasbora aurotaenia	12	Cyprinidae
Wallago attu	4	Siluridae
Xenetodon cancila	2	Belonidae

5.1.5 Smoking pre-treatment

Regarding the processing there are differences within the provinces but also within the fish species. Due to taste preferences and fish size, we can observe different approaches of treatment before smoking.

The typical smoking procedure starts with fish washing and cleaning in freshwater or salted water to remove slime from particular species; *Clarias spp.* or *Channa striata*. Subsequently, the cleaning consists of removing guts, this is also depends on the species, e.g., *Henicorhynchus caudimaculatus, Phalacronotus micronemus.* Removal of head is performed on small-scale fish species, where it is expected to be consumed whole, or due to taste preferences such as the bitterness of the head. This technique is used, for example, in the case of *Clarias spp..* During the field research a different approach was observed in the case of *Phalacronotus bleekeri* where the head was not removed, and its hard sculls were used for spiking (see Figure 45). After spiking, some of the species, mainly small-scale or fresh, were

placed on the mats, on the ground and left to sun-dry for 20 - 30 minutes. After drying, the grouped fish were placed on smoking trays and put into a smokehouse (see Figure 46).

Figure 45 Example of *Phalacronotus bleekeri* pretreatment, Tonlé Sap area, Cambodia

Some of the species were turned into half circles before sun-drying; this technique was used mainly for better storage of the final product and visual marketing preferences.

In Battambang province, which has a strong preference for *Clarias spp.*, they use different techniques. They cut the frontal part of the head, take out the guts through the head space, and spike them with wooden sticks, and clean with water 3 - 4 times to remove the slime before they start to smoke.

Figure 46 Fish smoked in a simple wooden smokehouse, Kampong Cham province.

5.1.6 Traditional technique of smoke-cured fish in Tonlé Sap area

A typical smoking procedure starts with pre-treatment as described above (5.1.5 Smoking), followed by fire preparation. Sometimes these steps may overlap. The most common approach is to prepare embers with a small or open fire, unlike the European technique of smoking where even with direct smoking the smoke is produced. However, fire-starting itself was produced by various techniques. The fire starters, which are important factors in PAH generation, mainly included the following techniques: preparation of net-like structures for better circulation of air (40%), plastic bags (>26% respondents) and use of sawdust (23%). Other reported fire starters were gas, coconut peel and oil-palm seeds (Figure 47 Examples of various fire starters, Tonlé Sap area, Cambodia, from right - plastic bags (1), oil-palm seeds (2), net-like structure (3).Figure 47).

Figure 47 Examples of various fire starters, Tonlé Sap area, Cambodia, from right - plastic bags (1), oil-palm seeds (2), net-like structure (3).

After the fish are grouped and put on sticks together, they are placed on smoking trays. All producers reported changing the position of the trays within the smokehouse. This provides an even distribution of heat and smoke in larger quantities of raw products. Generally, they are three techniques of rotation; a) from lower level to upper – vertical, b) change in order from left to right side within one level – horizontal, and c) front to back of each grouped fish plate/tray. The use of the technique for rotation mainly depends on the type of smokehouse, the number of levels, and the amount of smoked fish. Sometimes a combination of more than one technique occurs. Rotation is done regularly every 30 min to 1 h throughout the whole smoking process.

Although the temperature was not measured at all production sites, approximately 23% of the measured values for the first tray (next to the product) varied between 80 and 100 °C. The time of smoking reported was up to 16 hours (T1), 1 - 4 days (T2), one week (T3), and up to 10

days (T4) by 40%, 47%, 10% and 3% of the respondents, respectively (Figure 48). According to various authors, depending on whether the traditional or industrial technique (modern controlled environment and processing plants) was used, the presented time varies between hours (1 - 3) up to a maximum of 24 hours. It is considerably lower than those reported by Cambodian producers (Beltrfin & Universitaria 1991; Ciecierska and Obiedzinski 2007; Pöhlmann et al. 2013).

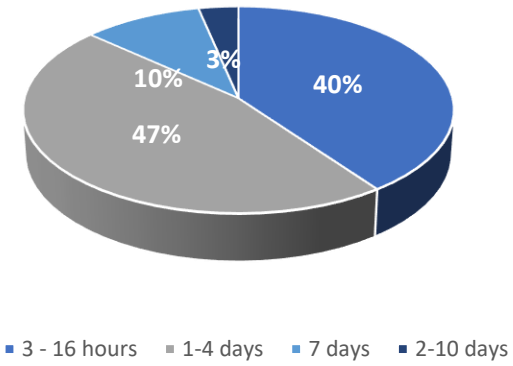

Figure 48 Distribution of responses: Length of smoking process reported by producers.

In addition, more than 50% of respondents also used materials such as paper cartons (69%), metal sheets (13%), grass mats (6%) or plastic rice bags (12%) to cover the product during the process of smoking, mainly when smoking kilns with open structures were employed (see Figure 49). This practice might cause additional contamination as glues and other substances are released from the covering material and burned. Indeed, practices such as burning any kind of waste to produce smoke can lead to increased concentrations of PAHs (Codex Alimentarius 2009; Ledesma et al. 2016).

Figure 49 Examples of additional techniques used during smoking, Tonlé Sap area, Cambodia; from right – paper carton (1), plastic mats (2), tarpaulin (3).

5.1.7 Selling practices of smoke-cured fish

Another aspect affecting PAH deposition and accumulation is the use of packaging and storage and selling practices. More than 44% of respondents stored their products hanging outside of the smokehouse; some stored them directly on smoking trays inside the smokehouse (less than 20%) or in the smokehouse itself (28%), where the continuous production and subsequent deposition of PAHs might occur; products were also stored in paper boxes where contamination from insects or rodents is common (Figure 50).

Figure 50 Examples of selling and transport practices, Tonlé Sap area, Cambodia; from right – paper boxes (1), hanging outside (2), plastic baskets (3).

Hanging the final product outside might help to reduce the final amount of PAHs due to photodegradation. Furthermore, according to Simko (1991), the concentration and distribution of BaP in smoked fish may change during storage due to diffusion and degradation, affected by the properties of the product and environmental factors (Stołyhwo & Sikorski 2005). On the other hand, most of the produces were hanging their products close to the main road where there was heavy traffic and subsequent deposition of toxic compounds and heavy metals might occur.

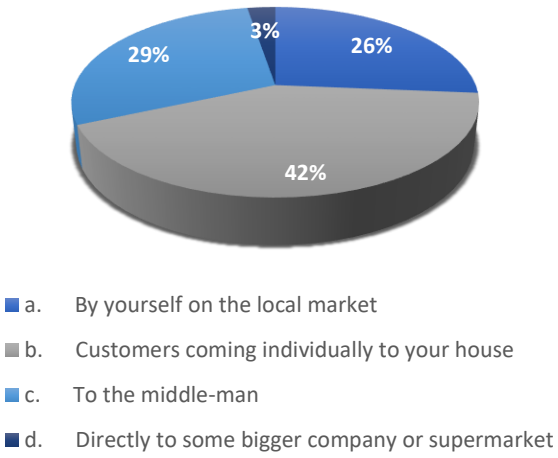

Figure 51 Distribution of responses**:** Selling practices of producers of smoked fish

Regarding the selling practices, producers responded that more than 40 percent of their goods are sold directly from households and the customers are come individually to purchase (42%). Almost evenly, the producers sell their goods to a middleman or personally on the local market, 29% and 26%, respectively (Figure 51).

Table 4 Selected parameters and relevant responses about traditional smoking of fish

Question	Responses	% of respondents	Question	Responses	% of respondents
Fuel	Wood	77%	**Fire-starting techniques**	Coconut peel	3%
	Charcoal	13%		Sawdust	23%
	Both	10%		Palm oil seeds	3%
Fuelwood species	*Havea brasiliensis*	20%		Plastic bags	26%
	Combertum trifoliatum	10%		Net structure - 0.5 m distance	40%
	Barringtonia asiatica	50%		Gas	3%
	Cashew tree	3%	**Frequency of smoking**	Daily	63%
	Barringtonia acutangula	7%		2 - 3 times a week	20%

	Mallotus anisopodus	7%		Weekly	10%
	Tamarindus indica	3%		Other - dependent on the raw material	7%
Distance from fire	< 50 cm	3%	**Storage**	At smokehouse	28%
	< 60 cm	23%		At smoking trays	17%
	< 70 cm	33%		Hanging	44%
	> 70 cm	33%		In boxes	11%
	N/A	7%	**Source of fish**	Fishing	40%
Temperature	40 - 80°C	17%		Buying	60%
	80 - 100°C	23%	**Daily production**	1 - 30 kg	37%
	> 100°C	7%		40 - 100 kg	23%
	N/A*	53%		100 - 500kg	33%
Length of smoking	3 - 16 h	40%		500 - 1000 kg	7%
	1 - 4 days	47%	**Selling practices**	Personally, on the local market	26%
	7 days	10%		Customers coming individually to your house	42%
	Up to 10 days	3%		Through the Middleman	29%
Use of additional technique	Carton	69%		Directly to some bigger company or supermarket	3%
	Grass mat	6%			
	Plastic Rice bag	12%			
	Metal sheet	13%			

5.2 Modified QuEChERS – EMR Lipid – DLLME method

5.2.1 Optimalisation of the sample preparation method

Figure 52 shows the comparison of PAHs recoveries obtained for the tested variants ("QuEChERS" (1), "EMR-Lipid" (2), and combined method QuEChERS - EMR Lipid (3) in combination with evaporation to dryness using the nitrogen stream). Generally, the recovery values within the acceptable range (50 - 120%, according to EU recommendation) were obtained only by the QuEChERS (1) method, but with the exception of naphtalene (NaP) and methylnaphthalene (MeNaP1), for which the recovery was below 50%. For two other compounds, anthracene (Ant) and benzo[a]anthracene (B[a]a), the results of the recovery were exceptionally high (120% and 119%, respectively), although they were still in the acceptable range (Figure 52). This presumed to be the impact of the sample preparation method.

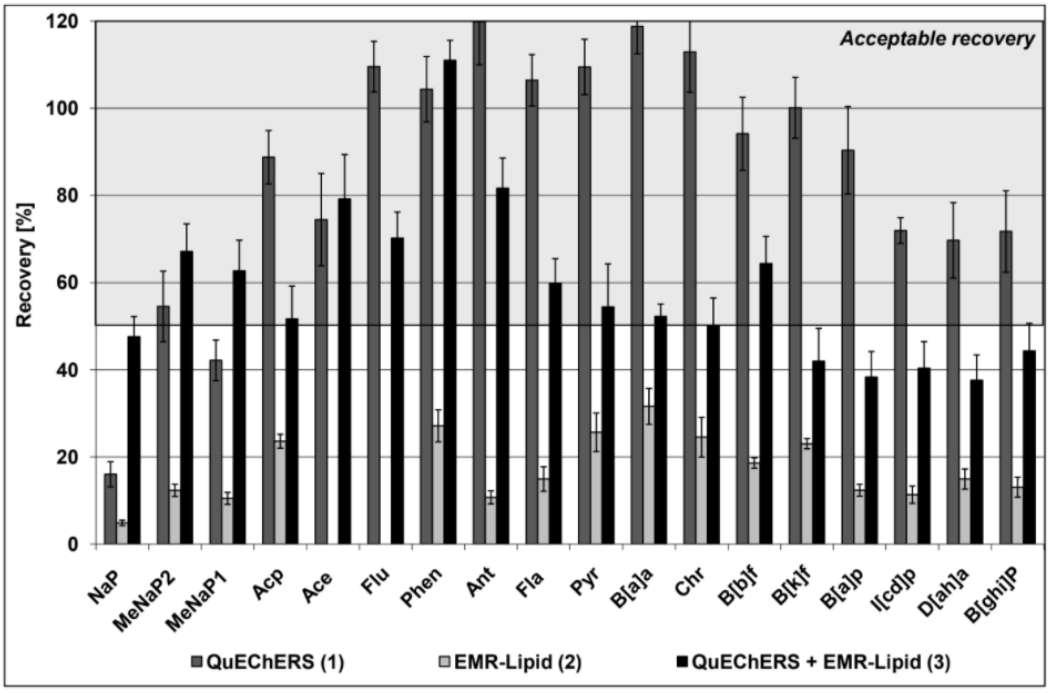

Figure 52 Comparaison of the PAHs recoveries obtained by QuEChERS (1), EMR Lipid (2) and combined method QuEChERS + EMR Lipid (3) in combination with evaporation to dryness using the nitrogen stream, for smoked mackerel.

Indeed, when analysing the chromatograms shown in Figure 53, it was found that the procedure with the application of freezing out and the use of conventional QuEChERS sorbents (Figure 53A), such as PSA and C_{18} did not provide sufficient removal of matrix co-extractives

from the sample. Additionally, these undesirable matrix residues influence the analytes, which can be seen in Figure 53D, leading as a consequence to the suppression or enhancement of recovery, which was mentioned previously. On contrary, in the second tested variant (EMR-Lipid (2) Figure 52), in which EMR Lipid was incorporated for clean-up step, obtained chromatogram (Figure 53B) was free from any undesirable compounds; however, the received recoveries were below 50% for almost all compounds, and acenaphthene (Ace) and fluorene (Flu) were not detected at all (Figure 52).

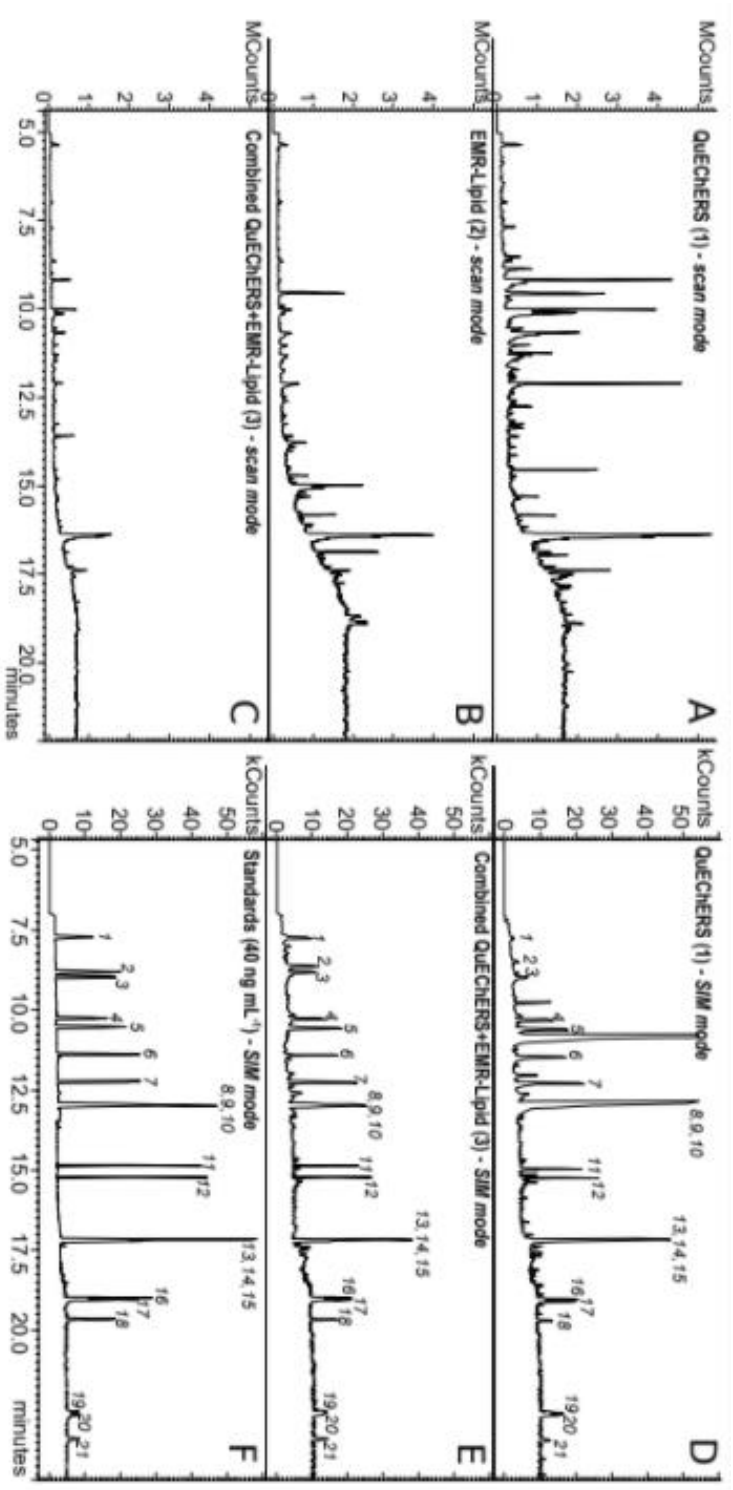

Figure 53 Comparison of GC-MS chromatograms (smoked mackerel) for three tested analytical procedures A– QuEChERS (1), full scan mode;. B – EMR-Lipid (2), full scan mode; C – Combination QuEChERS+EMR-Lipid (3), full scan mode; D - QuEChERS (1), sim mode; 1 – naphthalene; 2 –2-methylnaphthalene; 3 –1-methylnaphthalene; 4 – acenaphthene; 5 – acenaphthylene; 6 – fluorene; 7 – hexachlorobenzene (syringe standard); 8 – phenanthrene; 9 – anthracene-d10 (internal standard); 10 – anthracene; 11 – fluoranthene; 12 – pyrene; 13 – benzo[a]anthracene; 14 – chrysene-d12 (internal standard); 15 – chrysene; 16 – benzo[b]fluoranthene; 17 – benzo[k]fluoranthene; 18 – benzo[a]pyrene; 19 – indeno[1,2,3-c,d]pyrene; 20 – dibenzo[a,h]anthracene; 21 – benzo[g,h,i]perylene; E - Combination QuEChERS+EMR-Lipid (3), SIM mode: F – Standards, SIM mode

Regarding the sample preparation procedure, there are two significant steps influencing the yield of PAH recovery: extraction and clean-up step. The PAH extraction in the classical QuEChERS method is performed with acetonitrile (MeCN), usually in the presence of water, which releases the matrix components and improves the transfer of analytes into the solvent (Rejczak & Tuzimski 2015). In the second investigated procedure (with EMR-Lipid application), no water was used during the extraction step, as suggested by the manufacturer. Therefore, in this case, the lack of water can be a possible explanation for the low PAH recovery (Figure 52). However, the loss of compounds can also be a result of the use of inappropriate materials in the clean-up step, which can retain the analytes. Until now, the use of EMR-Lipid material for PAHs determination in food with high fat content has been reported in the studies conducted by Lucas and Zhao (2015), Han et al. (2016) and as well as by Urban and Lesueur (2017) who, however, did not noticed any PAHs losses when EMR-Lipid was applied, even when the extraction was performed without the addition of water. Nevertheless, it should be emphasised that the aforementioned experiments were carried out using untreated raw food samples, which, although containing a high level of fat, had not been previously smoked and therefore contained higher levels of water, which probably resulted in better recovery values. Hence, it was concluded that in the case of smoked food with a low water content, the sample preparation should be based on acetonitrile-water extraction of PAHs followed by a clean-up step with the application of EMR-Lipid material (QuEChERS + EMR Lipid (3)) (highlighted in blue Figure 37). Indeed, results confirming this hypothesis and the successful removal of matrix co-extractives were provided, but the recovery values for certain compounds were still below the acceptable limits (Figure 52).

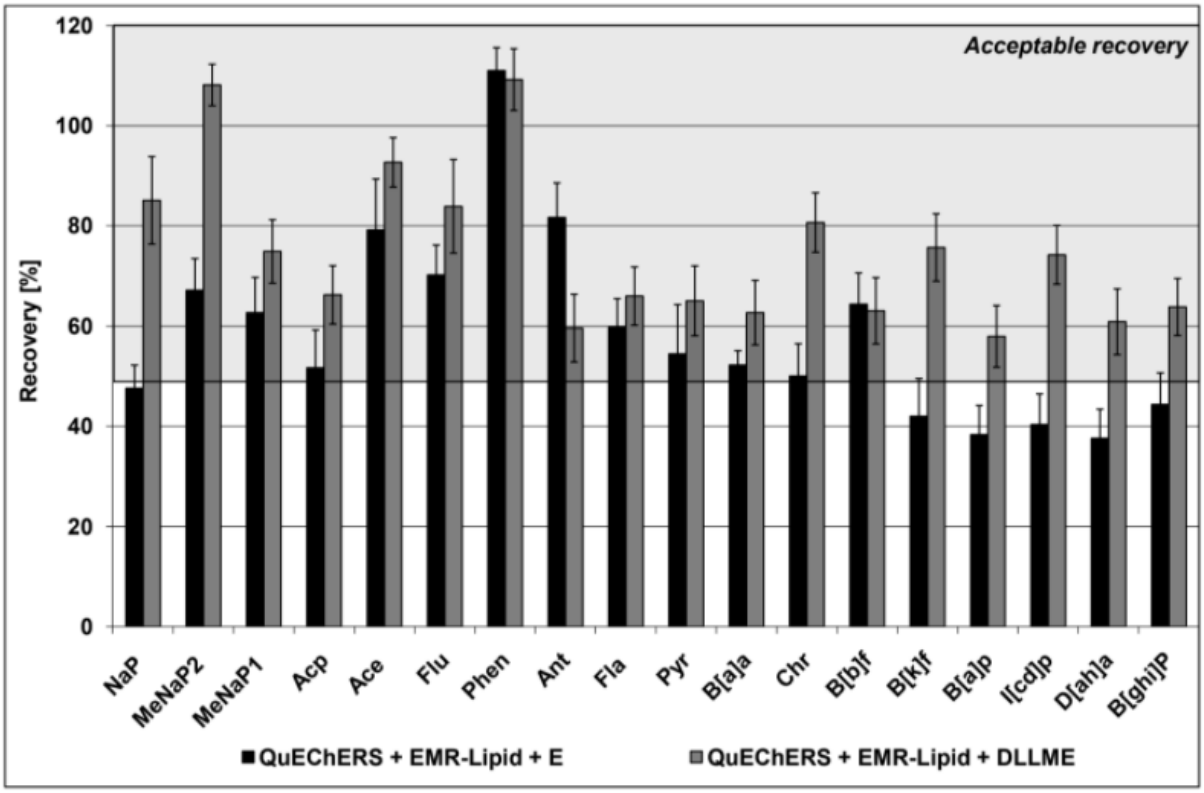

Figure 54 Comparaison of the PAHs recoveries obtained by evaporation (E) and dispersive liquid-liquid microextraction (DLLME) in the combination QuEChERS - EMR Lipid method (smoked mackerel).

Therefore, in order to investigate the potential cause of loss of PAHs and to improve the recovery rate of the compounds, we decided to include DLLME (see chapter Dispersive Liquid–Liquid Microextraction (DLLME)) as an alternative technique of extract preconcentration before the GC-MS analysis, instead of conventional evaporation to dryness by a stream of nitrogen (see Figure 37). Recovery rates of individual compounds you can see in the Figure 54. As expected, the application of DLLME (QuEChERS + EMR-Lipid + DLLME) contributed to the increase in the recovery values, especially for two of the lightest compounds: NaP and MeNap1 (Figure 54). This phenomenon can be explained by the implementation of chloroform, which can easily evaporate even without the incorporation of a stream of nitrogen, compared to acetonitrile that requires a longer time of evaporation process due to the higher boiling point. Additionally, the use of a stream of nitrogen (QuEChERS + EMR-Lipid + E) in the latter case might lead to the partial loss of light PAHs. DLLME method was also used in QuEChERS (1) and EMR-Lipid option (2), but it did not improve the quality of the sample clean-up (in the case of QuEChERS (1) method), and, in the case of EMR-Lipid option (2), the implementation of DLLME did not influence significantly on the PAHs recoveries, although the

values were slightly higher when compared to the option with the evaporation with nitrogen (data not shown in this study due to the lack of sufficient importance). This also suggests that the use of EMR-Lipid material without an effective extraction based on MeCN+H_2O, even if a DLLME preconcentration step is included in the procedure, does not contribute to an appropriate analyte recovery.

To summarise, the final version of the optimised sample preparation procedure composed of three steps (Figure 37, marked in blue colour): 1) QuEChERS extraction using water and acetonitrile followed by addition of NaCl and $MgSO_4$, 2) clean-up by EMR-Lipid material and 3) extract preconcentration by DLLME. This combination provided not only acceptable recovery data shown in the Figure 54 but also a satisfactory clean-up of the extract, which is shown in Figure 53C.

This protocol was also tested for other smoked fatty matrices, such as smoked cheese and smoked sausage. In each case, the recovery rates within the range of 50 – 120% were obtained for all compounds (Figure 56). Suggesting that the method is suitable not only for fish but other smoked fatty matrices.

5.2.2 Method performance

The developed analytical procedure was subjected to an in-house validation process that involved method linearity, limit of detection, limit of quantification, inter and intra-day precision, and accuracy according to the criteria established by the Commission Regulation 836/2011 (European commission (EC) 2011). Since a suitable reference material with certified content of the target analytes (to test the accuracy of the method) was not available for fish, meat and cheese products, a spiking procedure was used to calculate recoveries. The calculations were conducted for the spiked samples of smoked fish, sausage, and cheese, at the levels of 20 and 100 µg kg^{-1}. The results of the validation process are presented in Table 5 (to maintain the clarity of the table, the results are presented as the ranges of the values, obtained for all tested matrices) and Figure 53E shows the chromatogram of the sample of smoked fish, spiked at the level of 100 µg kg^{-1}, analysed in SIM mode.

To sum up, the linearity of the method was calculated based on the series of standard solutions in the range 2 – 400 ng ml^{-1}. The chromatograms of the PAH standards at the level of 40 µg ml^{-1} are presented in Figure 53F. The received values of correlation coefficient (r)

were higher than 0.99 for all compounds and matrices. Limit of detection (LOD) and limit of quantification (LOQ) were estimated on the basis of the signal of the background noise measured from the standard chromatograms at the lowest calibration level. The limit of detection was calculated as three times higher than the level of noise (S/N = 3), and the limit of quantification was equal to three times of the detection limit (LOQ = 3LOD). LOQs were lower than 0.9 µg kg^{-1} that is in accordance with the values established by EU (according to Commission Regulation 836/2011, LOQ should not exceed the level of 0.9 µg kg^{-1}).

Table 5 Parameters of in-house validation study of target PAHs for all tested matrices.

Compound	Calibration slope	Correlation coefficient, r	Repeatability* (RSD_r, n = 6) [%]	Reproducibility* (RSD_R, n = 6) [%]	Recovery* (level 20 µg kg^{-1}) [%]	Recovery* (level 100 µg.kg^{-1}) [%]	LOQ [µg.kg^{-1}]
NaP	220	0.9938	0.62-4.61	5.56-8.77	85-91	85-89	0.42
MeNaP2	153	0.9916	1.40-6.33	2.76-16.7	89-98	92-108	0.39
MeNaP1	143	0.9903	2.38-7.01	5.30-11.5	61-93	63-78	0.39
Acp	232	0.9975	1.59-7.55	3.49-9.28	69-71	60-66	0.38
Ace	157	0.9963	2.01-11.9	6.50-12.3	78-99	86-93	0.55
Flu	169	0.9986	2.36-9.58	3.18-10.6	74-82	69-84	0.47
Phen	225	0.9956	3.67-9.58	6.04-11.9	75-103	65-111	0.37
Ant	254	0.9940	4.49-6.39	5.55-14.7	63-93	60-81	0.49
Fla	289	0.9944	2.80-5.67	5.26-13.6	69-99	66-89	0.61
Pyr	341	0.9917	4.42-9.85	6.68-13.3	59-81	61-68	0.70
B[a]a	209	0.9936	4.12-9.87	4.67-10.2	59-84	63-80	0.82
Chr	282	0.9988	4.54-6.46	4.45-12.7	64-72	56-81	0.85
B[b]f	321	0.9930	1.20-6.25	4.41-8.38	67-69	61-64	0.76
B[k]f	410	0.9972	1.17-7.53	4.48-8.31	53-67	62-76	0.68
B[a]p	317	0.9924	1.36-5.84	2.95-8.19	63-75	58-70	0.67
I[cd]p	313	0.9982	1.98-6.10	7.03-13.9	59-72	69-74	0.89
D[ah]a	307	0.9990	2.31-10.7	6.99-12.0	67-76	61-73	0.81
B[ghi]P	393	0.9983	2.28-9.32	4.65-15.8	59-84	64-66	0.89

NaP – naphthalene; MeNaP2 – 2-methylnaphthalene; MeNaP1 – 1-methylnaphthalene; Ace – acenaphthene; Acp – acenaphthylene; Flu – fluorene; Phen – phenanthrene; Ant – anthracene; Fla – Fluoranthene; Pyr – pyrene; B[a]a – benzo[a]anthracene; Chr – chrysene; B[b]f – benzo[b]fluoranthene; B[k]f – benzo[k]fluoranthene; B[a]p – benzo[a]pyrene; I[cd]p – indeno[1,2,3-c,d]pyrene; D[ah]a – dibenzo[a,h]anthracene; B[ghi]P – benzo[g,h,i]perylene

* the results are presented as the ranges of the values obtained for all tested matrices

The repeatability expressed as a relative standard deviation (RSD_r) was calculated from six spiking samples analysed on the same day, whereas reproducibility (RSD_R) involved preparation and analysis from three different days. Received consistent deviations for all matrices were below 11.9% and 16.7%, respectively, with HORRAT values (calculated based on RSD_r and RSD_R, according to Horwitz equation; (EC 2011) lower 2 for each of the compounds which was in good agreement with EU criteria.

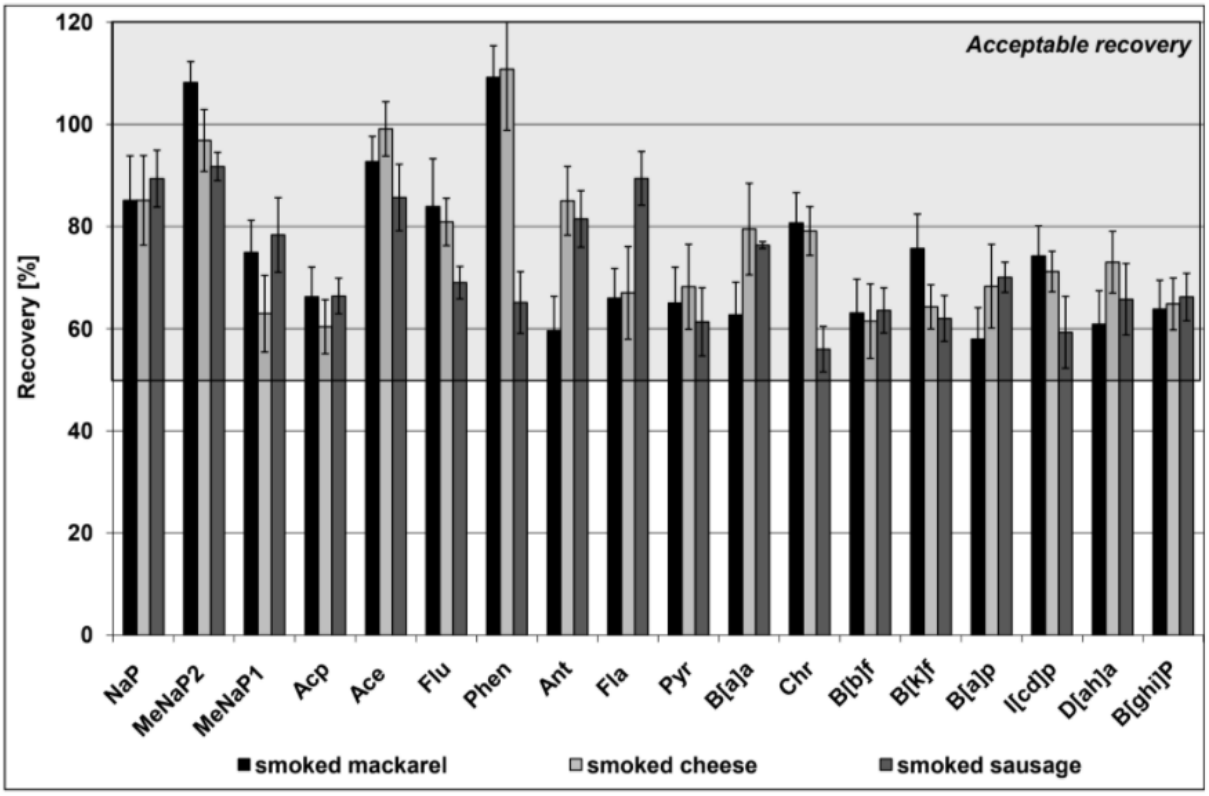

Figure 55 Recovery values for all tested matrices (smoked mackerel, smoked cheese, and smoked sausage) obtained at the level of 100 µg.kg-1 by the final version of the procedure.

The method accuracy was determined by recovery, using spiked samples, at two spiking levels. All results were found within acceptable limits and ranged from 55% to 103% for 20 $\mu g.kg^{-1}$ and 56 – 111% for 100 $\mu g.kg^{-1}$ (Figure 55 and Figure 56).

Based on the presented results of acceptable recovery values we can consider modified QuEChERS - EMR Lipid - DLLME method as suitable, fast and effective for PAHs determination in high fat content products of animal origin.

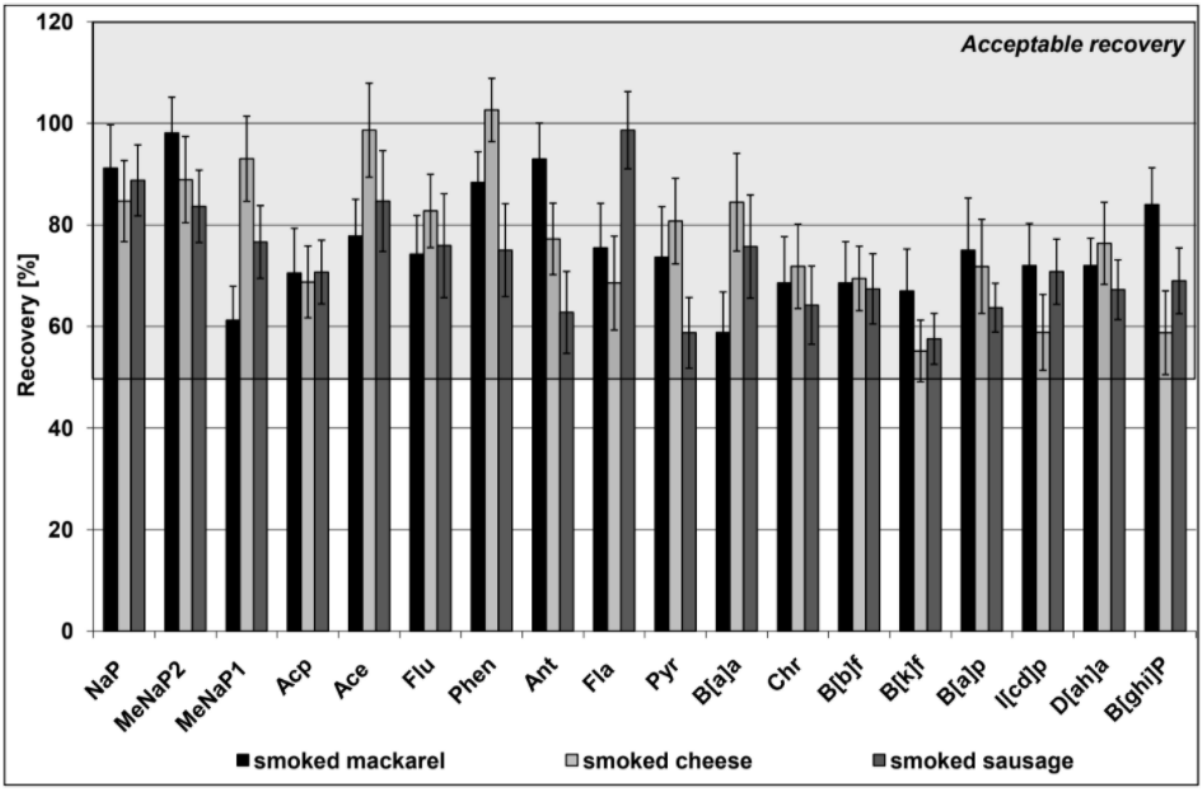

Figure 56 Recovery values for all tested matrices (smoked mackerel, smoked cheese, and smoked sausage) obtained at the level of 20 µg.kg^{-1} by the final version of the procedure.

5.3 General evaluation of total PAH concentrations in smoke-cured fish in Cambodia

In 2008, the European Food Safety Authority (EFSA) declared that by itself, BaP (benzo[α]pyrene) is not an appropriate marker for the occurrence of PAHs in food. Therefore, a combination of four specific PAHs, ΣPAH4 (benzo[a]pyrene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene), was introduced as a more accurate marker (EFSA 2008; Bansal & Kim 2015). In all studied smoked fish samples, the results for BaP, PAH4 (ΣPAH4) and the total PAHs (ΣPAH12) were expressed as the mean and standard deviation in µg PAHs per kg of dry fish matter. The dry weight of the samples was determined by oven-drying at 105 °C for 24 hours until a constant weight was achieved. In total, 57 fish samples (analysed in triplicates) from 18 species were tested. Detailed description of fish samples is in Table 2. The levels of BaP, ΣPAH4 and ΣPAH12 found in the samples of traditionally smoked fish using direct smoking from the Tonlé Sap area, Cambodia, are shown in Table 7. PAH concentrations are the mean of three analyses (triplicates for sample). The highest contents of ΣPAH4 and ΣPAH12 were determined to be 2701.45 µg.kg^{-1} and 16818.19 µg.kg^{-1} , respectively, in *Paralaubuca typus* smoked on wood for 3 - 16 hours (Producer 7 see Table 7) , followed by 3779.58 µg.kg^{-1} and 13547.89 µg.kg^{-1}, respectively, in *Labeo chrysophekadion* smoked on a combination of wood and charcoal for 3 – 16 hours (Producer 2 see Table 7). Interestingly, *Paralaubuca barroni* from the same producer (Producer 2) had the lowest measured mean values of ΣPAH4 and ΣPAH12 (76.33 µg.kg^{-1} and 536.95 µg.kg^{-1} , respectively). This might be explained by the different physical states of the samples, but also as a result of collecting samples on different smoking days to obtain a higher diversity of collected fish species. Because the effect of environmental factor such as decrease of BaP content with time and diffusion into the fish bulk was proven (Šimko 1991, 2005). Fish smoked under controlled conditions generally contains about 0.1 µg.kg^{-1} of BaP (Stołyhwo & Sikorski 2005). Compared to these data and those obtained from number of surveys on smoked fish and meat products from the European market (Moret et al. 1999; Yurchenko & Mölder 2005; EFSA 2008; Duedahl-Olesen et al. 2010), which rarely reported BaP concentration exceeding 1 µg.kg^{-1}. Samples from the Tonlé Sap area resulted in extremely contaminated fish samples with mean values ranging from 29.74 µg.kg^{-1} to 608.90 µg.kg^{-1} for benzo[a]pyrene used as biomarker in monitoring carcinogenic PAHs. These high levels recorded for BaP may pose an elevated cancer risk for consumers. Nevertheless, BaP values found in traditionally smoked samples of fish or meat from other areas were in agreement or were lower. Fasano et al. (2015) reported a comparably high total PAH content

in traditionally smoked sausages; they ranged from 313 to 3484 μg.kg^{-1}, with an average value of 1779 μg.kg^{-1} when the whole product (meat and casing) was taken into account. Several authors (Ciecierska and Obiedzinski 2007; Basak et al. 2010; Ledesma et al. 2014) found that skin serves as a barrier to PAHs in smoke. However, in this study, we used whole fish samples because smoke-cured fish products are consumed with the skin in Cambodia. This could also explain the higher total mean values compared with those of other studied products. In the same study by Fasano et al. (2015), smoked paprika was analysed in addition to traditional chorizo sausage. The results for paprika were significantly higher than those obtained for the chorizo sausage, and in principle, the technology used, were more similar to those of the fish samples in this study. The paprika was smoked by direct smoking, with smoke produced from oak wood for 10-15 days, and had no casings to protect the final product from smoke and PAHs. For chorizo sausage, the results for BaP, ΣPAH4 and ΣPAH8 ranged from 3.1, 38, and 41 μg.kg^{-1} to 98, 1370, and 1510 μg.kg^{-1}, respectively, and for paprika, the minimal and maximal values of PAH4 (ΣPAH8) were 593 μg.kg^{-1} (639 μg.kg^{-1}) and 3202 μg.kg^{-1} (3485 μg.kg^{-1}), respectively. Similarly high concentrations reported by Fasano et al. (2015) and in this research are also consistent with the results of Afolabi et al. (1983), Alonge (1988), Stołyhwo & Sikorski (2005) and Akpambang et al. 2011). A typical example of a product highly contaminated with PAHs is smoked dried bonito (katsuobushi). It is produced by repeated cycles of smoking for several hours at 80 – 120 °C, followed by overnight drying. The layer of tar that forms on the surface accounts for up to about 3 % of the fish weight. This layer of tar contains 20 – 40 times more BaP than the meat of the deeper layers with maximum levels of contamination reported for BaP 40 – 50 μg.kg^{-1} (Kikugawa et al. 1986; Stołyhwo & Sikorski 2005). Another example is a study of smoked fish from a Nigerian fishing settlement found to have a comparable high concentration of BaP in fish (6780 μg.kg^{-1}) (Anyakora et al. 2005). At the same time, the traditional method of smoking is also known to lead to higher PAH contamination than industrial/laboratory (controlled technological conditions) processes (Akpambang et al. 2011). Other studies also pointed out that compared with other meat and nonmeat foodstuffs, fish accumulates the most PAHs (Singh et al. 2016) and contamination was considerably higher than that in smoked meat (EFSA 2008; Plaza-Bolaños et al. 2010). This is because PAHs are lipophilic in nature and fish contains higher amounts of fat than other foodstuffs (e.g. vegetables, rice, wheat). Xia et al. (2010) published results for 25 food samples, this study indicated that fish contained the 3rd highest concentration of total PAHs,

160.30 µg.kg^{-1} (wet weight), compared to milk and vegetables but fish reached the highest in BaP content. Although the contamination of fish by PAHs from water and environment has been also discussed, it is worth noting that studies have confirmed that smoked and charbroiled/grilled products contain more PAHs than their uncooked counterparts (Rengarajan et al. 2015). The average natural content of PAHs is generally lower in fish musculature than in the liver and tissues of molluscs. Also, fish, in contrast to bivalves, have the ability to oxidize and further metabolise PAHs to water–soluble compounds that are excreted by the living organism (Stołyhwo & Sikorski 2005). Finally, very few studies have investigated the PAH content of smoked foods commonly consumed in Southeast Asian countries, especially in Cambodia, even though the results shown in the present study are alarming and indicate contamination levels comparable to those presented in other studies e.g. from industrial and heavily populated areas (Anyakora et al. 2005; Essumang et al. 2013).

Table 6 Mean (of triplicate analyses) BaP, PAH4, and PAH12 values of contentration in all samples within each fish species (µg.kg^{-1}).

Fish species	N	BaP*	SD***	PAH4**	SD	PAH12****	SD
Belodontichthys truncatus	2	79.99	±45.91	285.81	±166.17	2004.98	±747.84
Henicornhychus siamensis	10	182.00	±67.12	1183.03	±565.81	5518.28	±2055.12
Clarias batrachus	6	57.27	±32.35	182.56	±126.26	1164.73	±559.13
Hypsibarbus malcolmi	1	221.12	±22.02	1121.09	±113.95	4208.57	±438.83
Labeo chrysophekadion	2	394.37	±240.95	2154.22	±1581.58	8419.03	±5281.56
Micronema hexapterus	6	119.38	±73.95	592.47	±377.06	3215.40	±1712.31
Notopterus notopterus	1	46.16	±2.61	183.05	±159.12	1347.18	±77.13
Ompok bimaculatus	4	127.50	±81.27	296.72	±122.93	1277.39	±363.15
Osteochilus schlegeli	1	228.27	±9.66	1066.94	±62.97	4737.67	±362.15
Pangasius elongatus	1	124.75	±8.17	871.89	±80.69	4092.28	±436.26
Paralaubuca barroni	3	105.03	±60.15	506.97	±307.28	2299.45	±1359.74
Paralaubuca typus	3	247.63	±96.89	1644.19	±835.07	9171.56	±5784.68
Phalacronotus bleekeri	4	217.20	±40.62	1093.81	±197.03	3286.86	±1165.60
Phalacronotus micronemus	2	99.13	±31.77	461.95	±210.21	1729.73	±423.75
Puntioplites prostozystron	1	99.15	±48.76	425.67	±357.13	2887.15	±449.28
Rasbora hobelmani	7	179.12	±64.12	933.39	±392.99	6131.80	±3153.55
Wallago attu	2	43.87	±8.52	179.92	±30.81	1107.91	±207.41
Xenentodon cancila	1	91.40	±9.33	555.64	±2.26	2091.47	±302.62

*BaP = Benzo[a]pyrene; **PAH4 = Benzo[α]pyrene, Chrysene, Benzo[α]anthracene, Benzo[β]fluoranthene; ***SD = Standard Deviation; ****PAH12 = Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Chrysene, Benzo[a]anthracene, Benzo[b]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene and Benzo[ghi]perylene

The mean (triplicate analyses) of PAH concentrations for all samples within all collected fish species are displayed in the Table 6. Based on the results in Table 6 we can observe high variability between fish specieas in the sampling area. The high standard deviation levels are subject to high variability of PAHs within the species. Since each sample is from a different producer and the samples undergo different technological process (see Table 7). A similarly wide range of samples from different producers was reported by Hokkanen et al. (2018) during the evaluation of smoked product on the Finnish market. The highest mean values of ΣPAH4 and ΣPAH12 were measured in samples of *Paralaubuca typus* species (1644.19 $\mu g.kg^{-1}$ and 9171.56 $\mu g.kg^{-1}$, respectively), followed by samples of *Labeo chrysophekadion* species (2154.22 $\mu g.kg^{-1}$ and 8419.03 $\mu g.kg^{-1}$, respectively). Samples of *Wallago attu* species had the lowest mean ΣPAH4 and ΣPAH12 concentrations of 179.92 $\mu g.kg^{-1}$ and 1107.91 $\mu g.kg^{-1}$, respectively. The content of PAHs within one species varied greatly. This trend can be mainly explained by differences in e.g., age, season of catch or diet within each species (R.E.Rasoarahona et al. 2005). In addition to the fat content and species, it was reported that the size of the fish/sample might affect the level of contamination. In study of Hokkanen et al. (2018) was observed that small fish samples contained higher median PAH levels than larger fish samples. Since the collection of samples was real-time within restricted period of time the size of these samples was not possible to influence this parameter, although, samples of approximately same size were collected. Additionally, according to (Basak et al. 2010) PAHs content variations were attributed to non-homogenous smoke dispersion in traditional ovens. This might be an explanation of such variation in this study as well, since the traditional smokehouses are missing ventilation regulation systems and/or solid construction to prevent the fluctuation of dispersion of smoke.

Table 7 Overview of the results of mean and SD of BaP, SUM PAH4, SUM PAH12 in µg.kg^{-1} for all sample, fish species (n = 3).

Producer	Fish species	Province	Fuel	Time	BaP* Mean±SD	ΣPAH_4** Mean ±SD***	ΣPAH_{12}**** Mean±SD
P1	*Belodontichthys truncatus*	Kampong Cham	Wood	T2	113.50±2.02	407.03±8.16	2544.57±72.07
P3		Kampong Cham	Wood	T1	29.74±2.32	103.98±10.85	1195.60±207.36
P3	*Henicorhynchus siamensis*	Kampong Cham	Wood^{abcd}	T1^{abc}	211.96±5.00	1088.94±22.93	7027.38±116.94
P6		Kampong Cham	Wood^{aefgh}	T2^{adef}	274.45±11.21	1749.09±15.45	9515.35±180.59
P8		Kampong Chhnang	Wood^{bei}	T1	123.78±10.46	707.36±32.86	3568.98±987.98
P9		Kampong Chhnang	Wood	T3	90.89±12.28	549.88±24.77	2628.20±165.53
P13		Battambang	Both^{cfjk}	T1^{bdg}	85.98±7.16	549.06±61.27	4183.87±110.17
P16		Battambang	Wood	T2	193.09±19.32	1018.31±186.61	3860.58±499.29
P18		Battambang	Wood^{dgl}	T1^{cfh}	148.68±26.51	1165.42±75.93	4890.97±343.19
P19		Battambang	Wood	T1	257.83±44.28	2192.83±106.97	17160±430
P22		Battambang	Wood^{hk}	T1	139.13±4.59	1149.69±299.21	6062.81±245.19
P23		Battambang	Wood	T1	231.34±13.93	1531.68±201.91	7448.93±598.01
P2	*Clarias batrachus*	Kampong Cham	Both^{ab}	T2	47.27±5.33	109.51±14.03	690.09±17.12
P6		Kampong Cham	Wood	T2	38.99±3.66	141.68±12.54	822.55±29.81
P25		Battambang	Charcoal^{ac}	T2	112.27±0.74	408.42±7.94	1979.74±93.07
P26		Battambang	Charcoal^{bc}	T2	47.09±8.8	233.67±2	1400.45±12
P27		Battambang	Charcoal	T2	30.39±2.04	150.75±15.90	1650.94±88.27
P28		Battambang	Charcoal	T2	35.01±0.38	120.77±10.27	611.09±41.39
P8	*Hypsibarbus malcolmi*	Battambang	Wood	T1	221.12±22.02	1121.09±113.95	4208.57±438.83
P2	*Labeo chrysophekadion*	Kampong Cham	Both^{a}	T2	608.90±77.55	3779.58±302.93	13547.89±936.50
P6		Kampong Cham	Wood^{a}	T2	187.33±42.13	839.47±650.14	4229.21±3612.47
P2	*Micronema hexapterus*	Kampong Cham	Both^{abc}	T2^{abc}	273.42±8.38	1321.77±60.43	5249.82±679.23
P3		Kampong Cham	Wood^{adef}	T1^{adef}	57.94±6.97	214.94±16.66	1307.18±218.82
P4		Kampong Cham	Wood^{bdgh}	T2^{bdgh}	150.34±16.06	925.67±28.49	4057.79±199.93
P6		Kampong Cham	Wood^{cegi}	T2^{cegi}	122.94±3.10	441.97±16.86	1681.30±161.12

P18		Battambang	Wood	T1	44.97±1.86	313.85±11.66	2262.97±210.80
P23		Battambang	Wood[fhi]	T2[fhi]	80.13±2.41	573.30±1.80	5492.34±229.15
P2	*Notopterus notopterus*	Kampong Cham	Both	T2	46.16±2.61	270.05±19.12	1347.18±77.13
P5	*Ompok bimaculatus*	Kampong Cham	Both[ab]	T2[ab]	38.79±1.81	194.63±10.11	1389.68±47.80
P6		Kampong Cham	Wood	T2	94.26±17.41	398.81±59.52	1108.95±654.51
P7		Kampong Chhnang	Wood[ac]	T1[ac]	58.59±5.12	363.75±17.67	1950.63±20.85
P11		Kampong Chhnang	Wood[bc]	T3[bc]	196.41±28.21	1096.03±169.02	3072.97±339.20
P8	*Osteochilus schlegeli*	Kampong Chhnanhg	Wood	T1	228.27±9.66	1066.94±62.97	4737.67±362.15
P2	*Pangasius elongatus*	Kampong Cham	Both	T2	124.75±8.17	871.89±80.69	4092.28±436.26
P2	*Paralaubuca barroni*	Kampong Cham	Both	T2	29.93±0.63	76.34±3.08	536.95±30.92
P3		Kampong Cham	Wood[a]	T1[a]	163.10±3.09	757.35±27.30	2826.84±148.32
P5		Kampong Cham	Both[a]	T2[a]	93.04±0.93	562.01±4.83	3534.57±86.80
P7	*Paralaubuca typus*	Kampong Chhnang	Wood	T1	321.22±31.43	2701.45±162.24	16818.19±420.24
P8		Kampong Chhnang	Wood	T1[a]	299.57±4.17	1386.67±26.47	6139.54±212.25
P14		Battambang	Wood	T2[a]	122.09±27.08	844.46±163.75	4556.95±436.16
P7	*Phalacronotus bleekeri*	Kampong Chhnang	Wood	T1[abc]	157.98±25.81	749.53±97.45	1670.95±637.18
P10		Kampong Chhnang	Wood	T4[ad]	201.65±10.41	1094.81±53.75	4016.97±182.82
P11		Kampong Chhnang	Wood	T4[be]	241.33±10.28	1274.49±120.45	4484.51±133.20
P12		Kampong Chhnang	Wood	T3[cde]	256.13±14.31	1141.67±31.92	2975.02±38.13
P1	*Phalacronotus micronemus*	Kampong Cham	Wood[a]	T2	84.94±28.50	298.52±59.34	1371.35±44.68
P4		Kampong Cham	Wood[a]	T2	128.98±27.28	706.85±117.51	2191.71±242.50
P8	*Puntioplites prostozystron*	Kampong Chhnang	Wood	T1	71.56±13.80	415.86±40.76	2718.55±363.64
P13	*Rasbora hobelmani*	Battambang	Both	T1	163.00±48.06	459.77±63.06	5085.94±148.45
P17		Battambang	Wood	T1	326.47±6.13	1776.46±76.89	10900.81±720.05
P19		Battambang	Wood	T1	162.44±8.42	904.21±55.74	6579.45±367.08
P20		Battambang	Wood	T1	116.14±5.65	822.98±25.24	2539.73±114.53
P21		Battambang	Wood	T1	209.71±35.11	1205.47±109.94	5116.95±303.59
P23		Battambang	Wood	T1	191.86±25.45	1097.96±108.58	11641.20±1019.46
P24		Battambang	Wood	T1	125.17±2.00	559.78±23.98	4080.90±279.49

P1	*Wallago attu*	Kampong Cham	Wood	T2	51.27±9.35	154.61±4.37	922.99±38.31
P4		Kampong Cham	Wood	T2	38.94±3.17	196.80±28.65	1292.83±59.09
P4	*Xenentodon cancila*	Kampong Cham	Wood	T2	91.40±9.33	555.64±2.26	2091.47±302.62

*BaP = Benzo[a]pyrene; **ΣPAH_4 = sum of Benzo[α]pyrene, Chrysene, Benzo[α]anthracene, Benzo[β]fluoranthene; ***SD = Standard Deviation; ****ΣPAH_{12} = sum of Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Chrysene, Benzo[a]anthracene, Benzo[b]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene and Benzo[ghi]perylene; T = smoking time: T1 (3-16 hours) and T2 (1-4 days), and T2 (1-4 days) and T4 (up to 10 days); ^{abcdefghi} = refers to a statistically significant ($p<0.05$) difference in ΣPAH_{12} concentration in dependence on used fuel or smoking time within one species

5.3.1 Effect of the duration of smoking on PAH concentration

One of the parameters known to affect the level of contamination by PAHs is the period of the smoking process, which has a significant influence on both the quality and the levels of PAHs in smoked fish (Essumang et al. 2013). Longer smoking time is known to improve the shelf life of fish by significantly reducing the moisture and lipid contents, which would otherwise cause rancidification and spoilage of smoke-cured fish (Essumang et al. 2012, 2013; Viegas 2012), though the effect of loss of lipids and moisture cannot be evaluated since the collected samples were already smoke-cured. The data obtained for a given fish species from more than one producer were statistically compared to determine differences in the level of PAH contamination due to the length of smoking. In Table 7, the column "Time" shows the period for each fish sample and the fish species as declared by the producers. The index letters in column "Time" (Table 7) refer to statistically significant differences ($p<0.05$) between the different processing lengths and the mean values (n = 3) of ΣPAH12 for fish samples (more than one collected) within the fish species. Based on these results, significant differences were observed between T1 (3 - 16 hours) and T2 (1 - 4 days) for species of *Henicorhynchus siamensis*, *Micronema hexapterus*, *Paralaubuca barroni* and *Paralaubuca typus;* significantly higher mean values of ΣPAH12 were 9515.35 $\mu g.kg^{-1}$ (T2), 5492.34 $\mu g.kg^{-1}$ (T2), 3534.57 $\mu g.kg^{-1}$ (T2) and 6139.54 $\mu g.kg^{-1}$ (T1), respectively. In the case of species *Phalacronotus bleekeri*, significant differences were found between all the declared times (T1, T3, and T4). Smoking time T4 had the highest mean values; 1274.49 $\mu g.kg^{-1}$, 4484.51 $\mu g.kg^{-1}$ and 241.33 $\mu g.kg^{-1}$ for ΣPAH4, ΣPAH12 and BaP, respectively. For samples of *Henicorhynchus siamensis*, *Micronema hexapterus* and *Paralaubuca barroni* significantly higher mean values of ΣPAH12 were measured at T2, which might be explained by differences in the generation of PAHs over time. A study by Alomirah et al. (2011) suggested that low molecular weight PAHs (LMW, containing 2 - 3 aromatic rings), which are more volatile than high molecular weight PAHs (HMW, containing more than 3 aromatic rings), are predominant in the smoke generated by the pyrolysis of fat drippings over the heat source which occurs at the beginning of the process (Alomirah et al. 2011). On the other hand, all producers claimed to rotate the product on the smoking trays (if present); therefore, the distance from the fire varied depending on the stage of the smoking process. In contrast, a study by Ledesma et al. (2014) reported that the BaP content increased from less than 0.24 $\mu g.kg^{-1}$ to 0.75 $\mu g.kg^{-1}$ and finally

stabilized after 5 days of smoking. This trend was attributed to the fact that after 5 days, the natural pores of the casing or skin may be blocked by large tar particles in the smoke, preventing the continued penetration of PAHs. Additionally, Rose et al. (2015) reported that, contrary to expectations, the concentration of PAHs decreased with time in some cases. They attributed this result to differences in the surface area and surface texture of the food and how fat was lost during cooking. Similarly, Hokkanen et al. (2018) measured unexpectedly lower PAHs levels with longer time, explaining it by the type of smoking or size of the sample. According to Essumang et al. (2013) this trend may be attributed to the fact that PAHs adsorbed on fish surfaces were either easily detached or converted to volatile ones and released into the surroundings other than the fish when being heated for a long time. In the same study the highest mean values of all 16 PAHs ranging from 250.59 to 1143.51 $\mu g.kg^{-1}$ at 2 h, from 595.33 to 1315.66 $\mu g.kg^{-1}$ at 4 h, and from 574.97 to 1376.09 $\mu g.kg^{-1}$ at 8 h were reported. Chen & Lin (1997) also concluded that PAH contamination increased with smoking time. Roseiro et al. (2012) reported that the traditionally smoked meat sausages Painho and Paio tradicional had mean values of 1397.62 $\mu g.kg^{-1}$ and 2609.81 $\mu g.kg^{-1}$ after 15 and 30 days, respectively. In this study, approximately 40% and 47% of respondents declared the duration of smoking to be 3 – 16 hours and 1 – 4 days (see Table 4). This is still, however, a considerably longer period compared to usually reported smoking times, ranging between 2 – 12 hours (Bannerman & Horne 2001; Stołyhwo & Sikorski 2005; Essumang et al. 2013). However, the elevated concentrations are consistent with the above-mentioned studies in which the concentrations of PAHs increased and then stabilised with time. This could be explained by the fact that when smoking lasts longer than 4 days, the concentration of PAHs stabilises (due to blockage of the pores) or the increase in concentration is already statistically insignificant.

5.3.2 Effect of the fuel used for smoking on PAH formation and concentration

Table 7 lists the mean concentration levels for BaP, ΣPAH4 and ΣPAH12 obtained using various fuels in the smoking process for each fish sample and species. In Table 7, the column “Fuel” shows significant differences within the same species in the PAHs concentration depending on the type of fuel used for each sample (where more than one sample was collected). For samples of species of *Henicorhynchus siamensis*, *Labeo chrysophekadion*, *Micronema hexapterus*, *Ompok bimaculatus,* and *Paralaubuca typus*, levels of ΣPAH12 contamination were significantly higher when fuelwood (Wood) and a combination of fuelwood and charcoal

(Both) was used. Levels of ΣPAH12 were 9515.35 μg.kg^{-1} for *Henicorhynchus siamensis* (P6 - Wood), 13547.89 μg.kg^{-1} for P2 - Both for *Labeo chrysophekadion*, 5492.34 μg.kg^{-1} for *Micronema hexapterus* (P23 – Wood), 3072.97 μg.kg^{-1} for *Ompok bimaculatus* (P11 – Wood) and 2826.84 μg.kg^{-1} for *Paralaubuca barroni* (P7 – Both). A significant difference between charcoal and a combination of both fuels was only observed in the case of *Clarias batrachus* species, and the highest mean value of total PAHs was 1979.74 μg.kg^{-1} for P25 - Charcoal. In general, the chemical formation of PAHs during product smoking is due to the incomplete combustion or pyrolysis of wood (Ledesma et al. 2016). This is consistent with the results of Ross et al. (2002) and Han et al. (2020), who detected higher PAH emissions from the combustion of wood than from coal. In addition, more LMW PAHs were emitted in the early burning stage of wood, whereas more HMW PAHs were emitted in the later burning stage, in contrast to the trend for coal. Therefore, we can suggest that a combination of both fuels leads to increased contamination by PAHs. A study by Rose et al. (2015) indicated that preparing food over charcoal can lead to elevated levels of PAHs depending on the fat content. In comparison with this study, a report by Roseiro et al. (2012) also found a high level of contamination in traditional meat/blood sausages directly smoked over wood with a total PAH content of 2296.56 μg.kg^{-1}. They explained that the high levels of PAHs were caused by the higher temperature applied to these products at the beginning of the heat treatment. Additionally, grilling over an open fire and in direct contact with flames might result in extremely high PAH levels, as in this study. At the same time, Garcia-Perez (2008) found that softwood produces more PAHs than hardwood when burned because of its high lignin content. Using this type of fuel greatly increases the PAHs in meat products. Although Table 4 shows that fuelwood, such as Barringtonia asiatica (mangrove) and Havea brasiliensis (rubber tree), which are both classified as a hardwood, were mainly used for smoke curing. The results of Tekasakul et al. (2008) showed a correlation between PAHs concentrations and rubber-wood burning. However, a study by Essumang et al. (2013) noted that mangroves are considered as hardwoods and might have a lower lignin content due to the malfunctioning of water-transporting tissue. Therefore, the levels of PAHs in products smoked over this wood were lower than those in products smoked over other tested fuel woods (acacia and sugarcane bagasse). As shown in Table 4, use of various fire starters was reported in this study; even gas was used or plastic bags placed on wood piles to start fires. The co-combustion of plastics (polyethylene (PE) and polyethylene terephthalate (PET) with wood reportedly,

increased the total PAH7 (4 – 6-ring PAHs) by 43% and 71%, respectively, and the total PAH7 ranged from 4.5 to 11 $\mu g.kg^{-1}$ (Tomsej et al. 2018). In conclusion, co-combustion with PET resulted in a significant increase in the emissions of total PAHs. Chung et al. (2011) studied the PAH content of meat products grilled and roasted over charcoal with gasoline for 30 min, and reported a BaP content of 8.49 $\mu g.kg^{-1}$. However, our measured levels of contamination were considerably higher than those reported by other authors using fire starters. Therefore, we can assume that the use of inappropriate fire starters is not the only factor affecting the level of contamination by PAHs, but it might have an important effect on the final content.

5.3.3 Effect of temperature on PAH formation and concentration

As previously mentioned, the temperature is one of the factors affecting the level of contamination by PAHs. The composition of smoke is dependent on the temperature, which needs to be regulated to reduce the formation of PAHs (Codex Alimentarius 2009). According to a study by Ledesma et al. (2016), direct smoking can be classified as cold (15 – 25 °C temperature of the product) or hot (80 °C temperature of the product) smoking based on the temperature of the product. During the sampling measurements of temperature were done (see 4.1.3 Temperature measurements), however during the period of collection not all producers had production in operation, mainly due to shortage of raw material, fuel or because the production had already ended or not yet started. Although the general period of smoke-curing is known (October – December) it is highly affected by weather conditions since most of the producers are in the wetlands surrounding Tonlé Sap lake. From a total of 31 producers visited only 47% had production in operation. The range of the measured temperatures are presented in Table 4. In 23% of producers temperatures between 80 – 100 °C of the product were measured. Based on the measured temperatures from the producers with production in operation, we can describe the traditional smoking of fish in Cambodia as hot smoking according to Ledesma et al. (2016). With the temperatures of product at the first tray level of smoking kiln (50 – 100 cm from the base of the smoking kiln) being between 80 – 100 °C. A study by Han et al. (2016) focusing on the influence of combustion temperature and fuel type on PAH emissions reported that the temperature was the most important factor in PAH formation. According to a study by Hokkanen et al. (2018), lower amounts of PAHs were formed when the temperature was optimized than when it was not optimized, i.e., it might vary during the process, as in our case. The optimized temperature

in a later study in the thermal field was kept between 400 – 600 °C. This is in agreement with Šimko (2005) that the levels of PAHs are higher when the temperature regulation system is missing. Further temperature measurements focused on both the combustion temperature and the temperatures of the smoke and product throughout the whole process are recommended. To gather more robust data to evaluate the effect of temperature on final smoked product in this area.

5.4 Fish fat content and its association with PAH concentration

The fat content of fish species was evaluated by the modified microquantity colorometric sulfo-phospho-vanillin method method (SPV) and further correlation between fat content and PAH level was analysed. Figure 57 shows the mean fat content (in %) of sampled fish species from all producers. The *Paralaubuca typus* species had the highest mean value (50.96%), followed by *Clarias batrachus* (47.31%) and *Osteochilus schlegeli* (40.70%). As shown in Table 8, the fat content varied greatly within each species, fat conent measurements for each sample are given in Appendix II. This result might be attributed to different smoking processes, lengths of the process, and age and size of the fish. Also, according to a study by Taşbozan & Gökçe (2017), among vertebrates fish have the highest species diversity in fatty acid composition and the nutritional content. They explain this mainly by environmental factors as well as biological, physical and chemical factors. Another important factor in the relation to fish processing is the storage of fat within the fish's body (organs/muscle). To date, there are no reports summarizing the dependence of the concentration of PAHs on the fish species commonly consumed in Cambodia or Southeast Asian countries or on their fat content.

Table 8 Total fat content of samples of smoked fish within each fish species (%).

Fish species	**% fat (min)**	**% fat (max)**	**% Median fat**	**% fat SD***
Belodontichthys truncatus	18.7	44.9	33.2	±9.89
Henicorhynchus siamensis	1.43	52.9	30.9	±14.4
Clarias batrachus	31.6	64.2	48.9	±8.1
Hypsibarbus malcolmi	37.2	43.7	39	±3.36
Labeo chrysophekadion	0.09	25.3	23.9	±12.1

Micronema hexapterus	0.09	46.7	21.8	±13.6
Notopterus notopterus	0.46	2.35	1.76	±0.95
Ompok bimaculatus	0.09	30.6	1.89	±12.3
Osteochilus schlegeli	37.6	46.7	37.7	±5.23
Pangasius elongatus	31.5	32.2	31.5	±0.43
Paralaubuca barroni	1.99	60.8	40.2	±25.7
Paralaubuca typus	26	80.2	45.1	±20.9
Phalacronotus bleekeri	8.26	23.7	10.7	±5.3
Phalacronotus micronemus	6.53	11.5	9.89	±1.77
Puntioplites prostozystron	30.6	44.1	37.8	±4.41
Rasbora hobelmani	16.7	61	31.2	±11.7
Wallago attu	4.99	8.02	6.34	±1.32
Xenentodon cancila	9.71	10.6	10.4	±0.47

*SD = standard deviation

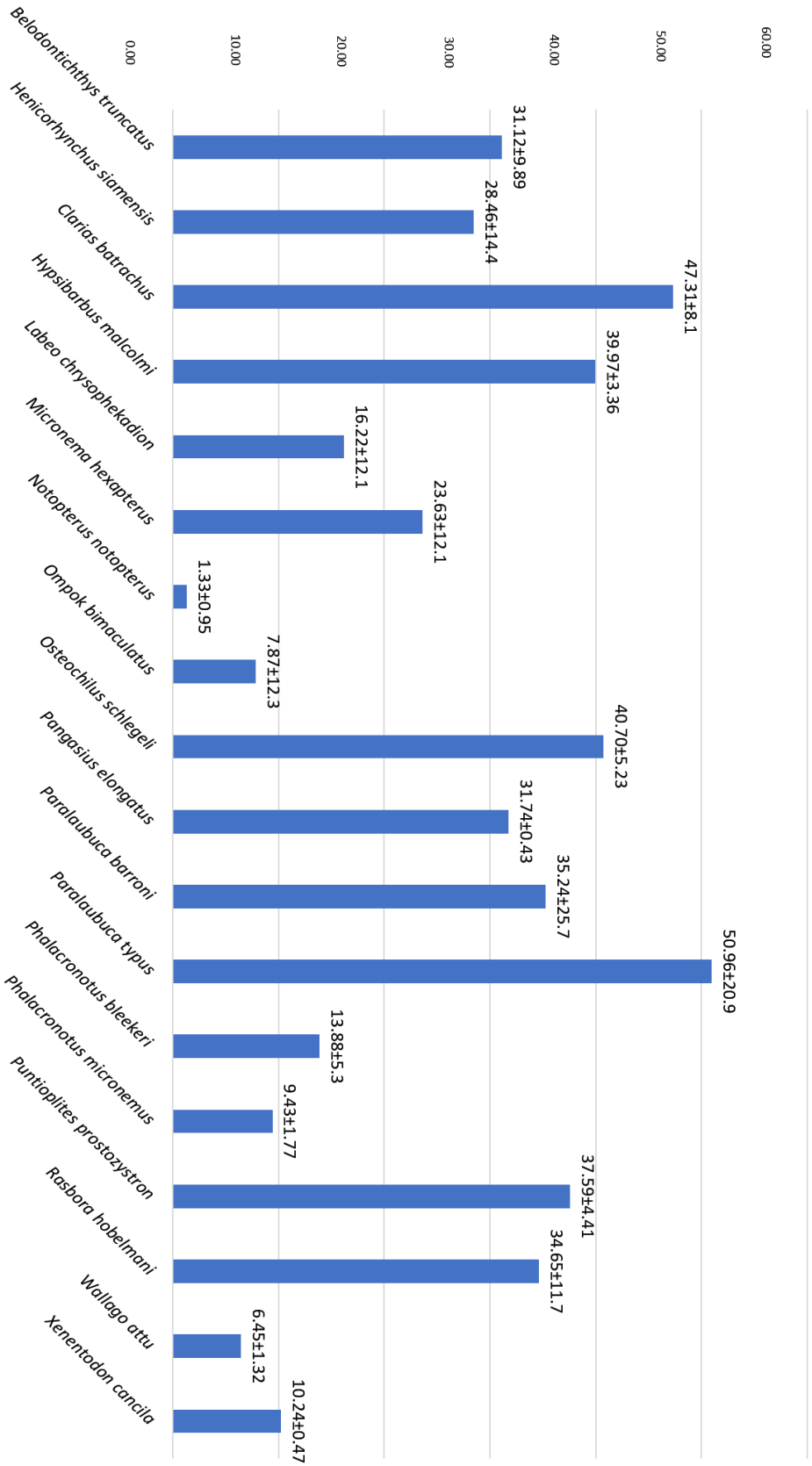

Figure 57 Mean total fat content in smoked fish samples within species (%).

However, some studies have discussed the correlation between fat and the concentration level of PAHs in smoked fish products, particularly in Europe (Duedahl-Olesen et al. 2010). Another study from Turkey measured the average total PAHs level of smoked rainbow trout (23.83%) and of smoked salmon (79.74%), which was supported by the average fat content of smoked salmon (6.57%), which was significantly higher than the average fat content of smoked rainbow trout (4.76%) proving the correlation between fat content and PAH contamination (Basak et al. 2010). Compared to current study the analyses were provided in a controlled environment with an even number of samples. Due to the limited amount of time for collection of the samples and real time on site field research, our number of samples within each species varied greatly and they cannot be compared to each other. However, correlation of more than one sample collected within one fish species were analysed for correlation between fat content and PAHs level contamination. However in this present study the correlation was not statistically proven. Our results are in agreement with previous studies by Akpambang et al. (2011) and Ghasemzadeh-Mohammadi et al. (2012) concluding that for the same conditions of smoking (wood used and temperature), the degree of smoking and product size were more important than fat content. This might be explained by a study by Friedman (1996) which showed that during the smoking process, fats and more water drips from the fish resulting in the physical loss of lipids, amino acids, and micronutrients (Abraha et al. 2018). At the same time as it was mentioned earlier, PAHs might be also trapped in the casing of product or skin of fish resulting in different levels of contamination in various depths of the final product. Since the smoked fish products in this area are mostly consumed with skin, and we analysed the whole product, we can explain the high level of PAHs contamination without correlation with fat content. Multiple species might occur at the same time in the smokehouse, therefore fat dripping from other species might take place and cause additional PAH genereation irrespective of the fat content of the original fish species. However, we can observe a certain trend of fish species with the highest mean percent of fat content being listed amongst the one with the highest PAH contamination (Table 9). For example, a sample of *Paralaubuca typus* species with 50.96±20.9% and 9171.56 $\mu g.kg^{-1}$, fat content and PAHs level, respectively. Therefore, we cannot completely reject the hypothesis that in the Tonle Sap area fish fat content correlates with PAH contamination. However, in the present study we cannot statistically prove it.

Table 9 Overview of mean fat content (in %) and level of PAH (mean values with SD for BaP, ΣPAH4 and ΣPAH12 in µg.kg^{-1}) contamination for all sampled fish species.

Fish	Mean %	SD %	BaP	SD	ΣPAH4	SD	ΣPAH12	SD
Belodontichthys truncatus	31.12	9.89	79.99	45.91	285.81	±166.17	2004.98	±747.84
Cirrhinus siamensis	28.46	14.35	182.00	67.12	1183.03	±565.81	5518.28	±2055.12
Clarias batrachus	47.31	8.10	57.27	32.35	182.56	±126.26	1164.73	±559.13
Hypsibarbus malcolmi	39.97	3.36	221.12	22.02	1121.09	±113.95	4208.57	±438.83
Labeo chrysophekadion	16.19	12.15	394.37	240.95	2154.22	±1581.58	8419.03	±5281.56
Micronema hexapterus	23.61	13.67	119.38	73.95	592.47	±377.06	3215.40	±1712.31
Notopterus notopterus	1.33	0.95	46.16	2.61	183.05	±159.12	1347.18	±77.13
Ompok bimaculatus	17.68	14.45	127.50	81.27	296.72	±122.93	1277.39	±363.15
Ompok bimaculatus	0.34	1.34	66.52	33.58	729.89	±434.02	2203.37	±902.83
Osteochilus schlegeli	40.70	5.23	228.27	9.66	1066.94	±62.97	4737.67	±362.15
Pangasius elongatus	31.74	0.43	124.75	8.17	871.89	±80.69	4092.28	±436.26
Paralaubuca barroni	35.24	25.68	105.03	60.15	506.97	±307.28	2299.45	±1359.74
Paralaubuca typus	50.96	20.88	247.63	96.89	1644.19	±835.07	9171.56	±5784.68
Phalacronotus bleekeri	12.89	6.20	217.20	40.62	1093.81	±197.03	3286.86	±1165.60
Phalacronotus micronemus	9.43	1.77	99.13	31.77	461.95	±210.21	1729.73	±423.75
Puntioplites prostozystron	37.59	4.41	99.15	48.76	425.67	±357.13	2887.15	±449.28
Rasbora hobelmani	34.65	11.70	179.12	64.12	933.39	±392.99	6131.80	±3153.55
Wallago attu	6.45	1.32	43.87	8.52	179.92	±30.81	1107.91	±207.41
Xenentodon cancila	10.24	0.47	91.40	9.33	555.64	±2.26	2091.47	±302.62

6 Conclusions

This purpose of this thesis was to monitor the traditional way of smoking fish products in the Tonlé Sap area, Cambodia, and evaluate the amounts of carcinogenic compounds in the traditionally smoked fish products in the selected region. Traditional smoking practices in the Tonlé Sap area in Cambodia, based on our results, might be described as direct, hot smoking (temperature between 80 – 100 °C), using predominantly wood as fuel, without a temperature regulating system and with the use of inappropriate fire-starting techniques, at a distance of 50 – 100 cm of the product from the fire with possible fat dripping and subsequent fat pyrolysis. For PAH determination in difficult fatty matrices of animal origin, such as our fish samples an effective and fast method was successfully developed and applied. This method combines QuEChERS extraction method with clean-up step by EMR-Lipid and DLLME step as an extract pre-concentration. This method resulted in an effective sample preparation procedure with less solvent and time input and successfully purified samples providing at the same time an acceptable recovery rate of PAHs in smoked fatty products. This study reported, for the first time the levels of BaP ΣPAH4 and ΣPAH12 in 18 species of smoked fish commonly consumed in Cambodia. Overall, determined PAH content highly exceeds the recommended levels of BaP and ΣPAH4 according to the European Commission regulation, 2 $\mu g.kg^{-1}$ and 12 $\mu g.kg^{-1}$, respectively. The highest PAH concentrations of BaP, ΣPAH4 and ΣPAH12 were detected in *Paralaubuca typus* samples 321.22 $\mu g.kg^{-1}$, 2701.45 $\mu g.kg^{-1}$ and 16818.19 $\mu g.kg^{-1}$ (smoked on wood for 3 – 16 hours), followed by Labeo chrysophekadion 608.90 $\mu g.kg^{-1}$, 3779.58 $\mu g.kg^{-1}$ and 13547.89 $\mu g.kg^{-1}$ (smoked on a combination of wood and charcoal for 1 - 4 days), respectively. The lowest mean values of BaP, ΣPAH4 and ΣPAH12 measured in Paralaubuca barroni were 29.93 $\mu g.kg^{-1}$ 76.33 $\mu g.kg^{-1}$ and 537.95 $\mu g.kg^{-1}$ (smoked on a combination of wood and charcoal for 1 - 4 days), respectively. Regarding the fish species, the highest mean values of ΣPAH4 and ΣPAH12 were measured for samples of Paralaubuca typus species (1644.19 $\mu g.kg^{-1}$ and 9171.56 $\mu g.kg^{-1}$, respectively). Although fish species might be a significant factor in the present study, high variability in PAH content within fish species was observed, explainable by the fluctuation of smoke and temperature (due to the smokehouse structure) during the process, and the physical state of the fish. The present study results show significant increase of ΣPAH12 mean values between smoking times T1 (3 – 16 hours) and T2 (1 - 4 days), explained by the increase of PAH contamination with time and by subsequent stabilisation when smoking lasts longer than 4 days (due to blockage of the pores). It was also

noted that consuming fish without the skin might decrease the level of PAHs ingested. Within the species, significantly higher PAHs concentrations were observed when fuel wood was used for smoke-curing. Finally, the total fat content was measured, and the correlation between fat content and PAH contamination was analysed. However, it was not proven, due to the high variability within the fish species. This variability is presumably produced by the physical state of the fish and environmental factors. Also, PAHs might be trapped in the skin, and pyrolysis of fat drippings from other fish species smoked at the same time can cause additional PAH generation irrespective of the fat content of the original fish species. The data analysis did not reveal any particular smoking parameter alone to be the reason for the higher PAH levels, but some factors were found significant in PAHs generation and smoked fish contamination, such as the wood being used as fuel and the length of the smoking process. Altogether, the extremely high concentrations of PAHs measured in this study are attributable to a combination of factors, such as the type of fuel used, the length of the process. But other factors such as the use of inappropriate fire-starting techniques, the use of a direct heat source, distance from the heat source, lack of temperature regulation systems and the size and physical state of the smoked fish cannot be excluded, although not supported by statistics in the present study. Such burden can lead to an elevated risk of the development of carcinogenic diseases and other diseases related to PAH exposure. However, by following good manufacturing practices, PAH contamination in smoked fish products can be controlled and decreased, maintaining the beneficial effects of smoking and preventing its undesirable effects.

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Appendix I Questionnaire for Producers of Smoked Fish Cambodia. 2018

Questionnaire for Producers of Smoked Fish Cambodia. 2018

បញ្ជីសំណួរសម្រាប់អ្នកធ្វើត្រីឆ្អើរនៅក្នុងប្រទេសកម្ពុជា ឆ្នាំ២០១៨

A. Introduction part of traditional smoking process

ផ្នែកទី១៖ ដំណើរការឆ្អើរត្រី

1. Location of the producer of traditional smoked fish.

   _____

   ទីតាំងរបស់អ្នកផលិតត្រីឆ្អើរ

2. Source of fish?

   ប្រភពត្រី

   a. Buying

      ទិញ

   b. Fishing

      នេសាទ

3. Location of fish collection

   ទីតាំងប្រមូល ឬយកត្រី

   a. Fisherman

      ពីអ្នកនេសាទ

   b. Port

      ពីផែ

   c. Market

      ពីផ្សារ

4. Name mainly smoked fish species:

   ប្រាប់ប្រភេទត្រីដែលគេនិយមយកមកឆ្អើរ_____

   a. _____
   b. _____
   c. _____
   d. _____

   e. _____
   f. _____
   g. _____
   h. _____
   i. _____

5. Where do you process the fishes?

   តើអ្នកឆ្អើរត្រីនៅកន្លែងណា ?

   a. Port

      នៅផែ

   b. Smokehouse

      ក្នុងទូឆ្អើរ

   c. Home

      នៅផ្ទះ

   d. Others

      កន្លែងផ្សេងទៀត

6. How do you sell the smoked fish?

   តើអ្នកលក់ត្រីឆ្អើរតាមរយៈនរណា ?

a. By yourself on the local market
លក់ដោយខ្លួនឯងនៅផ្សារក្នុងតំបន់

b. Customers coming individually to your house
អតិថិជនមកទិញផ្ទាល់ដល់ផ្ទះ

c. To the middle-man
លក់ឆាយឈ្មួញ

d. Directly to some bigger company or supermarket
លក់ផ្ទាល់ឆាយទៅក្រុមហ៊ុន ឬផ្សារទំនើបធំៗ

B. Technical part of traditional smoking process
ផ្នែកបច្ចេកទេស៖ ការផលិតត្រីឆ្អើរ

1. What material do you use for smoking?
តើអ្នកប្រើធនធានអ្វីដើម្បីឆ្អើរត្រី?

CHARCOAL/WOOD
ធ្យូង ឬ អុស

2. If wood what kind (name it/show)
ប្រសិនបើអុស តើជាប្រភេទឈើអ្វី? (ប្រាប់ឈ្មោះ ឬយកមកបង្ហាញ)

a. _____
b. _____
c. _____
d. _____

3. Do you know how much of fire wood or charcoal you use per day or per batch in kg or in m^3?
តើអ្នកប្រើអុស ឬធ្យូងប៉ុន្មានគីឡូក្រាម ឬម៉ែត្រគូបក្នុង១ថ្ងៃ?

a. Wood _____
អុស

b. Charcoal _____
ធ្យូង

4. How long is the fish in smokehouse (hours/days)? _____
តើអ្នកឆ្អើរត្រីប៉ុន្មានម៉ោង ឬថ្ងៃក្នុងឡឆ្អើរ?

5. How long does the whole process take including pre-treatment and fire preparation?
តើដំណើរការនៃការឆ្អើរមានរយៈពេលប៉ុន្មាន រួមបញ្ចូលទាំងការរៀបចំ និងការដុតភ្លើង?

a. Pre-treatment _____
រយៈពេលនៃការរៀបចំ

b. Fire preparation _____
រយៈពេលនៃការដុតភ្លើង

c. Smoking _____
រយៈពេលឆ្អើរ

6. Do you use trays? YES/NO
តើអ្នកប្រើចង្អេរដែរឬទេ?

a. If yes. do you change them regularly? YES/NO
ប្រសិនបើប្រើ តើអ្នកជាទៀងទាត់ដែរឬទេ?

b. Do you change trays in some order?
_____
តើអ្នកប្តូរចង្អេរតាមលំដាប់របស់វាដែរឬទេ?

7. How looks the fire during fish smoking?
_____
តើភ្លើងមានពណ៌អ្វី ឬសភាពបែបណា ក្នុងពេលកំពុងឆ្អើរ?

8. How do you prepare the fire before smoking?

   _____

   តើអ្នករៀបចំដុតភ្លើងយ៉ាងដូចម្តេចមុនពេលឆ្អើរ ?

9. Do you measure the right temperature? YES/NO

   តើអ្នកមានវាស់សីតុណ្ហភាពឆាយបានត្រឹមត្រូវដែរឬទេ ?

10. How do you estimate or recognize that the fish is ready (already smoked)?

    តើអ្នកមើលយ៉ាងដូចម្តេច មើបដឹងថាត្រីត្រូវបានឆ្អើរហើយត្រឹមត្រូវ ឬឆ្អើរហើយរួចរាល់ ?

    a. Visually

       មើលដោយភ្នែក

    b. Change in appearance

       ដោយសារមានការប្រែពណ៌

    c. Weight loss

       ត្រីមើលទៅក្រៀម ឬស្ងួត

    d. Others _____

       ផ្សេងៗ

11. Do you use any additional technique? (Usage of carton. covering.…) YES/NO What?

    _____

    តើអ្នកមានប្រើវិធីសាស្ត្រអ្វីផ្សេងទៀតដែរឬទេ ? (ប្រើក្រដាស ឬយកអ្វីមកគ្រប...) ប្រសិនបើប្រើ តើប្រើអ្វី ?

C. Marketing and selling practices of traditional smoking products

ការលក់ និងការធ្វើទីផ្សារផលិតផលត្រីឆ្អើរ

12. What is the amount of production per day? _____

    តើអ្នកផលិតផលត្រីឆ្អើរចំនួនប៉ុន្មានក្នុង១ថ្ងៃ ?

13. What amount of fishes is in one batch: _____

    បរិមាណត្រីក្នុង១ដុំ...

14. In which period of the year is main smoking season?

    តើខែណាដែលជារដូវធ្វើត្រីឆ្អើរ ?

January	February	March	April	May	June
July	August	September	October	November	December

    មករា - ធ្នូ

15. How often do you smoke?

    តើអ្នកឆ្អើរត្រីញឹកញាប់ប៉ុណ្ណា ?

    a. Daily

       រាល់ថ្ងៃ

    b. 2-3 times a week

       ២-៣ដង/សប្តាហ៍

    c. Weekly

       រាល់សប្តាហ៍

    d. Once per month

       ១ខែម្តង

    e. Other _____

       ផ្សេងៗ

16. Do you use any packaging of marketed smoked fish? YES/NO

    តើអ្នកមានប្រើអ្វីដើម្បីរាក់ត្រីដែលបានឆ្អើររួចទៅលក់នៅផ្សារដែរឬទេ ?

    a. Basket

       កន្ត្រកង

    b. Paper box

ប្រអប់ក្រដាស

17. Do you store final product before selling? YES/NO
    តើអ្នករក្សាទុកត្រីដែលបានឆ្អើរួចមុនពេលលក់ដែរឬទេ ?
18. Where do you store them?
    តើអ្នករក្សាត្រីឆ្អើរនោះនៅកន្លែងណា ?
    a. At smokehouse
       ក្នុងទូរឆ្អើរ ?
    b. At smoking trays
       ក្នុងកម្រៃងឆ្អើរ ?
    c. Hanging
       ព្យួរ ?
19. For how many days you store them? _____
    តើអ្នករក្សាវាទុកប៉ុន្មានថ្ងៃ ?

D. Consumption habits

ទំលាប់នៃការញុំាត្រីឆ្អើរ

1. How do you eat/prepare the smoking fish most often?
   _____
   តើអ្នកមៀបចំត្រីឆ្អើរយ៉ាងដូចម្តេចមុនពេលញុំា ?
2. How often do you eat smoked fish? _____
   តើអ្នកហូបត្រីឆ្អើរញឹកញាប់ប៉ុណ្ណា ?
3. How many smoked fish do you consume per month?
   _____
   តើអ្នកហូបត្រីឆ្អើរចំនួនប៉ុន្មានក្នុងរយៈពេល១ខែ ?

E. Ways of pretreatment's before smoking of fish (use of spices. salt. drying. brine. etc.)

ការមៀបចំមុនពេលឆ្អើរត្រី (ការប្រើគ្រឿងទេស អំបិល ហាល...)

_____
_____
_____
_____
_____

Appendix II Measured fat content in individual fish samples.

Fish sample No.	Fish species	Repetition	Fat content %
1	*Puntioplites prostozystron*	1	44.10
1	*Puntioplites prostozystron*	2	39.32
1	*Puntioplites prostozystron*	3	35.96
2	*Pangasius elongatus*	1	31.48
2	*Pangasius elongatus*	2	32.24
2	*Pangasius elongatus*	3	31.51
3	*Clarias batrachus*	1	51.76
3	*Clarias batrachus*	2	43.32
3	*Clarias batrachus*	3	45.32
4	*Cirrhinus siamensis*	1	35.31
4	*Cirrhinus siamensis*	2	31.34
4	*Cirrhinus siamensis*	3	29.99
5	*Cirrhinus siamensis*	1	45.73
5	*Cirrhinus siamensis*	2	52.90
5	*Cirrhinus siamensis*	3	50.61
6	*Micronema hexapterus*	1	36.92
6	*Micronema hexapterus*	2	34.83
6	*Micronema hexapterus*	3	36.35
7	*Labeo chrysophekadion*	1	0.09
7	*Labeo chrysophekadion*	2	0.09

7	*Labeo chrysophekadion*	3	0.09
8	*Xenentodon cancila*	1	9.71
8	*Xenentodon cancila*	2	10.42
8	*Xenentodon cancila*	3	10.59
9	*Cirrhinus siamensis*	1	38.55
9	*Cirrhinus siamensis*	2	43.32
9	*Cirrhinus siamensis*	3	39.41
10	*Ompok bimaculatus*	1	1.32
10	*Ompok bimaculatus*	2	1.42
10	*Ompok bimaculatus*	3	1.89
11	*Micronema hexapterus*	1	24.94
11	*Micronema hexapterus*	2	29.15
11	*Micronema hexapterus*	3	26.33
12	*Ompok bimaculatus*	1	27.30
12	*Ompok bimaculatus*	2	26.65
12	*Ompok bimaculatus*	3	30.59
13	*Paralaubuca barroni*	1	40.15
13	*Paralaubuca barroni*	2	38.49
13	*Paralaubuca barroni*	3	50.29
14	*Paralaubuca barroni*	1	3.60
14	*Paralaubuca barroni*	2	2.60
14	*Paralaubuca barroni*	3	1.99
15	*Cirrhinus siamensis*	1	28.99

15	*Cirrhinus siamensis*	2	32.79
15	*Cirrhinus siamensis*	3	27.51
16	*Notopterus notopterus*	1	2.35
16	*Notopterus notopterus*	2	1.18
16	*Notopterus notopterus*	3	0.46
17	*Cirrhinus siamensis*	1	2.59
17	*Cirrhinus siamensis*	2	2.27
17	*Cirrhinus siamensis*	3	2.60
18	*Clarias batrachus*	1	64.16
18	*Clarias batrachus*	2	54.45
18	*Clarias batrachus*	3	57.36
19	*Phalacronotus micronemus*	1	8.71
19	*Phalacronotus micronemus*	2	10.02
19	*Phalacronotus micronemus*	3	9.24
20	*Clarias batrachus*	1	52.64
20	*Clarias batrachus*	2	50.29
20	*Clarias batrachus*	3	44.66
21	*Wallago attu*	1	5.13
21	*Wallago attu*	2	4.99
21	*Wallago attu*	3	7.90
22	*Cirrhinus siamensis*	1	1.54
22	*Cirrhinus siamensis*	2	1.43
22	*Cirrhinus siamensis*	3	1.84

23	*Cirrhinus siamensis*	1	29.10
23	*Cirrhinus siamensis*	2	22.99
23	*Cirrhinus siamensis*	3	30.50
24	*Paralaubuca typus*	1	35.46
24	*Paralaubuca typus*	2	26.04
24	*Paralaubuca typus*	3	27.12
25	*Ompok bimaculatus*	1	0.09
25	*Ompok bimaculatus*	2	0.09
25	*Ompok bimaculatus*	3	0.09
26	*Ompok bimaculatus*	1	1.90
26	*Ompok bimaculatus*	2	1.98
26	*Ompok bimaculatus*	3	1.08
27	*Phalacronotus bleekeri*	1	9.01
27	*Phalacronotus bleekeri*	2	9.26
27	*Phalacronotus bleekeri*	3	8.26
28	*Puntioplites prostozystron*	1	30.58
28	*Puntioplites prostozystron*	2	38.05
28	*Puntioplites prostozystron*	3	37.53
29	*Rasbora hobelmani*	1	23.77
29	*Rasbora hobelmani*	2	20.92
29	*Rasbora hobelmani*	3	16.66
30	*Labeo chrysophekadion*	1	23.94
30	*Labeo chrysophekadion*	2	23.95

30	*Labeo chrysophekadion*	3	24.74
31	*Clarias batrachus*	1	43.78
31	*Clarias batrachus*	2	37.55
31	*Clarias batrachus*	3	50.91
32	*Phalacronotus micronemus*	1	10.88
32	*Phalacronotus micronemus*	2	9.89
32	*Phalacronotus micronemus*	3	11.46
33	*Phalacronotus micronemus*	1	6.53
33	*Phalacronotus micronemus*	2	6.96
33	*Phalacronotus micronemus*	3	11.19
34	*Micronema hexapterus*	1	36.79
34	*Micronema hexapterus*	2	36.74
34	*Micronema hexapterus*	3	46.65
35	*Rasbora hobelmani*	1	35.99
35	*Rasbora hobelmani*	2	30.16
35	*Rasbora hobelmani*	3	32.74
36	*Clarias batrachus*	1	39.88
36	*Clarias batrachus*	2	50.85
36	*Clarias batrachus*	3	50.52
37	*Cirrhinus siamensis*	1	24.35
37	*Cirrhinus siamensis*	2	20.68
37	*Cirrhinus siamensis*	3	21.98
38	*Rasbora hobelmani*	1	31.17

38	*Rasbora hobelmani*	2	37.57
38	*Rasbora hobelmani*	3	23.38
39	*Wallago attu*	1	8.02
39	*Wallago attu*	2	5.99
39	*Wallago attu*	3	6.69
40	*Belodontichthys truncatus*	1	36.04
40	*Belodontichthys truncatus*	2	34.99
40	*Belodontichthys truncatus*	3	44.85
41	*Cirrhinus siamensis*	1	22.14
41	*Cirrhinus siamensis*	2	27.53
41	*Cirrhinus siamensis*	3	23.84
42	*Cirrhinus siamensis*	1	31.93
42	*Cirrhinus siamensis*	2	32.94
42	*Cirrhinus siamensis*	3	32.20
43	*Paralaubuca typus*	1	80.23
43	*Paralaubuca typus*	2	73.94
43	*Paralaubuca typus*	3	75.28
44	*Micronema hexapterus*	1	0.09
44	*Micronema hexapterus*	2	0.09
44	*Micronema hexapterus*	3	0.09
45	*Paralaubuca barroni*	1	58.90
45	*Paralaubuca barroni*	2	60.76
45	*Paralaubuca barroni*	3	60.37

46	*Rasbora hobelmani*	1	28.36
46	*Rasbora hobelmani*	2	26.08
46	*Rasbora hobelmani*	3	27.76
47	*Osteochilus schlegeli*	1	46.74
47	*Osteochilus schlegeli*	2	37.73
47	*Osteochilus schlegeli*	3	37.63
48	*Cirrhinus siamensis*	1	38.12
48	*Cirrhinus siamensis*	2	46.87
48	*Cirrhinus siamensis*	3	32.89
49	*Phalacronotus bleekeri*	1	19.84
49	*Phalacronotus bleekeri*	2	23.74
49	*Phalacronotus bleekeri*	3	19.13
50	*Rasbora hobelmani*	1	61.04
50	*Rasbora hobelmani*	2	51.76
50	*Rasbora hobelmani*	3	58.45
51	*Micronema hexapterus*	1	18.81
51	*Micronema hexapterus*	2	18.08
51	*Micronema hexapterus*	3	17.05
52	*Rasbora hobelmani*	1	30.10
52	*Rasbora hobelmani*	2	25.60
52	*Rasbora hobelmani*	3	25.61
53	*Belodontichthys truncatus*	1	31.37
53	*Belodontichthys truncatus*	2	18.69

53	*Belodontichthys truncatus*	3	20.77
54	*Cirrhinus siamensis*	1	44.92
54	*Cirrhinus siamensis*	2	36.81
54	*Cirrhinus siamensis*	3	36.02
55	*Paralaubuca typus*	1	44.17
55	*Paralaubuca typus*	2	45.07
55	*Paralaubuca typus*	3	51.36
56	*Hypsibarbus malcolmi*	1	37.23
56	*Hypsibarbus malcolmi*	2	43.73
56	*Hypsibarbus malcolmi*	3	38.96
57	*Labeo chrysophekadion*	1	24.53
57	*Labeo chrysophekadion*	2	23.20
57	*Labeo chrysophekadion*	3	25.31
58	*Rasbora hobelmani*	1	36.59
58	*Rasbora hobelmani*	2	31.28
58	*Rasbora hobelmani*	3	46.55
59	*Rasbora hobelmani*	1	48.57
59	*Rasbora hobelmani*	2	44.03
59	*Rasbora hobelmani*	3	37.46
60	*Phalacronotus bleekeri*	1	16.75
60	*Phalacronotus bleekeri*	2	16.88
60	*Phalacronotus bleekeri*	3	14.62
61	*Phalacronotus bleekeri*	1	10.70

61	*Phalacronotus bleekeri*	2	8.96
61	*Phalacronotus bleekeri*	3	9.35
62	*Clarias batrachus*	1	35.04
62	*Clarias batrachus*	2	31.62
62	*Clarias batrachus*	3	47.55
63	*Micronema hexapterus*	1	20.88
63	*Micronema hexapterus*	2	21.77
63	*Micronema hexapterus*	3	19.73