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Mirja Hokkanen

Polycyclic aromatic hydrocarbons in foods and their mitigation, food mutagenicity and children's dietary exposure in Finland

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POLYCYCLIC AROMATIC HYDROCARBONS IN FOODS AND THEIR MITIGATION, FOOD MUTAGENICITY AND CHILDREN'S DIETARY EXPOSURE IN FINLAND

MIRJA HOKKANEN

DOCTORAL DISSERTATION

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ABSTRACT

Polycyclic aromatic hydrocarbon (PAH) contamination in food poses a potential risk to human health. PAHs are formed primarily as a result of incomplete combustion of organic material and can enter the food chain either from the environment or from food manufacturing processes such as smoking, roasting, drying and grilling. As some of them are known to cause cancer, it is important to reduce the PAH levels in foods as low as is reasonably achievable. The European Food Safety Authority (EFSA) has evaluated the sum of PAH4 compounds (=benzo[a]pyrene (BaP), benz[a]anthracene (BaA), chrysene (CHR) and benzo[b] fluoranthene (BbF)) to serve as a suitable indicator of both the occurrence and toxicity of the PAHs.

The primary purpose of this dissertation study was to assess for the first time Finnish children's potential health risk caused by dietary exposure to PAH4. In order to complete this task, the applied analytical method must be appropriately validated to be able to produce precise occurrence data. In this study, the gas chromatography-tandem mass spectrometry method was successfully validated according to legislative requirements and the occurrence of PAH4 in a wide range of food groups (fish, meat, fat and oil, bread, cereal and muesli) was determined. Generally, the concentrations of PAH4 in foods were low and below the prevailing maximum levels. That being said, in smoked fish and meat products, the variation of the detected PAH4 levels was high, up to 200 µg/kg in smoked ham. It appeared that the smoking process was either controlled or not, and the science-based guidance is required to prevent and reduce contamination of PAHs. Therefore, our goal was to investigate the critical smoking factors that affect the PAH4 formation in fish and meat products in order to provide tools for manufacturers to produce safer smoked products, thereby decreasing human dietary exposure and adverse health effects. Based on our results, indirect smoking, smoking in less than five hours, optimised smoke generation temperature between 400 and 600 °C and distance more than five metres between the food and the smoke source led to reduced PAH4 levels in smoked fish and meat products.

This study aimed to gain a better understanding of the potential mutagenicity of the processed foods, and therefore selected fish and meat products were tested by the Ames test in *Salmonella* TA 100 and TA 98 strains with or without metabolic activation. The outcome was further compared to the chemical PAH4 analyses. The statistically significant mutagenic response was observed in all three lots of smoked Baltic herring, which also indicated higher PAH4 concentrations than other samples. By contrast, the tested meat products were not mutagenic and the corresponding individual PAH4 concentrations were, for the most part,

undetectable. Despite the challenges in food mutagenicity testing, our results provided more information on the potential mutagenic activity of various foods. Specifically, based on our findings, a combination of both biological assays and chemical analyses can improve the interpretation of the findings regarding mutagenicity.

Lastly, in reference to our primary purpose of considering potential safety concerns of PAH4 in food, Finnish children's dietary exposure to PAH4 was evaluated by combining the acquired occurrence and food consumption data. Utilising that data, a margin of exposure (MOE) was calculated in order to be used to provide relative indication of the level of health concern and support prioritisation of possible risk management actions. Our results demonstrated that bread, smoked ham, fat and oil and sausage contributed the most to BaP and PAH4 exposure. Even though the mean PAH4 levels in bread were below the limit of detection, its consumption volumes are high, which explains the contribution. Children's total mean dietary exposure was estimated to BaP 1,500 pg/kg bw/day and to PAH4 8,100 pg/kg bw/day. The total margins of exposure (MOEs) for children were 482,000 for BaP and 42,000 for PAH4. Furthermore, the calculated MOEs for highly exposed children were also above the reference value 10,000, which caused them to be considered of low concern. Taking into account uncertainties and limitations, this study indicated no health risk to Finnish children aged three to six years.

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Helsinki, December 2021
Mirja Hokkanen

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (I-III), which are referred to in the text by their Roman numerals:

I. Hokkanen, M., Luhtasela, U., Kostamo, P., Ritvanen T., Peltonen, K., Jestoi, M. 2018. Critical effects of smoking parameters on the levels of polycyclic aromatic hydrocarbons in traditionally smoked fish and meat products in Finland. *Journal of Chemistry*, 2018: 1-14.

II. Omoruyi, I.M., Hokkanen, M., Pohjanvirta, R. 2020. Polycyclic aromatic hydrocarbons (PAHs) in select commercially processed meat and fish products in Finland and the mutagenic potential of these food items. *Polycyclic Aromatic Compounds*, 40 (4): 927-933.

III. Hokkanen, M., Mikkelä, A., Pasonen, P., Tuominen, P., Uusitalo, L., Erkkola, M., Korkalo, L., Hirvonen, T. 2021. Children's dietary exposure to polycyclic aromatic hydrocarbons in Finland. *Polycyclic Aromatic Compounds*. Published. https://doi.org/10.1080/10406638.2021.1903951

AUTHOR CONTRIBUTION TO PAPERS I-III

I. Mirja Hokkanen took on an active role in the planning and organisation of the study, performed the chemical analyses and interpreted the results. She was responsible for the statistical data analyses, wrote the manuscript for the most part with the other co-authors and was the corresponding author of this article.

II. Mirja Hokkanen took on an active role in the planning and organisation of the study, performed the chemical analyses and interpreted the chemical results. She wrote parts of the manuscript in each section related to chemical analyses of PAHs. Mirja Hokkanen actively commented on and modified the article before submission and after the peer review. Some of the data has previously been used as part of the doctoral thesis by Iyekhoetin Matthew Omoruyi (2015, University of Helsinki).

III. Mirja Hokkanen took on an active role in the planning and organisation of the study and collected all the samples from the grocery stores. She validated the analytical method, performed the analyses and interpreted the chemical results. Mirja Hokkanen wrote the manuscript for the most part with the other co-authors and was the corresponding author of this article.

ABBREVIATIONS

3-MCPD	3-monochloropropane-1,2-diol
AKR	aldo-keto reductase
ALARA	as low as reasonably achievable
ASE®	accelerated solvent extraction
BaA	benz[a]anthracene
BaP	benzo[a]pyrene
BbF	benzo[b]fluoranthene
BMD	benchmark dose
BMDL	benchmark dose lower confidence limit
bw	body weight
CAC	Codex Alimentarius Commission
CAS	Chemical Abstracts Service
CHR	chrysene
CI	chemical ionisation
CONTAM	Scientific Panel on Contaminants in the Food Chain of EFSA
CRM	certified reference material
CYP	cytochrome P450
DAGIS	Increased Health and Wellbeing in Preschool -study
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC	European Commission
EFSA	European Food Safety Authority
EH	epoxide hydrolase
EI	electron ionisation
EU	European Union
Evira	Finnish Food Safety Authority
EVO	National Food Control Program
FAO	Food and Agriculture Organization
FBO	food business operator
FID	flame ionisation detector
FLD	fluorescence detector
GC	gas chromatography
GC-QTOF-MS	gas chromatography quadrupole time-of-flight mass spectrometry
GPC	gel permeation chromatography
his	histidine
HorRat	Horwitz ratio
HPLC	high performance liquid chromatography

HRMS	high resolution mass spectrometry
IARC	International Agency for Research on Cancer
ISO	International Organization for Standardization
ISTD	internal standard
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JRC	Joint Research Centre
LC	liquid chromatography
LD50	lethal dose 50%
LOD	limit of detection
LOQ	limit of quantification
MAE	microwave-assisted extraction
ML	maximum level
MOE	margin of exposure
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSPE	magnetic solid phase extraction
MU	measurement uncertainty
NOEL	no-observed-effect-level
PAC	polycyclic aromatic compound
PAH	polycyclic aromatic hydrocarbon
PDA	photo-diode array
PLE	pressurised liquid extraction
$PRSD_R$	predicted relative standard deviation (reproducibility)
PT	proficiency test
QTOF	quadrupole time-of-flight
QuEChERS	quick, easy, cheap, effective, rugged and safe
ROS	reactive oxygen species
RSD	relative standard deviation
RSD_r	relative standard deviation (repeatability)
RSD_R	relative standard deviation (reproducibility)
SCF	Scientific Committee on Food
SD	standard deviation
SFE	supercritical fluid extraction
SME	small to medium-sized enterprise
SPE	solid phase extraction
TPH	triphenylene
UV	ultraviolet
WHO	World Health Organization

It's the possibility of having a dream come true that makes life interesting.

— Paulo Coelho

1 INTRODUCTION

Safe and nutritious food is a key to promote human health and all parties involved in the food production chain are responsible for the safety of the product. Potential risks related to our daily diet are of great interest to researchers, policymakers, food business operators (FBOs) and consumers, because the presence of harmful chemicals, microorganisms, foreign material or cross-contamination in food may pose health threats to consumers and unwanted challenges in food production. Of these listed hazards, chemical contaminants are one of the serious sources of food contamination. They are unintentionally added to food from various sources such as environment, food processes and packaging materials (Rather et al. 2017). Some of them, such as polycyclic aromatic hydrocarbons (PAHs), can enter the food chain both from the environment and food processes as a result of the incomplete combustion of organic material at high temperature (≥500 °C) (Bamforth and Singleton 2005; Wenzl et al. 2006). In particular, food preparation in grilling, roasting, frying, drying and smoking can generate PAHs (Dennis et al. 1991; Duedahl-Olesen et al. 2006; Rose et al. 2015).

Food smoking belongs to one of the oldest preservation techniques that maintains microbiological stability and extends the shelf life of the food product. Today, the focus of the smoking has shifted from inactivating enzymes to providing foods with favourable taste, colour and aroma (Theobald et al. 2012; Fasano et al. 2016). However, while achieving these organoleptic characteristics, PAHs are preferably generated during a smoke production (Šimko 2005; Purcaro et al. 2006). Traditional smoking is a widely used technique throughout Finland, which can further be classified as direct and indirect techniques. Besides the smoking technique, the smoking process contains a large variety of variables to be controlled in order to minimise the amounts of PAHs in the final food product (CAC 2009). Our research contributes to this area by examining the critical smoking factors affecting the PAH4 formation in smoked fish and meat products. Aiming to provide a realistic basis for guidance and mitigation tools for manufacturers, this study was carried out in real-life smoking conditions in smokehouses. The main objective was to improve the understanding of the smoking process in order to reduce the dietary exposure to PAHs.

PAHs may occur in various complex food matrices and in order to determine them, effective and reliable methods are required. Method validation provides evidence that the method can be used for its intended purpose and meets the quality demands set out in legislation or elsewhere. Regarding PAHs, the legislative method requirements by the European Commission (EC) for specific PAHs, namely benzo[a]pyrene (BaP) and the sum of PAH4 (=BaP, benz[a]anthracene

(BaA), chrysene (CHR) and benzo[b]fluoranthene (BbF)), define the frames for the method validation (EC 2007, 2011b). Nowadays, the trend in analytics is towards tandem mass spectrometry (MS/MS) techniques, which enable more accurate confirmation compared to other analytical techniques. Specifically, gas chromatography (GC) coupled with MS/MS is preferred in PAH analyses (Plaza-Bolaños et al. 2010). In this study, the aim was to validate a GC-MS/MS method for PAH4 determination and to generate reliable data on the occurrence of PAH4 in selected foods on the Finnish market. In the absence of other Finnish studies focusing on PAH4 levels in foods, these results would benefit manufacturers, policymakers and consumers as well as serve risk assessment.

Identifying potentially mutagenic foods could help to target the measures to specific foods on the market, but earlier research can only be seen as first steps aimed at understanding mutagenic activity in foods. In order to provide more information on the mutagenic activity of processed foods, our purpose was to measure the mutagenic activity of the selected fish and meat products using an Ames test and compare the outcome with the corresponding chemical PAH4 data. As PAH4 compounds have genotoxic, mutagenic and carcinogenic properties, a dietary PAH exposure can be a public health concern, particularly for vulnerable groups (e.g. females in fertile age, pregnant women, the elderly and children) (Polanska et al. 2014; Yebra-Pimentel et al. 2015). In order to address the risk resulting from dietary exposure to PAHs for consumers, a risk assessment is a tool utilising existing data and providing scientific evidence to risk managers. The Scientific Committee on Food (SCF) of the European Food Safety Authority (EFSA) recommends applying a margin of exposure (MOE) approach for safety evaluations of genotoxic and carcinogenic substances such as PAHs (EFSA 2005, 2008). To date, EFSA has performed risk assessment of PAHs using MOE for consumers but not for subpopulations such as children or elderly (EFSA, 2008). Otherwise, MOE has not been widely used, resulting in a lack of comparable PAH data (Veyrand et al. 2013; Wu et al. 2016). The primary purpose of this dissertation was to evaluate for the first time Finnish children's dietary exposure to PAH4 using the MOE approach. Use of the MOE can help risk managers in defining possible actions required to keep exposure to PAHs as low as possible. To achieve this goal, data on PAH4 occurrence, food consumption and exposure assessment were utilised.

2 LITERATURE REVIEW

2.1 Physicochemical properties of PAHs

Polycyclic aromatic compounds (PACs) include a wide variety of heterocyclic aromatic compounds and PAHs. Hundreds of organic PAHs consist of at least two aromatic rings linked together. The general characteristics of PAHs are their high boiling points as well as low vapour pressures. Moreover, they are lipophilic, non-polar and uncharged hydrocarbons comprising solely carbon and hydrogen joined by covalent bonds to form aromatic rings. Aromaticity is a characteristic of cyclic and planar structures, which are highly stable, and therefore have low reactivity. However, PAHs are susceptible to oxidation and photo-degradation in the presence of light (EC 2002c; Andersson 2009). Depending on the number of aromatic rings, PAHs can be divided into light (<four rings) or heavy (≥four rings) PAHs. Their physical and chemical properties are defined by the size and shape of each molecule. Heavy PAHs are more stable, lipophilic and toxic compared to those of lighter PAHs. At room temperature, their colour varies from colourless to yellow solids (Purcaro et al. 2013).

PAHs are a major group of chemical substances as cancer inducing agents and several of them have been assessed as carcinogens by the International Agency for Research on Cancer (IARC) (IARC 2010). Of the large number of PAHs, BaP is the most studied and used as a marker of toxicity and occurrence for PAHs in food. However, EC amended legislation on PAHs in 2011, concluding that the PAH4 compounds are more suitable indicators of PAHs in food (EC 2011a). A closer look at their chemical structures in Figure 1 illustrates the structural similarities between individual PAH4 compounds, all of which contain either four or five benzene rings. Furthermore, the molecular weights are identical for BaP and BbF as well as for BaA and CHR (Table 1). PAHs are characterised by containing multiple isomers that refer to the same molecular formula but distinct configurations.

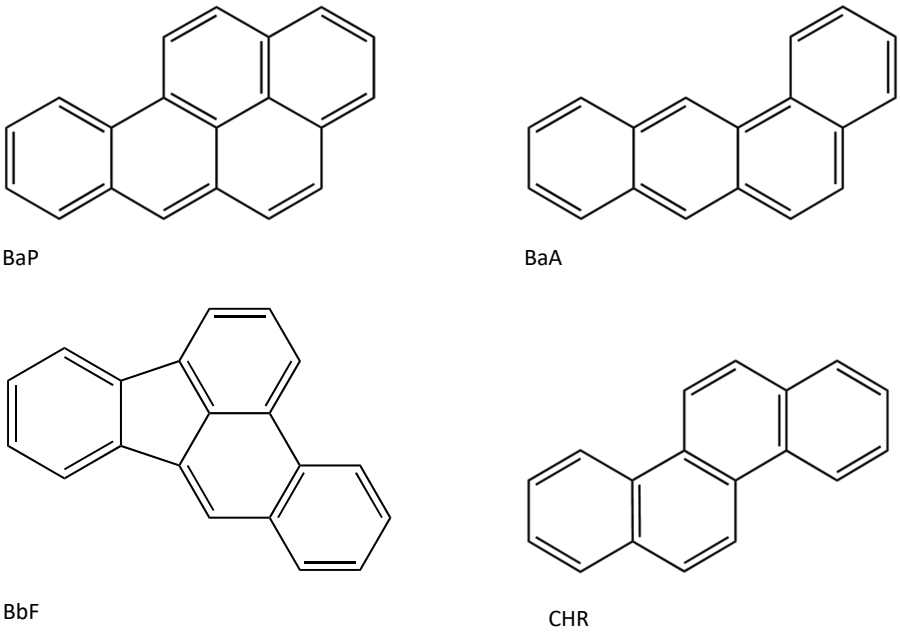


Figure 1. The chemical structures of PAH4 compounds.

Table 1. Identification of the polycyclic aromatic hydrocarbons (IARC 2010; NCBI 2021).

Polycyclic aromatic hydrocarbon	CAS No.	Molecular formula	Molecular weight (g/mol)	Boiling point (°C)	IARC classification*
Benzo[a]pyrene	50-32-8	$C_{20}H_{12}$	252.3	495.0	1
Benz[a]anthracene	56-55-3	$C_{18}H_{12}$	228.3	437.6	2B
Chrysene	218-01-9	$C_{18}H_{12}$	228.3	448.0	2B
Benzo[b] fluoranthene	205-99-2	$C_{20}H_{12}$	252.3	481.0	2B

*IARC: 1 carcinogenic; 2B possibly carcinogenic.

2.2 Formation and sources of PAHs

The long history of PAHs dates back to the year 1775, when Sir Percivall Pott discovered soot as the cause of scrotal cancer among chimney sweeps (Brown and Thornton 1957). However, it took more than a century before researchers succeeded in determining certain PAHs, e.g. BaP and BaA, and demonstrated PAHs causing cancer in experimental animals. Workers may be subject to occupational exposure to PAHs in areas such as coke or asphalt production, roofing, oil refining, coal gasification and by inhaling exhaust fumes (IARC 2012; Lawal 2017).

PAHs are ubiquitous in the environment, originating either from natural (biogenic and geochemical) or anthropogenic sources and occur as components of complex mixtures including even hundreds or thousands of compounds. Their formation occurs in the incomplete combustion of organic material involving two reaction steps, pyrolysis and pyrosynthesis. During pyrolysis at high temperature (approx. 500-700 °C) in the absence of oxygen, organic material breaks into small fragments that recombine in pyrosynthesis to form relatively stable PAHs (EFSA 2008; Purcaro et al. 2013; Zelinkova and Wenzl 2015b). In regard to wood combustion, two formation mechanisms of PAHs may occur: 1) breakdown reactions of lignin followed by further condensation reactions and 2) ring formation reactions of molecules from acetylene and butadiene. Low molecular weight PAHs and other molecules can serve as precursors for higher molecular weight PAHs as higher temperature leads to the formation of larger PAHs (Orasche et al. 2013). As the temperature continues to rise, soot formation increases and the yields of PAHs increase. In wood combustion emissions, both gaseous and particle-bound PAHs exist (Eriksson et al. 2014).

Oil spills, forest fires and volcanic eruptions are regarded as notable sources of PAHs as well as use of the fossil fuels (e.g. coal and oil), bitumen and rubber material (Bamforth and Singleton 2005; Purcaro et al. 2013; Yebra-Pimentel et al. 2015). Due to their hydrophobic properties, PAHs are often detected in water only in negligible amounts, whereas they tend to remain in a solid state in soil and sediments and are particle-bound in the atmosphere. Thus, the amounts of PAHs released into the atmosphere are generally remarkably higher than those in water (EFSA 2008; Cousin and Cachot 2014). Environmental PAHs may further enter the food chain through plants and animals (Yebra-Pimentel et al. 2015). Raw, unprocessed food should not entail notable concentrations of PAHs unless the environment is contaminated. For instance, leafy vegetables situated close to roads are likely to contain PAHs (Phillips 1999).

Human exposure to PAHs occurs via various pathways, mostly by ingestion, skin contact, and inhalation (EC 2002b; Purcaro et al. 2013). Inhaled tobacco smoke is the most substantial source of PAHs for smokers, whereas for non-smokers diet plays a major role (>70%) as a PAH source (Falcó et al. 2003). As process contaminants, PAHs can be formed in foods as a result of their manufacturing processes. Food contamination originates from certain home cooking practices at high temperatures as well as industrial food production (Dennis et al. 1991; Duedahl-Olesen et al. 2006; Rose et al. 2015). PAHs may often form if parts of food or fat drippings encounter charcoal or very hot surfaces as well as in direct contact with the flame. In general, home cooking practices vary a lot, and therefore the levels of PAHs may fluctuate (CAC 2009). The formed PAH levels in the cooked food depend on food, fuel and the cooking method (Rose et al. 2015). Consistent

practices, including controlled heating and regular turning of the food, may decrease the amounts of PAHs (Aaslyng et al. 2013; Duedahl-Olesen et al. 2015).

Industrial processes, such as food drying and smoking, are also possible routes for PAHs to enter the food chain (Speer et al. 1990; EC 2002c). The drying process takes place either by using the combustion gas to dry the food or by drying directly in the fire or the sun (CAC 2009; de Lima et al. 2017). According to Bansal and Kim (2015), the PAH contamination of edible oils is associated with the drying of the oilseeds and the solvent extraction applied, the package materials and the residues of mineral oils. Extra virgin olive oil is extracted from olive fruits, applying mechanical and physical processes without requiring any high-temperature roasting or refining. Under these conditions only trace amounts of PAHs are produced. In contrast, when manufacturing olive pomace oil, the olive pomace is dried through burning smoke and hot air and further extracted with solvent resulting in high PAH concentrations (Sun et al. 2019). Another well-known process in the food industry that causes PAH formation is traditional smoking. The smoke is generated as an outcome of thermal wood combustion when the amount of oxygen is limited (Ŝimko 2002; Stołyhwo 2005). Depending on the smoking technique, the smoke is produced either from an open fire in a same chamber as the food (direct smoking) or in an external chamber, from which the smoke is passed through a pipe to the surface of the product (indirect smoking) (Wretling et al. 2010; Ledesma et al. 2016). Penetration of the smoke components, and thus PAHs, into foods is dependent on factors such as fat content and casing. The highest levels of PAHs typically exist on the surface of the food product but they can migrate through the surface to the inner parts due to the lipophilic nature of PAHs (García-Falcón and Simal-Gándara 2005).

In fact, the traditional smoking procedure involves various smoking parameters to be adjusted and controlled, including the smoking technique, the type of wood, the smoking time, the smoke generation temperature and the distance between the food and the smoke source, all of which affect the PAH levels formed (CAC 2009; Purcaro et al. 2013; Ledesma et al. 2016). Alongside traditional smoking techniques, liquid smoking can be regarded as a noteworthy option. The smoke is mostly filtered and purified of PAHs and other contaminants prior to use, and therefore liquid smoking is considered safer to use than traditional smoking (Ledesma et al. 2016). However, both traditional and liquid smoking techniques are used extensively throughout Finland in the smoking of fish and meat, and therefore the influence of smoking factors on forming PAH levels is of great importance.

2.3 Quantitative PAH analysis

2.3.1 Sampling and sample preparation

Analytical measurements for food analysis are primarily carried out of a small quantity of sample used to represent the entire sample. Sophisticated sampling techniques are demanded for a large variety of food matrices to ensure that each incremental sample represents the original lot in order to measure the magnitude of human exposure to BaP and other PAH4. Without proper sampling, the subsequent steps aimed at achieving reproducible data are useless (Ridgway et al. 2007). For the official controls, the principles of PAH4 sampling and method performance criteria are laid down in Commission Regulation (EC) No 333/2007 with amendments. The competent authorities shall follow this regulation to guarantee the representativeness and transparency of the sampling. Therefore, depending on the lot size, the lot may be divided into sublots and the required number of incremental samples is dependent on the size of the lot or sublot. The incremental samples shall be of equal weight and at least 100 g or 100 ml to form an aggregate sample of at least 1 kg or 1 litre. In general, a homogeneous distribution of PAHs is presumed within a lot or sublot. Special care must be taken to avoid the sample contamination during sampling or sample preparation. Since PAHs can adsorb onto plastics, preferably aluminium, glass or polished stainless steel should be used in contact with the samples and keep them protected from the light during the whole sample process. Containers must also be rinsed with high-purity solvents (acetone or hexane) before use. When the samples arrive at the laboratory, the edible parts of the incremental samples are combined to form an aggregate sample and homogenised as finely ground as possible. Furthermore, the aggregate sample will be divided into three parts for enforcement, defence and referee purposes (EC 2007, 2011b).

Owing to highly sensitive and accurate analytical techniques, the focus of the development has shifted more towards sample preparation methods. The appropriate sample preparation is required to achieve reproducible data and fulfil the requirements of the legislation. However, due to the complexity of the food matrices and the target compounds typically present at relatively low levels in relation to the remaining sample constituents, the sample preparation can be laborious and usually requires thorough extraction and many alternative methods to prepare them. Food samples vary from solid biological matrices, such as meats and fats, to liquid vegetable oils. These matrices differ greatly in composition, and the PAH contamination varies from trace levels up to thousands of µg/kg in product (Vaessen et al. 1988). The high amount of fat of several food matrices belongs to one of the major challenges in the laboratory analysis of contaminants, including PAHs. Sample preparation is defined as a series of operations starting with the

extraction, in which PAHs and fat are extracted from the sample matrix, followed by the sample purification prior to detection (Duedahl-Olesen et al. 2020). As Ridgway et al. (2007) aptly describe, sample preparation is a common bottleneck of the analysis causing a huge need to increase its effectiveness. The preparation steps should be effective, taking into account the applied instrumentation, the food matrix and the compounds to be determined. The advantages of the method optimisation are a shorter analysis time, better quality and eco-friendly methods with less solvent and smaller sample quantities (Ridgway et al. 2007).

Solvent extraction remains the most frequently used extraction technique for organic contaminants in foods (Andreu and Picó 2019). In saponification, a reaction with potassium or sodium hydroxide breaks down the proteins and lipids in food matrix and releases the adsorbed PAHs. Liquid-liquid extraction with organic solvents is used to isolate PAHs and remove impurities (Sampaio et al. 2021). However, due to their large amounts of solvent and laborious, time-consuming steps, other options have become more popular (Wenzl et al. 2006). Today, most extraction procedures shift towards automated systems, low solvent volumes, efficient extraction time and green chemistry (Veyrand et al. 2007; Andreu and Picó 2019). Modern extraction applications for PAHs in foods include methods such as supercritical fluid extraction (SFE) (Hawthorne et al. 2000), solid phase extraction (SPE) (Stumpe et al. 2008), microwave-assisted extraction (MAE) (Purcaro et al. 2009) or pressurised liquid extraction (PLE; also known by the trade name ASE®, accelerated solvent extraction) (Andreu and Picó 2019). The principle of PLE is to use solvent at elevated temperature and pressure to extract the selected analytes. The high temperature increases the sample solubility and further enhances the diffusion rate, whereas the high pressure maintains the solvent below its boiling point. The automated PLE system saves time and solvent and, very importantly, results in higher yield. PLE has been successfully employed to extract contaminants, such as PAHs, in complex matrices. Challenges naturally occur as the temperature rises, because the undesirable food matrix components are also extracted. The elevated temperature can also increase the degradation rate of the analyte, and hence the extraction time must be optimised (Andreu and Picó 2019).

In general, PAH extracts require extensive clean-up and enrichment prior to liquid chromatography (LC) or GC determination. There are several approaches to remove interferences and isolate the analytes, but the main techniques for PAHs are SPE, column chromatography and gel permeation chromatography (GPC) (Suchanová et al. 2008). According to several studies, GPC has been used regularly for PAH analyses in foodstuffs (Fromberg et al. 2007; Wang and Guo 2010; Jung et al. 2013; Duedahl-Olesen et al. 2020). SPE is another widely employed technique for PAHs, which involves partitioning between a liquid (sample matrix or solvent with analytes) and a solid sorbent phase (Moret and Conte 2000). Multiple samples can

be processed parallel by SPE either offline, i.e. the sample preparation is separated from the analysis step, or online mode, where SPE is directly connected to the chromatographic LC or GC separation. Apart from online SPE, the emerging trend is to develop new concepts in order to provide faster and more efficient sample preparation methods. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method has been successfully applied, for instance, in pesticide food analysis (Payá et al. 2007) and recently also for PAHs in selected matrices, such as smoked meat (Surma et al. 2014), dried and smoked fruits (Surma et al. 2018), smoked fish, cheese and malt (Duedahl-Olesen et al. 2020; Slámová et al. 2020) by GC-MS detection. Duedahl-Olesen et al. (2020) validated the QuEChERS method successfully for screening purposes, focusing on PAH4 in smoked fish and malt. Another promising technique for sample preparation is a magnetic solid phase extraction (MSPE) based on the magnetic or magnetisable adsorbents, which can be isolated from the sample matrix using an external magnet (Zhao et al. 2011).

2.3.2 Analytical techniques for PAH determination

The most common analytical techniques for PAH analysis refer to an LC coupled with a fluorescence detector (FLD) and a GC-MS (Veyrand et al. 2007; Suchanová et al. 2008; Plaza-Bolaños et al. 2010). In the past, high performance LC (HPLC) with an ultraviolet (UV) or photo-diode array (PDA) detector and GC coupled with a flame ionisation detector (FID) were used, but the selectivity and sensitivity no longer meet today's demands (EFSA 2008; Plaza-Bolaños et al. 2010). Therefore, the LC-FLD has been widely applied for PAHs in foods due to its better selectivity and sensitivity compared to UV detection (Ishizaki et al. 2010; Plaza-Bolaños et al. 2010). In addition to the traditional analytical LC methods, modern LC-MS/MS methods have been developed (Smoker et al. 2010; Hollosi and Wenzl 2011).

As a principal alternative to LC-FLD, GC combined with MS is a widespread analytical technique for the identification and quantification of organic compounds in a large variety of food matrices. In fact, the recent PAH studies indicated an increased interest in GC-MS(/MS)-based techniques (Lee et al. 2019; Duedahl-Olesen et al. 2020; Slámová et al. 2020). Due to an urge for a more accurate confirmation of PAHs, GC provides high chromatographic resolution while MS offers high mass selectivity and structural identification. The GC-MS is applicable for volatile and thermally stable compounds, thus excluding a number of compounds to be determined by other techniques (Fialkov et al. 2003; Plaza-Bolaños et al. 2010). The GC separation is initiated by the sample volatilisation in a heated injector, which is operated either in split or splitless mode. For PAHs, the splitless mode is more suitable, as the loss of analytes is prevented. All the sample analytes are directed by a carrier gas (e.g. hydrogen or helium) into the column, where the analytes are retarded by the stationary phase of the column.

The oven temperature is raised until those higher boiling and strongly retained PAHs are also released from the stationary phase. The separation is based on the partitioning of each component between the mobile phase (carrier gas) and the stationary phase of the capillary column (Sparkman et al. 2011). Considering the selection of different stationary phases, methyl- and phenyl-substituted polysiloxanes appear to be the most applied stationary phases for PAH separation, typically containing 5% phenyl and 95% methylpolysiloxane (Zelinkova and Wenzl 2015b). However, in order to achieve an accurate resolution for each PAH4 compound, more polar selective column phase is necessitated due to isomers and co-elution in the stationary phase, such as triphenylene (TPH) and CHR as well as benzo[b,k,j]fluoranthenes (Veyrand et al. 2007; Plaza-Bolaños et al. 2010; Hollosi and Wenzl 2011). An in-depth comparison of the stationary phases revealed a mid-polar 50%-phenyl-methylpolysiloxane column solving the resolution problems of benzo[b,k,j]fluoranthenes (Gómez-Ruiz and Wenzl 2009). Nowadays, there are at least a few column brands offering enhanced separation between TPH and CHR and even more columns capable of separating benzo[b,k,j]fluoranthenes.

After the PAHs are eluted from the GC column, they are mostly detected by the FID or MS. Certainly, MS has become popular thanks to its versatile properties including high selectivity and sensitivity (Plaza-Bolaños et al. 2010). Primarily, two types of ionisation techniques connected to GC–MS are used: electron ionisation (EI, formerly known as electron impact ionisation) and chemical ionisation (CI). Up to the present day, the most commonly used technique for PAH fragmentation is EI, which typically employs the electron energy 70 eV to produce molecular ions with a positive charge. The high stability hampers the PAH fragmentation, although EI is a harsh ionisation technique compared to CI (Veyrand et al. 2007). The inadequate fragmentation leads to difficulties in identification of PAHs at trace levels, and therefore modern techniques such as the GC-MS/MS with EI, are recommended. The MS/MS analyser consists of two quadrupoles, a hexapole in the middle and a photomultiplier detector positioned after the second quadrupole. The greatest benefit of the MS/MS is the extensive fragmentation in the collision cell with argon collision gas resulting in specific product ions of PAHs. In GC-MS/MS, two specific transitions are requested in order to provide accurate identification for each PAH4 (Veyrand et al. 2007). The dedicated PAH databases will further improve the accuracy of the analysis process. Alongside GC-MS/MS, other techniques have emerged for PAH determinations in foods, such as GC coupled with high-resolution MS (HRMS) (Rozentale et al. 2017), GC quadrupole time-of-flight MS (GC-QTOF-MS) (Duedahl-Olesen et al. 2020), and GCxGC-TOF-MS (Purcaro et al. 2007).

2.3.3 Role of validation in quality assurance

The growing interest in food safety has led to the development of methods capable of tackling the analytical challenges. While the PAH4 methods should be rapid and effective, they have to meet the quality requirements. Laboratories employ accreditation according to International Organization for Standardization ISO 17025 to implement a quality system which aims to improve their ability to continuously produce accurate results (ISO 2005). In Commission Decision 2002/657/EC, the general guidelines on the performance of the analytical methods were introduced (EC 2002a). Even though this guidance is aimed mostly at veterinary medicinal products in live animals and animal products, where applicable, it is also suitable for organic contaminants. A specific guidance for contaminants was published by the Joint Research Centre (JRC) focusing on measurement of the limit of detection (LOD) and the limit of quantification (LOQ) (Wenzl et al. 2016).

To implement European Union (EU) legislation and produce high-quality data, a thorough method validation for PAH4 is demanded. The objective of the validation process is to prove that the analytical method is fit for its purpose, fulfilling the agreed criteria or requirements. A validation plan includes the chosen validation parameters and their acceptance criteria, which may differ depending on the method. Typically, the validation parameters shown in Table 2 are determined within the conventional validation process (EC 2002a). The legislative method performance criteria for PAH4 were set in Commission Regulation (EU) No 836/2011 amending (EC) No 333/2007 (EC 2007, 2011b). A Horwitz ratio (HorRat) is defined as a performance parameter that indicates the acceptability of a chemical method of analysis in relation to precision. According to Horwitz and Albert (2006) the Horwitz ratio is the ratio of the relative standard deviation (RSD_R) calculated from the reproducibility data divided by the RSD predicted from the Horwitz equation ($PRSD_R$). The applied criteria are intended for official food control laboratories analysing official samples. To ensure the validity of the analytical method, various internal quality control measures are performed on a regular basis. The quality parameters to be monitored are selected on a method basis and they often include controls for both the method and the analytical instrument (e.g. control standards, spiked and replicate samples).

In addition to the common method performance characteristics obtained in the process of validation, the laboratory shall employ procedures for estimating measurement uncertainty (MU). Based on the utilised information, MU characterises the dispersion of the values that are associated with the measurand (NMKL 2004). In addition, the influence of the potential matrix interferences must be considered within the method validation process. The matrix effect is defined as a difference in mass spectrometric response between an analyte in standard solution and in a biological matrix. The phenomenon is caused by co-eluting matrix components, which alter ionisation and lead to ion suppression or

ion enhancement (Panuwet et al. 2016). While for the most part the tools to reduce matrix effects in GC-MS and LC-MS are alike, the causes are different. In the GC system, active surfaces may cause retention and/or degradation of compounds, and thereby matrix-induced signal enhancement. In case of complex extracts, the active sites are occupied by matrix components that increase the efficiency of the analyte move to the detector. In practice, the comparison between the matrix-matched and solvent-based calibration curves reveals whether the matrix effects occur (Kwon et al. 2012). The recommended approach to compensate for this effect is to apply isotopically labelled internal standards (ISTDs) to increase the reliability of the results (EC 2002a; Ridgway et al. 2007; Kwon et al. 2012). The purpose of ISTD is to behave similarly to the analyte of interest but produce a signal which can be distinguished from that of the analyte. Considering individual PAH4 compounds, both the isotope 13C-labelled and the deuterated ISTDs are on the market.

Table 2. Validation parameters and corresponding legislative performance criteria of PAH4 methods.

Validation parameter	**Definition** (Wessman et al. 2001; EC 2002a; NMKL 2009)	**Legislative criterion (EC) No 333/2007 with amendments** (EC 2007)
Ruggedness/Applicability	The susceptibility of an analytical method to changes in experimental conditions under which the method can be applied as presented or with specified minor modification	Foods specified in Commission Regulation (EC) No 1881/2006 with amendments
Specificity	The ability of an analytical method to separate the analyte to be analysed from other substances present in the sample	Free from matrix or spectral interferences, verification of positive detection
Selectivity	The extent to which a method can determine specific analytes in mixtures or matrices without interferences from other components	
Repeatability (RSD_r)	Precision under repeatability conditions, where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment	$HorRat_r$ less than 2 HorRat= Horwitz Ratio $HorRat_r$=observed RSD_r/predicted RSD_r (Horwitz) RSD is calculated using the Horwitz equation: $RSD=2C^{(-0.15)}$ C=concentration expressed as a mass fraction
Reproducibility (RSD_R)	Reproducibility under reproducibility conditions, where test results are obtained using the same method in different laboratories and with different operators and equipment.	$HorRat_R$ less than 2 $HorRat_R$=observed RSD_R/predicted RSD_R (Horwitz) RSD is calculated using the Horwitz equation: $RSD=2C^{(-0.15)}$ C=concentration expressed as a mass fraction
Within-laboratory reproducibility (RSD_R) (Internal reproducibility)	Precision obtained using the same method in the same laboratory at different times and by different operators	
Recovery	The percentage of the true concentration of an analyte recovered during the analytical procedure	50–120%
Limit of detection	The lowest quantity of an analyte which can be detected with an acceptable statistical significance	≤0.30 µg/kg for each of the four substances
Limit of quantification	The lowest analyte concentration which can be detected quantitatively with a closely defined confidence	≤0.90 µg/kg for each of the four substances
Linearity	Function which reflects the correlation between the content of an analyte in a sample, and the resulting measurement response	
Trueness	The closeness of agreement between a sample's assigned value of a specific analyte and the result of the analysis	
Measurement uncertainty	Parameter which characterises the dispersion of the values which could be attributed to the measurand	$Uf=\sqrt{\left(\frac{LOD}{2}\right)^2 + (aC)^2}$ Uf=maximum standard measurement uncertainty (µg/kg) C=concentration of interest (µg/kg) a= factor, which is dependent on the concentration. When C ≤50 µg/kg, a=0.2

2.4 Foods related to PAH contamination

The presence of PAHs has been reported in all types of foods, comprising raw, unprocessed, processed and cooked foodstuffs (Phillips 1999). Of the large number of the reported data, PAHs have been measured in varying combinations. The changing PAH legislation naturally reflects the compounds being analysed and their methods of analysis. Taking into account EFSA's opinion on PAH4 published in 2008 and the legislative requirements for PAH4 in foods implemented in 2011, relevant data for PAH4 have been mostly determined after 2008 (EFSA 2008; EC 2011b). In order to form an overall picture of the presence of PAH4 in food, a literary search was conducted that primarily focused on 2009-2020 (Table 3). A few exceptions were made as older data from either Finland or neighbouring countries with similar products were considered relevant.

Generally, the PAH contamination in a non-smoked fish is lower compared to smoked fish even though seafood may be polluted and contain PAHs (e.g. bivalve molluscs) (Hellou et al. 2005; Drabova et al. 2013). The occurrence of PAH4 in smoked fish and meat varies greatly from not detected to hundreds of µg/kg PAHs. This could be due to the variation in smoking process resulting in PAH profiles with no clear trend (Duedahl-Olesen et al. 2010; Roseiro et al. 2012). Elevated PAH4 levels were observed in traditionally smoked meat products (Rozentale, Zacs et al. 2018). By contrast, another study reported 64% of the determined smoked meat samples containing BaP levels below the LOD of 0.3 µg/kg (Reinik et al. 2007). Regarding smoked fish samples, the determined BaP concentrations remained mostly below 5 µg/kg in a Danish study (Duedahl-Olesen et al. 2010). Different fish species were more predominant in Nordic countries than in other countries. For instance, a Swedish study demonstrated the highest BaP level in smoked herring (Wretling et al. 2010), whereas in Poland the highest BaP concentration was obtained in smoked canned sprat (Zachara et al. 2017).

In Finland as well as in Sweden, the traditional "sauna"-smoked food products are national delicacies. The smoke sauna differs from a regular wood-fired sauna in that it lacks a chimney, enabling the smoke rise from the stove to fill the room. The analyses of a Swedish study indicated 10 out of 11 traditionally sauna-smoked meat and fish samples containing BaP exceeded 5 µg/kg. However, the other analysed smoked meat and fish products were primarily in compliance with the prevailing legislation (Wretling et al. 2010). In Finland, the concentrations of 11 PAHs in a large variety of food groups were determined and traditionally smoked pork products contained the highest BaP levels, varying between 5.6 and 13 µg/kg (Hietaniemi et al. 1999). Apart from smoking, grilled food may contain higher PAH amounts caused primarily by the pyrolysis of the fat. The generation of PAHs is dependent on the food type, fuel, and cooking method (Rose et al. 2015).

Another food group of concern comprises vegetable oils and fats consisting of raw materials processed in many ways (Hopia et al. 1986). The majority of the published oil and fat studies concern olive oils, which can be classified according to their quality from low-quality olive pomace oil to high-quality extra virgin olive oil (Sun et al. 2019). In crude edible oils, the PAH contamination appears to vary a lot, while refined oils generally contain low levels of PAHs (Cejpek et al. 1998). Elevated amounts of PAH were detected in pomace olive oil (Ergönül and Sánchez 2013; Rascón et al. 2018; Sun et al. 2019). Moreover, relatively high BaP and PAH4 concentrations were found in canola oil (known also as rapeseed oil) (Yousefi et al. 2018), whereas some other studies confirmed mostly BaP levels below 2.0 µg/kg (Larsson et al. 1987; Fromberg et al. 2007). For the most part, low BaP levels were observed in Finnish butter, margarines, vegetable oils and their raw materials. The only exception was coconut oil, which contained up to 24 µg/kg of BaP (Hopia et al. 1986).

Despite the fact that cereal products contain mostly low levels of PAHs, they can be considered a major source of dietary exposure due to their high consumption volumes (EFSA 2008). The contamination occurs through the drying process if the grains are in direct contact with the combustion gases (Tuominen et al. 1988; Dennis et al. 1991). When using direct gas heating, the BaP levels were generally lower than 0.1 µg/kg (Larsson et al. 1991). Other pathways for contamination can be the flour and the baking process (Ciecierska and Obiedziński 2013). Drying and roasting practices may have an impact on PAH contamination in cocoa and coffee beans. The reported PAH4 levels are still relatively low in cocoa products and only trace levels in coffee beans (Ciecierska 2020; Rattanarat et al. 2021). Food supplements consist of a large product group and PAHs have been found in products containing edible oils as well as propolis, spirulina and St. John's worth (Danyi et al. 2009; Zelinkova and Wenzl 2015a). Dried herbs and spices may also contain varying PAH levels (Rozentale, Yan Lun et al. 2018). In addition, PAH contamination may occur in baby foods including variable ingredients. It is assumed that origins of milk-based products containing PAHs are located in the area near the potential environmental contamination source of PAHs (Grova et al. 2002). Depending on the ingredients in the baby foods, the contamination varies (Santonicola et al. 2017). Environmental contamination may also occur in fruits and vegetables besides cooking procedures. However, compared to other food groups, PAH levels in fruits and vegetables are typically below 0.5 µg/kg (Paris et al. 2018).

Table 3. Overview of the BaP and PAH4 concentrations (µg/kg) in major foodstuffs related to PAH exposure.

Food matrix	Mean BaP (µg/kg)	Max BaP (µg/kg)	Mean PAH4 (µg/kg)	Max PAH4 (µg/kg)	Number of samples (n)	Country	Reference
Meat and fish							
Ham	0.4	0.5			6	Estonia	(Reinik et al. 2007)
Smoked sausage	0.4	1.3			13	Estonia	
Smoked chicken	0.4	0.8			3	Estonia	
Smoked meat		36 (ham)			38	Sweden	(Wretling et al. 2010)
Smoked fish		14 (herring)			39	Sweden	
Smoked fish		11 (cod roe)			180	Denmark	(Duedahl-Olesen et al. 2010)
Meat and blood sausage		5.7 (blood sausage, highest mean value)		300 (meat sausage, highest mean value)	110	Portugal	(Roseiro et al. 2012)
Processed beef		5.4 (smoked beef)			34	South Africa	(Olatunji et al. 2014)
Processed pork		10 (smoked pork)			34	South Africa	
Processed chicken		5.9 (smoked chicken)				South Africa	
Smoked meat		6.2 (smoked sausage)	24	36 (smoked sausage, highest mean value)	60	Poland	(Zachara et al. 2017)
Smoked fish		4.8 (smoked canned sprat)		73 (smoked canned sprat)	60	Poland	
Smoked fish (Cambodia)	55	120	250	600	23	Czech Republic	(Slámová et al. 2017)
Smoked meat (Latvia)	8.1	120	54	630	52	Latvia	(Rozentale, Zacs, et al. 2018)
Smoked meat (Lithuania)	1.5	7.1	9.5	56	17	Latvia	
Smoked meat (Estonia)	2.8	17	26	180	8	Latvia	
Smoked fish		0.9 (smoked sprat)		6.8 (smoked sprat)	54	Poland	(Malesa-Ciećwierz et al. 2019)
Meat		4.8 (dry sirloin)		13 (dry sirloin)	99	Croatia	(Bogdanović et al. 2019)
Fish	0.3	-		3.2	18	Croatia	

Table 3. Continued.

Food matrix	Mean BaP (µg/kg)	Max BaP (µg/kg)	Mean PAH4 (µg/kg)	Max PAH4 (µg/kg)	Number of samples (n)	Country	Reference
Fat and vegetable oil							
Fats and oils		24 (crude coconut oil)			25	Finland	(Hopia et al. 1986)
Rapeseed oil		2.1			23	Sweden	(Larsson et al. 1987)
Olive oil		0.4 (extra virgin olive oil)			52	Denmark	(Fromberg et al. 2007)
Vegetable oil other than olive oil		1.8 (grape seed oil)			17	Denmark	
Olive oil		14 (crude olive pomace oil)			31	Turkey and Spain	(Ergönül and Sánchez 2013)
Vegetable oil		11 (canola oil)		84 (canola oil)	40	Iran	(Yousefi et al. 2018)
Olive oil		150 (olive pomace oil, highest mean value)		3,400 (fried virgin olive oil, highest mean value)	18	Spain	(Rascón et al. 2018)
Vegetable oil other than olive oil		1,900 (coconut oil, highest mean value)		3,300 (coconut oil, highest mean value)	31	Spain	
Bread and cereal							
Bread	1.9	9.4			15	Italy	(Orecchio and Papuzza 2009)
Bread	0.2	0.3	0.3	0.5	20	Turkey	(Kacmaz 2016) (Kacmaz 2016)
Cereal	0.2	0.30	0.4	0.9	18	Turkey	
Cereal	0.06	0.07	0.6	0.7	3	Latvia	(Rozentale et al. 2017)
Rye bread	0.08	0.2	0.7	1.6	20	Latvia	
Wheat bread	0.06	0.2	0.5	1.1	12	Latvia	
Bread		1.0			4	Iran	(Rostampour et al. 2017)

Table 3. Continued.

Food matrix	Mean BaP (µg/kg)	Max BaP (µg/kg)	Mean PAH4 (µg/kg)	Max PAH4 (µg/kg)	Number of samples (n)	Country	Reference
Baby food							
Milk-based baby food	0.5	2.1		16	22	Italy	(Santonicola et al. 2017)
Meat- and fish-based baby food	0.2	1.7		7.3	18	Italy	
Grilled foods							
Home-grilled meat	0.4	3.3	2.3	19	26	Sweden	(Wretling et al. 2013)
Commercially grilled products		18 (hamburger)		48 (hamburger)	200	Denmark	(Duedahl-Olesen et al. 2015)
Grilled food	3.7	31	14	96	140	United Kingdom	(Rose et al. 2015)
Other foods							
Food supplement		12 (black radish)			20	Belgium	(Danyi et al. 2009)
Food supplement		87 (propolis intense)		710 (propolis liquid)	110	Belgium	(Zelinkova and Wenzl 2015a)
Herbs and spices		6.6 (black pepper)		25 (black pepper)	150	Latvia	(Rozentale, Yan Lun, et al. 2018)
Cocoa beans and their derived products				1.6 (roasted cocoa beans)	340	Poland	(Ciecierska 2020)

2.5 Methods for mutagenicity testing

In order to identify and measure the mutagenic activity of the chemical, various methods are available for testing such as a bacterial reverse mutation assay (the Ames test) (Ames et al. 1975), the single cell gel electrophoresis assay (the comet assay) (Singh et al. 1988) and the spectrophotometry method (Zhang et al. 2000). The Ames test appears to be one of the most widely applied screening tests for the identification of the mutagenic substances owing to its inexpensive, rapid and handy use. Chemicals are tested according to international guidelines in the chemicals, cosmetics, pharmaceuticals and agriculture industries as part of genetic toxicity testing (Levy et al. 2019). The test protocol utilises multiple strains of *Salmonella typhimurium*, which each carry different mutation in their histidine amino acid (Ames et al. 1975; Maron and Ames 1983). TA98 (frameshift mutation) and TA100 (base-pair substitution) are common strains assessed in the Ames test. All *Salmonella* strains are histidine auxotrophs, and thus cannot grow and generate colonies without it. However, if the bacteria are exposed to a mutagenic chemical during their growth, the mutagen induces the organism to reverse the mutation in the histidine operon and to synthesise this essential amino acid required for growth. The detection of revertant bacteria is based on their ability to grow and form revertant colonies, which indicate the mutagenic activity of the test substance (Ames et al. 1975; Maron and Ames 1983; Mortelmans and Zeiger 2000). A large number of mutagenic substances, including PAHs, require metabolic activation in order to bind to deoxyribonucleic acid (DNA) and induce a mutation. As such, the bacterial strains do not contain eukaryotic metabolic enzymes. To mimic mammalian metabolic conditions, rat liver extract (S9-mix) can be added before culturing to the top agar *in vitro* containing both the bacterium and the test sample (Maron and Ames 1983; Guengerich 2000; Mortelmans and Zeiger 2000). Nevertheless, metabolism differs between rat and human and that may influence the mutagenicity of the substances. In general, an *in vitro* human liver should be more representative compared to a rat liver and thus, human S9 fractions could be used to confirm the positive findings of the test (Hakura et al. 1999).

Although mutagenicity studies have been performed for a while, only a few papers address the mutagenic potential of the chemicals in foods. The mutagenic activity of the white grape juice using several *Salmonella* strains was tested by the Ames test. Based on the results, a mutagenic response was observed with or without the activation indicating that the grape components of the juice are directly acting mutagens. Furthermore, it was shown that the mutagenic activity was not caused by the presence of histidine. As the mutagenic response was also found in freshly prepared juice, the commercial production process was excluded, and the chemicals may be inherent in grapes (Patrineli et al. 1996). Another Ames study focusing on the sunflower oil showed no mutagenic potential in the *Salmonella*

strains TA97a, TA98, TA100, TA102 and TA1535 (de Mello Silva Oliveira et al. 2016). Moreover, other Ames study investigated the mutagenicity of heat-treated meat products. Chicken and beef steak broiled in high temperature as well as hamburgers showed a wide range of mutagenicity towards *Salmonella* TA 1538 (Bjeldanes et al. 1982). Two studies examined the genotoxicity of Finnish processed food products and ready-to-eat snacks employing the Ames test (Tikkanen 1991; Omoruyi and Pohjanvirta 2014) along with the complementary biological assays (comet assay, methylcellulose overlay, treat and wash assay) (Omoruyi and Pohjanvirta 2014). Most of these food products showed mutagenic activity via the Ames test. However, due to the possible limitations of this test, other complementary mutagenicity assays and/or chemical analyses can improve the reliability of the results.

As an alternative mutagenicity test, a comet assay can be applied to measure DNA strand breaks in individual eukaryotic cells. Briefly, cells are embedded in agarose on a glass slide lysed with detergent and salt. Thus membranes, soluble cell constituents and histones are removed leaving the DNA supercoiled and linked to the nuclear matrix. Under electrophoresis at high pH, DNA loops with breaks move towards the anode forming a "comet tail", whereas the undamaged DNA remains within the cell generating a "comet head". The relative content of DNA in the comet tail indicates the frequency of DNA breaks (Ostling and Johanson 1984; Singh et al. 1988). The comet assay has advantages due to its simple and rapid performance, high sensitivity for detecting DNA breaks, the analysis of data at the level of the individual cell and the use of small samples. In addition, DNA repair is possible to monitor with the comet assay. This assay has a huge number of applications in genotoxicity testing to screen novel drugs, or chemicals as well as in human biomonitoring and ecogenotoxicology (Azqueta and Collins 2013; Bajpayee et al. 2019). Furthermore, the comet assay has been applied in the detection of irradiation treatment of foods (Khan and Delincée 1998) or testing specific compounds in foods such as antioxidants and food additives (Wasson et al. 2008; Peycheva et al. 2014). In a previous Finnish study, the mutagenicity results obtained by the Ames test in heat-treated foods were re-evaluated using the comet assay. In contrast to the Ames test, none of the food products appeared to be mutagenically active in the comet assay (Omoruyi and Pohjanvirta 2014). False positive and negative results are mainly due to the differences in levels of sensitivity and specificity within mutagenicity methods. Other potential mutagenicity tests include treat-and-wash and methylcellulose overlay methods, which are valid modifications of the bacterial mutation assay for avoiding false positive results related to released amino acids (Thompson et al. 2005) as well as the spectrophotometry that measures bacterial growth (Zhang et al. 2000).

2.6 Toxicological features of PAHs

2.6.1 Metabolism of PAHs

The presence of PAHs in food is of public concern, as they belong to a group of toxic substances. Their biological activity is related to structural properties when aromatic rings form so-called fjord or bay regions (Figure 2). The reactivity is dependent on the electron charge density, but also geometric distortions in molecules affect the charge distribution and further reactivity in certain positions. PAHs containing a fjord region are nonplanar binding in DNA mainly to the N6 of adenine nucleotides, while those with a bay region are planar attacking the N2 of guanine nucleotides. As a result, a PAH-DNA adduct is formed, which refers to the mutagenic and carcinogenic potential of PAHs (Błaszczyk and Mielżyńska-Švach 2017).

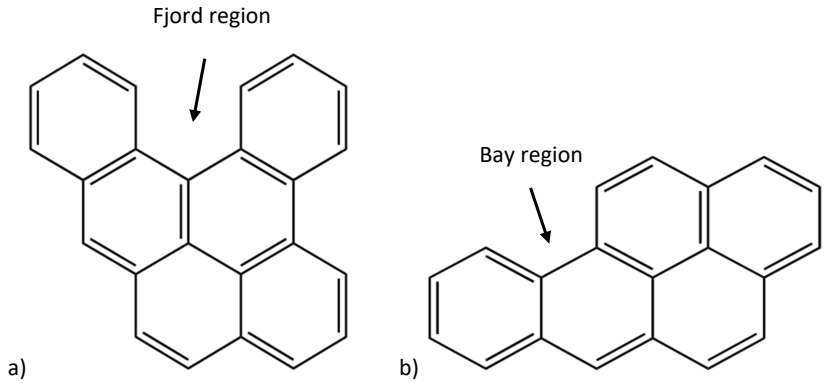


Figure 2. Fjord and bay regions illustrated in a) dibenzo[a,l]pyrene and b) benzo[a]pyrene.

DNA adducts play a role in the early stages of carcinogenesis, but several other factors are also involved in this process (Błaszczyk and Mielżyńska-Švach 2017). Any substance capable of modifying or causing a permanent change in the genetic structure of a cell by altering the DNA structure can be considered a mutagen. If only a single base is altered or one or few nucleotide bases are inserted or deleted, mutations are gene (point) mutations. It leads to an alteration in the cell growth, which can be measured in bacteria and other cell systems. Some mutagens, such as PAHs, are biologically inactive procarcinogens and require activation in order to be metabolised to their active forms. The main metabolic enzymes catalysing the oxidation of PAHs belong to a cytochrome P450 (CYP) family. Although PAHs are not considered to be liver carcinogens, the CYP metabolic oxidation system is located in the liver and to a lesser extent in the lungs and kidneys (Mortelmans and Zeiger 2000). When PAHs enter into the body, they undergo multiple metabolic

pathways. The activity mechanisms are complex and uncertain, but the principles of the three major pathways are known and illustrated in Figure 3: 1) CYP450 peroxidase pathway, 2) the CYP450 1A1/1B1 and epoxide hydrolase pathway (CYP/EH pathway) and 3) aldo-keto reductases pathway (AKR pathway) (Fishbein and Heilman 2013; Moorthy et al. 2015). In particular, the CYP/EH pathway is considered the most important metabolic route for PAHs (EFSA 2008; Benford et al. 2010). These metabolic pathways result in DNA adduct formation, inactivation or detoxification. A procarcinogen may be metabolised in such a way that instead of generating an active metabolite, a metabolite is processed for elimination (Gonzalez and Gelboin 1994). Most of the detoxified PAHs and their metabolites are excreted and eliminated in urine, bile and faeces after metabolism (Yebra-Pimentel et al. 2015). In urine, the main metabolites of BaP are 3-hydroxy- and 9-hydroxybenzo[a]pyrene. It is noteworthy that the metabolic conditions are much more complex, because the reaction products of PAHs can have different spatial conformations and enzymes catalyse stereoselective reactions, leading to optically active products (EFSA 2008). Moreover, PAH metabolites can induce an increase in the number of reactive oxygen species (ROS) causing oxidative stress, which may directly impact DNA, lipids or proteins and initiate carcinogenesis (Moorthy et al. 2015). Despite the role of the PAH-DNA in carcinogenesis, determination of DNA adducts in whole tissues is only an estimate of the cancer risk (Yebra-Pimentel et al. 2015). In addition to DNA-adduct formation, local cell proliferation can be a critical factor in tumour development by BaP (Goldstein et al. 1998; Kroese et al. 2001).

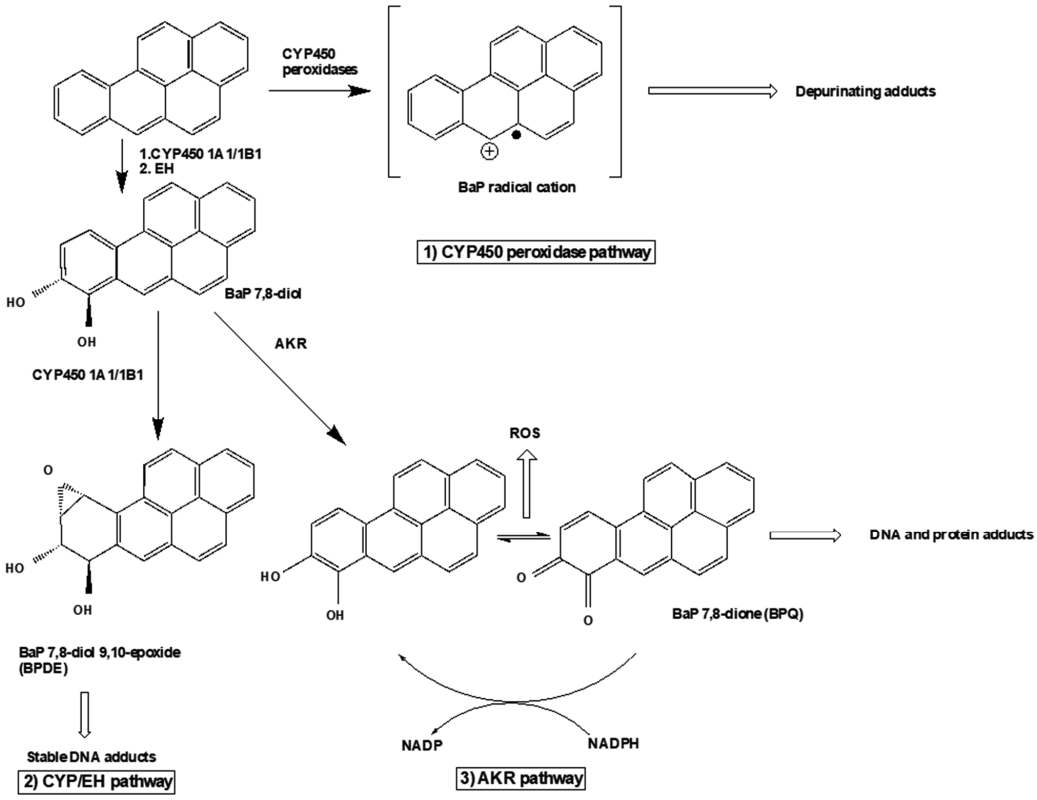


Figure 3. Metabolic pathways of PAHs, of which benzo[a]pyrene is an example (Fishbein and Heilman 2013).

2.6.2 Toxicological effects of PAHs

Human exposure to PAHs can cause diverse adverse health effects with cancer being the most substantial. The main pathways to carcinogenic exposure are inhalation, dermal contact and ingestion (WHO/IPCS 1998). Usually, the route of administration determines the location of tumour growth such as gastric tumours following oral exposure. Still, tumours can be detected at the point of contact as well as in remote locations (EFSA 2008). The oral exposure is considered the main pathway in human exposure. Although 15 PAHs are known carcinogens, the existing oral studies concern mostly BaP (EFSA 2008; Benford et al. 2010). The PAH absorption through the diet is dependent on the molecule size and its lipophilic properties, the appearance of bile in the digestive tract, the dose and the lipid content in the diet. In a rat study, BaP was absorbed orally within two to four hours and was assessed to vary between 35% and 99% after dietary or gavage exposure (Ramesh et al. 2004). Based on the limited research data, the

acute oral toxicity of PAHs appears to be moderate to low. A lethal dose of 50% (LD50) of >1600 mg/kg bw was reported for BaP in mice and rats (WHO/IPCS 1998). The existing oral exposure studies performed in animal experiments have proved that the ingested BaP may generate tumours in the gastrointestinal tract, liver, lungs and mammary glands of mice and rats (EFSA 2008). A study compared tumours induced by coal tar mixtures and BaP in mice indicating that BaP in the coal tar diets might be responsible for the forestomach tumours (Culp et al. 1998), whereas another study investigated BaP in rats resulting in tumours in many organs and tissues, with forestomach and liver as main target organs (Kroese et al. 2001). The no-observed-effect-level (NOEL) of 3 mg/kg bw/day for BaP in rats was assessed in a 90-day study based on liver toxicity (Kroese et al. 2001). Detrimental haematological impacts, such as myelotoxicity in the presence of BaP, have been demonstrated in short-term studies. The information related to the systemic effects of the long-term PAH exposure is scarce since carcinogenicity is often the endpoint of the study. In mice and rats, BaA and BaP were found to be embryotoxic and BaP was also had found to have teratogenic and reproductive effects. Fetal deaths and malformations were observed only in those cases when the cytochrome P450 monooxygenase system was induced, either in the mother or in the embryo (WHO/IPCS 1998).

2.7 PAH legislation in food

As genotoxic carcinogens have potential adverse effects, it is advised to keep their exposure at the lowest possible level according to the ALARA principle ("as low as reasonably achievable") based upon good manufacturing, drying and agricultural/fishery practices (EFSA 2005). With regard to the risk assessment of PAHs to human health by SCF, BaP was proposed as a marker in foods; additionally, a list of 15 priority PAHs was introduced (EC 2002b). However, the Joint Food and Agriculture Organization (FAO) and World Health Organization (WHO) Expert Committee on Food Additives (JECFA) re-evaluated PAHs and highlighted that not only 15 PAHs are genotoxic carcinogens, but also benzo[c]fluorene was appointed as an additional compound into the group named as 15+1 EU PAH (Table 4) (EC 2005).

Maximum levels (MLs) have been set for BaP in foods considered to be most at risk of contamination in Commission Regulation No 1881/2006 (EC 2006). Nevertheless, the Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) of EFSA decided that BaP alone was not a suitable indicator for the occurrence of PAHs in food, and therefore either the sum of eight PAHs (PAH8) or PAH4 is preferred (EFSA 2008). Table 4 lists the PAH compounds which are included in these groups. Since PAH8 does not provide any added benefit over

PAH4, the EC laid down new MLs in Commission Regulation (EU) No 835/2011 amending (EC) No 1881/2006 for BaP and the sum of PAH4. The separate ML for BaP was kept in order to compare the previous and future data. Moreover, the scope was extended to cover a wide range of food groups (EC 2006, 2011a). Table 5 summarises the MLs for PAH4 in foods, according to Commission Regulation (EC) No 1881/2006 with amendments (EC 2006).

However, the new stricter MLs for PAHs in smoked fish and meat products as of 1.9.2014 appeared to be unachievable for certain traditional smoked products in some Member States, including Finland. As a consequence, EC established a derogation for three years (EU) No 1327/2014 amending Commission Regulation (EC) No 1881/2006, allowing appointed Member States (e.g. Finland) to manufacture and consume traditionally smoked fish and fishery products as well as meat and meat products within their nation, complying with higher MLs (BaP 5.0 μg/kg and PAH4 30.0 μg/kg). The nominated countries shall proceed to monitor PAHs in traditionally smoked fish and meat and further create programmes to implement good smoking procedures (EC 2006, 2014). Finland applied for a permanent derogation, which was accepted and implemented in Commission Regulation (EU) No 2020/1255 amending (EC) No 1881/2006. As a result, Finnish traditionally hot smoked meat and meat products as well as traditionally hot smoked small fish and fishery products consisting of small fish, can be placed on the domestic market with higher MLs for PAHs (EC 2006, 2020).

Table 4. The identification of 15+1 EU PAH, EU PAH8 and EU PAH4 compounds (EC 2005; EFSA 2008).

15+1 EU PAH	EU PAH8	EU PAH4
benzo[a]pyrene	x	x
benz[a]anthracene	x	x
chrysene	x	x
benzo[b]fluoranthene	x	x
benzo[k]fluoranthene	x	
benzo[ghi]perylene	x	
dibenz[a,h]anthracene	x	
indeno[1,2,3-cd]pyrene	x	
benzo[j]fluoranthene		
dibenzo[a,e]pyrene		
dibenzo[a,h]pyrene		
dibenzo[a,i]pyrene		
dibenzo[a,l]pyrene		
cyclopenta[cd]pyrene		
5-methylchrysene		
benzo[c]fluorene		

Table 5. Overview of the maximum levels for BaP and PAH4 in foods set in Commission Regulation (EC) No 1881/2006 with amendments (EC 2006).

Product	**Maximum level (µg/kg wet weight)**	
	BaP	**PAH4**
Oils and fats (excluding cocoa butter and coconut oil) intended for direct human consumption or use as an ingredient in food	2.0	10.0
Cocoa beans and derived products	5.0 µg/kg fat	30.0 µg/kg fat
Coconut oil intended for direct human consumption or use as an ingredient in food	2.0	20.0
Smoked meat and smoked meat products	2.0	12.0
Muscle meat of smoked fish and smoked fishery products	2.0	12.0
Smoked sprats and canned smoked sprats (Sprattus sprattus); Smoked Baltic herring ≤ 14 cm length and canned smoked Baltic herring ≤ 14 cm length (Clupea harengus membras); Katsuobushi (dried bonito, Katsuwonus pelamis); bivalve molluscs (fresh, chilled or frozen); heat-treated meat and heat-treated meat products sold to the final consumer	5.0	30.0
Bivalve molluscs (smoked)	6.0	35.0
Processed cereal-based foods and baby foods for infants and young children	1.0	1.0
Infant formulae and follow-on formulae, including infant milk and follow-on milk	1.0	1.0
Dietary foods for special medical purposes intended specifically for infants	1.0	1.0
Cocoa fibre and products derived from cocoa fibre, intended for use as an ingredient in food	3.0	15.0
Banana chips	2.0	20
Food supplements containing botanicals and their preparations Food supplements containing propolis, royal jelly, spirulina or their preparations	10.0	50.0
Dried herbs	10.0	50.0
Dried spices with the exception of cardamon and smoked Capsicum spp.	10.0	50.0
Powders of food of plant origin for the preparation of beverages	10.0	50.0

2.8 Risk assessment of PAHs in food

Risk assessment is part of a risk analysis consisting of three critical components: risk assessment, risk management and risk communication (FAO/WHO 2013). It can be defined as a scientific evaluation of known or potential adverse health effects connected to biological, chemical, and physical agents possibly present in food. The aim of the risk assessment is to provide scientific evidence for risk managers in order to decide whether the risk is acceptable or what kind of control measures must be taken if the risk is not acceptable. Furthermore, risk communication is related to the information exchange of risk between risk assessors, risk managers and all stakeholders. The purpose and findings of the risk assessment are communicated to all relevant parties clearly and effectively (IPCS 2009; FAO/WHO 2013).

Chemical risk assessment is focused on the human exposure to chemicals through food within a selected period. The difference between the hazard and the risk must be highlighted because the hazard can potentially harm human health, whereas the risk refers to the likelihood that this effect will occur. Even if a hazardous chemical is detected in food, it does not automatically pose a risk to the consumer. Risk assessment can be regarded as a scientifically based stepwise process including four steps illustrated in Figure 4. First, a hazard identification is the process of determining whether the exposure to a specified chemical in food or food groups can cause adverse health effects in humans such as cancer, reproductive toxicity, respiratory problems and allergic reactions. It is based on available scientific data from exposure studies in humans, experimental animals and/or *in vitro*, leading to an identification of the affected target organs or tissues (IPCS 2009). Specifically, PAH-related adverse health effects were presented in the context of the toxicological features of PAHs.

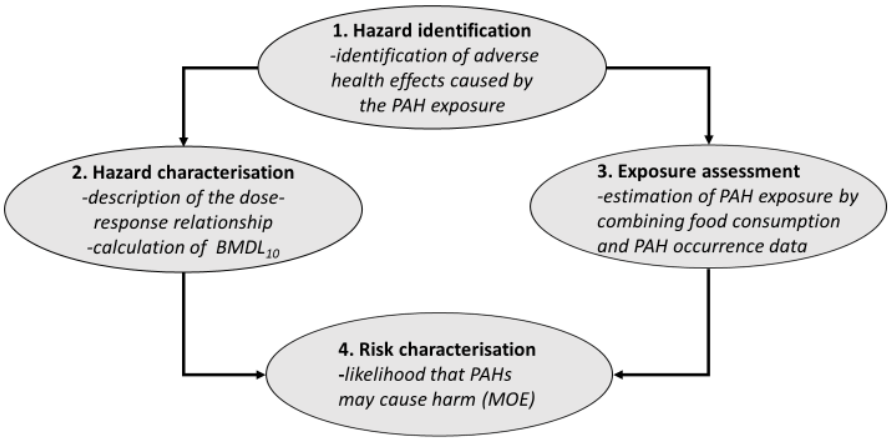


Figure 4. An overview of the risk assessment process of PAHs (EFSA 2008).

Secondly, a hazard characterisation involves a quantitative evaluation of the adverse effects, and thus determines the association between the administered dose of a chemical or chemical exposure dose–response data *in vivo* or *in vitro* studies and the incidence of the adverse health effect (dose-response relationship) (IPCS 2009). EFSA has provided a benchmark dose (BMD) model, which is based on a mathematical model and aims to estimate a dose that corresponds to a low but measurable change in response, typically at a 5% or 10% incidence level above the control. A lower confidence limit of the benchmark dose (BMDL) can be regarded as the lower limit of the 90% or 95% confidence interval of BMD. For genotoxic and carcinogenic compounds, the use of the BMDL of a 10% response level ($BMDL_{10}$) is considered a relevant reference point (EFSA 2005, 2017). The CONTAM Panel of EFSA considered the 2-year carcinogenicity mice study on coal tar mixtures (Culp et al. 1998) as the most relevant study for dose-response modelling (EFSA 2008). The BMD modelling was performed based on the total number of tumour-bearing mice as reported by Schneider et al. (2002). As a result, the calculated accepted BMD_{10} values showed the best model fitting for BaP from 0.13 mg/kg bw/day to 0.14 mg/kg bw/day, whereas the best fitting for PAH4 was from 0.60 mg/kg bw/day to 0.61 mg/kg bw/day. Furthermore, the $BMDL_{10}$ values 0.12 mg BaP/kg bw/day and 0.52-0.53 mg PAH4/kg bw/day represented the best fittings. The CONTAM panel, however, utilised the lowest accepted $BMDL_{10}$ values in order to be cautious. Thus, the $BMDL_{10}$ of 0.07 mg/kg bw/day and 0.34 mg/kg bw/day for BaP and PAH4 were selected as markers for the carcinogenic PAHs in food and were applied to the risk characterisation, respectively. These values correspond to levels at which the risk of various cancers in the population increases by 10% in the long term compared to a population not exposed to PAHs (EFSA 2008). In addition to the dose-response relationship, possible species differences can be identified, the mode of action can be characterised and the extrapolation from experimental animals to humans and from high to low dose can be calculated (Dybing et al. 2002).

Third, the exposure assessment is defined in the procedural manual of the Codex Alimentarius Commission (CAC) as "the qualitative and/or quantitative evaluation of the likely intake of biological, chemical and physical agents via food as well as exposures from other sources if relevant" (FAO/WHO 2009). Regarding food chemicals, dietary exposure assessment combines the data on the concentrations of chemicals in specific foods and the food consumption recorded in dietary surveys. Depending on the study design, and therefore on the food groups selected for the study, the foods contributing most to PAH exposure may vary (Table 6). According to EFSA, the highest contributors to the dietary exposure for consumers in EU Member States are cereals and cereal products as well as seafood and seafood products (EFSA 2008). A previous Finnish study of PAHs and their exposure from foods to humans covered 19 PAHs in several matrices. The

exposure to 19 PAHs for the Finnish population was up to 8800 ng/day, whereas the exposure to carcinogenic PAHs (BaA, benzo[b,k,j]fluoranthene, BaP, CHR, indeno[1,2,3-cd]pyrene and dibenz[a,h]anthracene) was 600 ng/day. Individual food products such as talkkuna, i.e. powdered mixture of dried oats, barley, and peas, contained a very high BaP level of 265 μg/kg, but the actual consumption of this product is negligible among the population. However, due to the different combinations of PAHs and incomplete separation of the critical pairs, the results of this study cannot be compared (Hietaniemi et al. 1999).

Among the population, the nutrition of adults and children differs from each other. Compared to adults, children have lower body weight but higher food consumption with relation to their body weight, which is required for growth and development (Rey-Salgueiro et al. 2009; Domingo and Nadal 2015). In addition, children have a high capacity to absorb nutrients, but a reduced ability of detoxification and they are thus more susceptible to exposure (Santonicola et al. 2017). Generally, dietary exposure estimates are provided for mean/median and high consumers in relevant population subgroups like the elderly, adults or children. The obtained estimates should be highly protective of health and performed using suitable statistical methods. An overview of the total dietary exposures to BaP and PAH4 (ng/kg bw/day) for different subgroups in selected studies are shown in Table 7. Those studies containing only one category of food, such as meat products, were omitted. Up to now, only a few assessments for children have been carried out showing variable dietary exposures to BaP and PAH4. Therefore, it is necessary to carry out national exposure assessments.

As the final step, risk characterisation integrates the information from the hazard characterisation and the exposure assessment to provide an estimate of the potential risk to human health. Concerning genotoxic and carcinogenic compounds, communicating on the risks to human health is a complicated task, as there is no threshold dose. Therefore, the ALARA principle has been applied to PAHs among risk assessors. The weakness of this approach lies in the fact that it does not take into account the potency and efficacy of the compound, the actual concentrations detected in foods, and risk comparisons between other compounds cannot be made to target risk management actions. Instead, the MOE approach is recommended for safety evaluations of genotoxic and carcinogenic substances in food. This approach is defined as a ratio between the reference value (e.g. $BMDL_{10}$) and the estimated dietary exposure (EFSA 2005, 2008). A few elements are worthy of consideration when interpreting the MOE: interspecies and intraspecies differences (human variability), the character of the carcinogenic process and the reference point. In general, the MOE of 10,000 or higher based on a $BMDL_{10}$ is of low concern from a public health standpoint (EFSA 2005, 2008). A list of relevant studies using the MOE approach is shown in Table 8.

Table 6. Major contributors to the BaP and PAH4 exposure in selected studies.

Food group	**Type of study**	**Population subgroup**	**Country**	**BaP exposure**	**PAH4 exposure**	**Reference**
Cereals	EFSA	Consumer	European Union	67 ng/day	260 ng/day	(EFSA 2008)
Seafood	EFSA	Consumer	European Union	36 ng/day	290 ng/day	
Egg-based products	Survey	Children	Italy	70 ng/day	280 ng/day	(Cirillo et al. 2010)
Fats and oils	Market basket study	Woman	Spain	13 ng/day	-	(Martorell et al. 2010)
Meat and meat products	Market basket study	Man	Spain	19 ng/day		
Meat and meat products	Market basket study	Girl	Spain	16 ng/day	-	
	Market basket study	Boy	Spain	17 ng/day	-	
Oils	Total diet study	Adult	France	-	0.2 ng/kg bw/day	(Veyrand et al. 2013)
	Total diet study	Children	France	-	0.3 ng/kg bw/day	
Sugar and sweets	Market basket study	Consumer	Sweden	0.2 ng/kg bw/day	1.0 ng/kg bw/day	(Abramsson-Zetterberg et al. 2014)

Table 7. Total dietary exposures to BaP and PAH4 (ng/kg bw/day) for different population subgroups in selected studies.

	Mean exposure (ng/kg bw/day)								
Subgroup	Consumer		Adult		Consumer	Children			
Country	European Union	Finland	Spain	France	Sweden	United Kingdom	Spain	Italy	France
	Median	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
BaP	3.9	3.1	1.0	0.2	0.7	3.3	3.0	6.0	0.3
PAH4	20	16	-	1.5	3.9	-	-	47	2.3
	High exposure (ng/kg bw/day)								
	Median	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
BaP	6.5	3.9	-	0.4	-	5.0	-	39	0.6
PAH4	35	19	-	3.0	-	-	-	340	4.7
Reference	(EFSA 2008)	(EFSA 2008)	(Martorell et al. 2010)	(Veyrand et al. 2013)	(Abramsson-Zetterberg et al. 2014)	(COT 2002)	(Martorell et al. 2010)	(Cirillo et al. 2010)	(Veyrand et al. 2013)

Table 8. Margins of exposure for the mean/median and high population subgroups in selected studies.

Country	**Subgroup, mean level**	**Mean MOE, BaP**	**Mean MOE, PAH4**	**Reference**
European Union	Consumer	17,900 (median)	17,500 (median)	(EFSA 2008)
France	Adult	-	230,000	(Veyrand et al. 2013)
Italy	Children	15,474 (median)	12,128 (median)	(Cirillo et al. 2010)
France	Children	-	150,000	(Veyrand et al. 2013)
Country	**Subgroup, high population**	**Mean MOE, BaP**	**Mean MOE, PAH4**	**Reference**
European Union	Consumer	10,800 (median)	9,900 (median)	(EFSA 2008)
France	Adult	-	113,000	(Veyrand et al. 2013)
Italy	Children	1,812 (median)	1,012 (median)	(Cirillo et al. 2010)
France	Children	-	72,000	(Veyrand et al. 2013)

3 AIMS OF THE STUDY

The principal aim of this dissertation study was to investigate whether dietary exposure to PAH4 poses a risk for children's health in Finland. Our results provide FBOs, policymakers and consumers valuable data on PAH4 levels in various foods and their impacts on children's possible exposure. By way of this information, risk management can be targeted in the industry appropriately. In order to reduce dietary exposure, the smoking factors affecting the PAH4 formation in foods were examined. The purpose was to provide tools and guidance for manufacturers on how to control and mitigate PAHs during the smoking process. Furthermore, our aim was to acquire more information and hence a better understanding of the potential mutagenicity of the processed foods on the market.

The task was accomplished by:

1) validating a GC-MS/MS method for determining PAH4 occurrence in specified foods (I, III)

2) examining the influence of the critical smoking factors on PAH4 formation in smoked fish and meat products (I)

3) investigating whether the selected processed foods show mutagenic activity via the Ames test and comparing the outcome with the corresponding chemical PAH4 data (II)

4) performing a scientific risk assessment of Finnish children's dietary exposure to PAH4 by combining the data on PAH4 occurrence, food consumption and exposure assessment (I, III)

4 MATERIALS AND METHODS

4.1 Chemicals and reagents

4.1.1 Chemical analysis

Dichloromethane, hexane, acetone, cyclohexane, methanol, and toluene were either HPLC or ultra resi-analysed grade by J.T. Baker (Deventer, Netherlands). Ethyl acetate (HPLC grade) was provided by VWR Chemicals (Fontenay-sous-Bois, France) and ethanol of Aa grade (≥99.5%) by Altia Oyj (Rajamäki, Finland). For accelerated solvent extraction (ASE 200®, Dionex Corporation), utilised Celite® 545 and florisil (100–200 mesh) were acquired from Merck (Darmstadt, Germany) and from Sigma-Aldrich (Steinheim, Germany), respectively. SPE cartridges (Supelclean ENVI-Chrom P, 6 mL, 500 mg) comprising styrene/divinyl benzene co-polymer were obtained from Supelco (Bellefonte, PA, USA).

In Study I, the employed analytical standard mixture (PAH-Mix 183, 10 µg/ml) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), whereas in Studies II-III, the standard mixture (15+1 EU PAH Cocktail, 100 µg/ml) was provided by Chiron AS (Trondheim, Norway). Moreover, the isotope-labelled internal PAH standards ($^{13}C_6$ BaA, $^{13}C_6$ CHR, $^{13}C_6$ BbF, $^{13}C_4$ BaP), 100 µg/ml each, were sourced from LGC Standards GmbH (Wesel, Germany). The stock solutions of individual ^{13}C PAHs were combined in toluene to form a mixture. The stock standard mixtures were diluted to provide appropriate working solutions. All stock and working standard solutions were maintained at +4 °C and protected from the light.

4.1.2 Mutagenicity testing

In Study II, all chemicals provided were of analytical grade. Dimethyl sulfoxide (DMSO), 2-aminoanthracene and sodium azide were from Sigma–Aldrich (Steinheim, Germany). Aroclor-induced S9 from rat liver was obtained from Trinova Biochem (Giessen, Germany). *Salmonella enterica sv. Typhimurium* strains TA 100 and TA 98 were purchased from Pasteur's Institute (Paris Cedex, France).

4.2 Samples

All samples analysed within this thesis are described in Table 9. As part of the National Food Control Program (EVO), a national project on PAHs in smoked fish and meat products was implemented and the samples were collected by the municipal food control authorities and the inspection veterinarians of the Finnish Food Safety Authority (Evira). The sampling from smokehouses and grocery stores having their own smoking chamber complied with the Commission Regulation (EC) No 333/2007 with amendments and followed a risk-based sampling plan (EC 2007). Therefore, only so-called worst-case samples were collected that represent traditionally smoked fish and meat products, which would most likely contain PAHs. The smoking process varied between the smokehouses, as the applied smoking equipment are mainly self-made with differences in factors such as the length of the smoking tube as well the model of the filter. The in-depth information on the smoking procedure was collected by the authorities whenever possible. However, some information was lacking or unclear (as it did not refer to that particular sample) and had to be omitted.

Food control authorities took rapeseed oil and butter products as official control samples from grocery stores in compliance with the EU legislation (EC 2007). In addition to official control samples, monitoring samples were taken for mutagenicity studies and to supplement the risk assessment. The processed fish and meat products were collected from a grocery store. Samples were taken from two or three different batches for each product and analysed separately. Other samples comprising bread, cereal, muesli, fluid vegetable oil, margarine and vegetable fat spread were purchased from three grocery stores. Two different lots of each product were taken and combined in the laboratory. The same samples were utilised both for validation studies and thereafter for sample analyses.

Table 9. The foods analysed within studies I-III.

Food group	Food product	Number of samples (n)	Year	Purpose of use*	Study
Smoked fish	Salmon, rainbow trout, whitefish, Baltic herring, vendace, mackerel, roach, canned fish (vendace, roach)	80	2012	a,c	Study I
Smoked meat	Pork, salami, sausage, reindeer, turkey, horse	62	2012	a,c	Study I
Processed fish	Baltic herring	3	2014	b	Study II
Processed meat	Chicken, turkey, salami, beef, ham	17	2014	b	Study II
Fat and oil	Rapeseed oil, fluid vegetable oil, butter, margarine, vegetable fat spread	17	2016	c	Study III
Bread	Crispbread, rye crispbread, multigrain bread and roll, wheat bread, potato flatbread	20	2016	c	Study III
Cereal and muesli	Corn flakes, wheat flakes, oat flakes, Rice Krispies, muesli with yoghurt and dried berries, muesli with dried fruits, cereal muesli	10	2016	c	Study III
Total		**209**			

*a=national EVO project, b=mutagenicity study, c=risk assessment study

4.3 Sample preparation and detection of PAH4

All samples were stored in sales packages or packed in aluminium foil and depending on the product, stored either at +5 °C or at room temperature protected from the light until preparation. Only the edible parts were processed further due to the fact that MLs apply for them. If the fish or meat skin was not edible (information provided by the manufacturer), the skin was removed. The samples were homogenised in a food mixer, followed by freeze-drying of the solid samples, whereas oils and fats were ready for further sample preparation steps according to the PAH method utilised. The canned fish samples were analysed by dividing them into fish and oil parts and analysing both separately. Processed fish and meat products were prepared in replicate samples, with one sample prepared according to the method and the other conducted for the Ames test without the addition of ISTD. The quantification of PAH4 analytes was conducted by a GC-MS/MS method, which is accredited in accordance with ISO 17025 (ISO 2005).

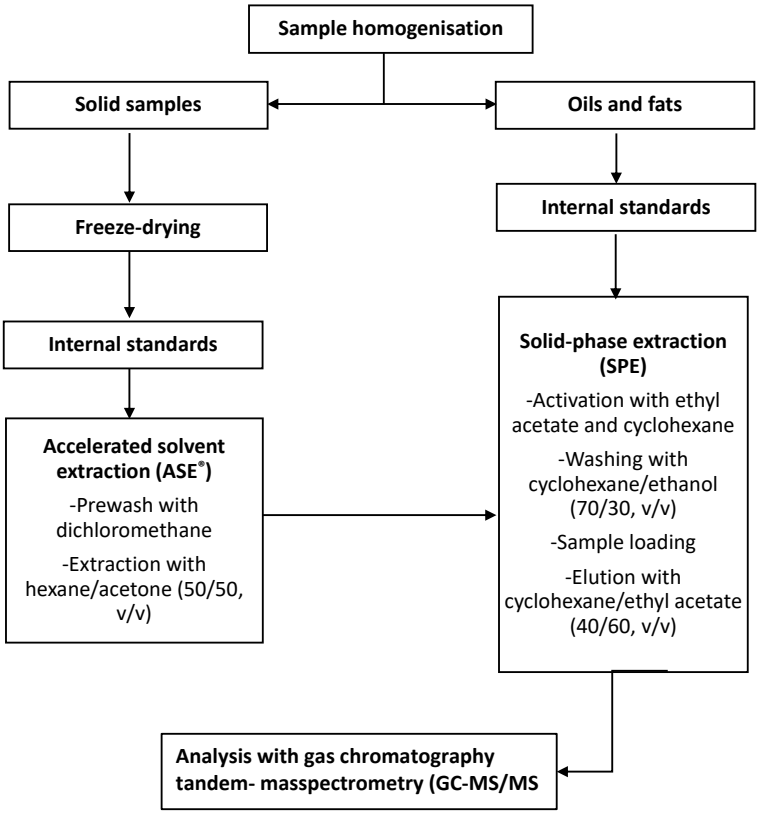


Figure 5. A flowchart of the PAH method.

It was based on the method by Veyrand et al. (2007) with minor modifications such as the ASE® extraction system, the analytical column, GC and MS conditions. This method was used for the PAH4 quantification of each sample type present in our study. A flowchart of the method is described in Figure 5.

Considering the wide concentration range at which PAHs are present in samples, calibration curves were prepared using the standard solutions with known concentrations of the analytes. Altogether, six to eight calibration points were prepared to obtain the final concentrations of native PAH4 in a range from 0 to 0.8 µg/ml in toluene depending on the working range of the matrix. Internal quality control comprised the isotope-labelled analogues, which were used in each calibration standard at a level of 0.017 µg/ml of each individual PAH4. In addition, each batch of samples included calibration check standards, spiked samples documented in the control chart. Purified sample extract, along with the calibration standards and quality control samples, were injected into the GC injector and PAH4 compounds were quantified by monitoring two specific

transitions in the multiple reaction monitoring (MRM) mode. MS identifies compounds by the mass of the analyte molecule. The instrumental analysis of PAH4 was conducted using a gas chromatograph (Agilent, 6890N) equipped with a triple quadrupole analyser (Waters, Micromass Quattro Micro® GC). Details of the GC and MS conditions are listed in Table 10. The data processing was performed using MassLynx® V4.1 software supplied by Waters (Manchester, United Kingdom) and Microsoft Excel® 2010 (Microsoft Co., Redmond, WA, USA). Lower bound concentrations were calculated based on the assumption that all the values of the PAH4 compounds below the LOQ are zero (EC 2011a).

Table 10. Gas chromatograph and mass spectrometer conditions.

Column Dimensions	Agilent J&W Select PAH 30 m x 0.25 mm x 0,15 µm
Injector	300 °C, splitless mode
Injection volume	1 µl
Carrier gas and flow rate	Helium, 1.0 ml/min
Oven program	110 °C, 0.7 min 85 °C/min→180 °C 3 °C/min→230 °C, 7 min 28 °C/min→280 °C, 15 min 14 °C/min→350 °C, 5 min
Detector	Electron ionisation 70 eV, ion source 275 °C, transfer line 300 °C
Collision gas	Argon
Analyte and precursor ion (m/z)	**Product ion (m/z) Quantifier ions underlined**
Benz[a]anthracene 228.2	202.2, <u>226.2</u>
ISTD benz[a]anthracene $^{13}C_6$ 234.0	<u>232.2</u>
Chrysene 228.2	202.1, <u>226.1</u>
ISTD chrysene $^{13}C_6$ 234.0	<u>254.1</u>
Benzo[b]fluoranthene 252.3	226.0, <u>250.1</u>
ISTD benzo[b]fluoranthene $^{13}C_6$ 258.0	<u>256.2</u>
Benzo[a]pyrene 252.3	226.2, <u>250.1</u>
ISTD benzo[a]pyrene $^{13}C_4$ 256.0	<u>254.1</u>

4.4 Method validation

A large range of food matrices (fish, meat, vegetable oil, fat, bread, cereal and muesli) were validated prior to sample analyses according to the parameters demonstrated in Table 11. For practical reasons, the common MU was calculated for vegetable oils, butter and margarine based on the validation and proficiency test (PT) data. In some cases, suitable blank samples were lacking, and thus low contaminated samples (pseudo-blanks) were also used to prepare spiked samples. In-house validations were performed from one to three different concentration levels, representing either 0.5 ML, 1.0 ML, 1.5 ML or another relevant level if MLs do not exist for a given matrix. Six replicate samples per day were performed at selected concentrations within two to three different days. Additionally, at least ten blank samples or pseudo-blanks were analysed. Matrix-matched calibration curves in each matrix were prepared alongside the solvent calibration curves.

Table 11. Validation procedure (EC 2002a; NMKL 2009).

Validation parameter	Testing protocol
Ruggedness	• The degree of reproducibility of the results were tested under a variety of conditions such as different analysts, different lots of reagents, different days or different assay.
Specificity	• Specificity of the method was performed by testing blank samples for possible interferences with the analyte from other compounds. • Retention times of an analyte in a sample and in a standard solution were compared. • Ion ratio between the quantifier and qualifier ion for each PAH4 in a matrix-matched calibration curve was calculated and the maximum permitted tolerance range of quantifier/qualifier ion ratios were defined. • Spiked samples were further tested to fulfil the defined tolerance range, which was specific to each analyte and matrix.
Selectivity	• Solvent calibration curves were analysed in parallel with the matrix-matched calibration curve. The applied concentrations were dependent on the working range of the matrix, but at least six calibration points were used. • A t-test was employed to compare the coefficient slope of the solvent calibration curve and the matrix-matched calibration curve. The matrix effect occurs in cases where $p<0.05$.
Linearity	• Standard calibration curves were prepared on three different days comprising at least six calibration points between zero and 0.8 µg/ml. The residuals were calculated using the MassLynx program.
Repeatability	• Spiked samples were analysed from one to three different concentration levels (0,5 ML, 1,0 ML and 1,5 ML) on two or three different days, having six replicate samples for each day at every level.

Table 11. Continued.

Validation parameter	Testing protocol
Within-laboratory reproducibility	• The samples from repeatability analyses were used to calculate the within-laboratory reproducibility between different days.
Recovery	• The samples from repeatability analyses were used. The recovery was calculated using individual ISTDs in both calibration and validation samples.
Limit of detection	• LOD= LOQ/3
Limit of quantification	• The lowest quantitation point within the calibration curve, six to ten repeated determinations.
Trueness	• Participation in PTs or certified reference material (CRM) when available. Otherwise, recovery results were used.
Measurement uncertainty	• Standard deviation from within-laboratory reproducibility combined with the bias obtained from the PT. The expanded MU was measured by multiplying the combined MU with a coverage factor of two, which yields a level of confidence of approximately 95% (EC 2007).

4.5 Smoking parameters

The effect of 1) the smoking technique, 2) the smoking time, 3) the optimisation of the smoke generation temperature and 4) the distance between the food and the smoke source on formed BaP and PAH4 levels in traditionally smoked fish and meat products were examined based on the in-depth information on the smoking procedure. Furthermore, in order to eliminate the potential interferences, such as the effect of the other fish species and the fish skin, smoked salmon fillets (n=57) were evaluated separately from the other smoked fish samples. Due to the targeted sampling, only traditional smoking techniques (direct and indirect techniques) were examined, thus excluding liquid smoking. The impact of the direct and indirect smoking technique was investigated dividing the results based on the applied technique. Regarding the smoking time, BaP and PAH4 results were grouped according to whether the smoking was performed in less than or more than five hours. To study the effect of the smoking time further, salmon fillets were divided into cold- and warm-smoked salmon fillets. Moreover, small and larger fish species were separated with regard to their smoking time. In optimisation of the smoke generation temperature, the smoke generation temperature between 400 and 600 °C was considered optimised in our study. By contrast, temperatures outside the defined range were treated as non-optimised. The fourth parameter,

the distance between the smoke source and the food product, was counted either less or more than five metres.

4.6 The Ames test

The mutagenic potential of the smoked meat and fish products was tested by the Ames test. The assay followed the procedure by Maron and Ames (1983), which employed *Salmonella* strains TA 100 and TA 98 with and without metabolic activation (S9 mix). A flowchart of the procedure and principle of the Ames test are displayed in Figure 6. The selected negative controls for TA 100 and TA 98 were water and DMSO, whereas sodium azide (0.04 mg/ml) and 2-aminoanthracene (0.02 mg/ml) served as positive controls for TA 100 and TA 98, respectively. Triplicate determinations (50 µl/plate) were performed at four concentration levels (25, 50, 100 and 200 mg/ml) of the meat and fish products in each experiment, with the highest concentration (200 mg/ml) corresponding to one gram of the food sample. The incubation time for plates was 48 h at 37 °C. The number of spontaneously induced revertant colonies per plate is relatively constant, whereas a possible addition of mutagen to the plate will increase the amount of revertant colonies, normally in a dose-related manner. It should be emphasised that the food samples were considered mutagenic only if the sample extract at the highest concentration level produced at least twice as many revertants as the negative control (DMSO or water).

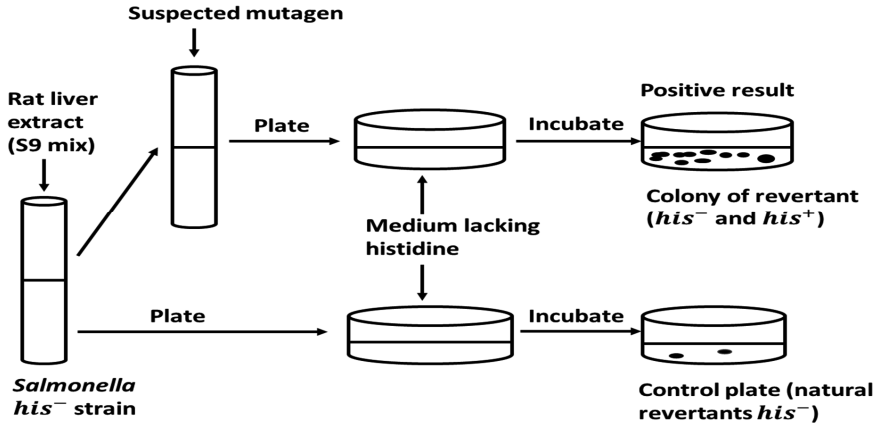


Figure 6. A flowchart depicting the steps involved in the Ames test.

4.7 Statistical analyses

In order to visualise and obtain information on the variability of the data sets, a statistical analysis was conducted by IBM SPSS® Statistics, version 25 (SPSS Inc., Chicago, Illinois). Boxplots were displayed as a five-number summary of the data set: minimum, first quartile, median, third quartile and maximum. The mutagenicity of the studied food products was examined from the linear slope of the dose-response curve. Software program Prisma 4.0 (GraphPad Software Inc., San Diego, CA, USA) was applied to perform a linear regression analysis.

4.8 The methodology of the exposure estimation

The food consumption data of a cross-sectional Increased Health and Wellbeing in Preschools study (DAGIS) were used in our exposure estimations. The DAGIS research project was implemented in cooperation with the Folkhälsan and the University of Helsinki, where 864 children (3-6 years of age) represented 66 Finnish preschools in eight different municipalities in Southern Finland and South Ostrobothnia. The food consumption data (n=815) including two food diary periods and food records were completed between years 2015 and 2016. Not all children were involved in both periods, and therefore the number of children recorded in consumption data was lower than the original turnout. Data were collected through questionnaires to which both parents and personnel responded. Finally, each food record was checked and entered into Aivo Diet software at the University of Helsinki utilising the Fineli Food Composition Database (THL 2019).

Alongside the concentration data of PAH4 from the analysed food products, the data were collected from a peer-reviewed journal (Ciecierska and Obiedziński 2013) as well as from an official report of the Swedish national food agency (Wretling et al. 2013). These literature data are illustrated in Table 12. The data of the exposure estimation was based on the food record data from 815 children as well as PAH4 concentration data from 271 samples (consisting of the analysed data from studies I and III and the literature data). Four non-compliant smoked meat samples were excluded from the exposure estimates because these products were withdrawn from the market and would have distorted the exposure estimation. In addition, several food groups (e.g. dairy products, vegetables, fruits, chocolate and cocoa) were not involved in our estimations. These exclusions were made either due to lack of published PAH4 data or challenges estimating the proportion of chocolate and cocoa in products.

The Bayesian statistical model was used to evaluate the PAH4 exposures from studied foods, taking into account individual variation in food consumption.

Children's exposure assessment was performed by applying a gamma distribution to model the BaP concentration in each food group. However, in some food groups, the majority of the BaP levels were below the LOD. Therefore, the BaP results below the LOD or between the LOD and the LOQ were added in the statistical model as censored observations. In other words, all observations followed the same probability distribution. The concentration of the PAH4 was estimated as a gamma distribution for the sum of PAH4 compounds. The middle bound was employed in the case where one or more of the individual PAH4 compounds was below the LOD or between the LOD and the LOQ. The long-term mean daily serving of each food group was modelled as a hierarchical log-normal model considering both the within-individual and between-individual variation and the consumption frequency applying the binomial-logit model with random effects for individuals. For the individual parameters (mean daily serving and frequency) of the Bayesian model, multinormal prior distributions were set to consider the correlation in consumption between different food groups. Lastly, a chronic exposure of BaP and PAH4 resulting from consumption of each food product was reported as a combination of the mean concentration and long-term consumption. The resulting posterior distribution of the Bayesian model depicts the variation and uncertainty in the chronic exposure and other quantities of interest. In the risk characterisation, the $BMDL_{10}$ of 0.07 mg/kg bw/day and 0.34 mg/kg bw/day were applied to BaP and PAH4, respectively. Finally, the safety margin of MOE was calculated according to an equation $MOE=BMDL_{10}/exposure$ (EFSA 2008).

Table 12. Concentrations of selected food groups (µg/kg) from the literature for use in the assessment of dietary exposure.

Food group	**Number of samples (n)**	**Mean concentration (variation range) µg/kg**		**Reference**
		BaP	**PAH4**	
Fat and oil	50	0.19 (0.03–1.41)	1.21 (0.19–6.82)	(Wretling et al. 2013)
Grilled meat	15	0.48 (0.03–3.29)	3.41 (0.34–18.9)	(Wretling et al. 2013)
Grilled sausage	11	0.21 (0.05–0.67)	1.35 (0.35–3.80)	(Wretling et al. 2013)
Bread	6	0	0.36 (0.26–0.59)	(Ciecierska and Obiedziński 2013)
Total	**82**			

5 RESULTS

5.1 Quantitative determination of PAH4 in selected foods

5.1.1 Method performance

The performance of the PAH4 method using GC-MS/MS was evaluated by means of the selected validation parameters. The results varied slightly between different matrices such as recoveries from 76% to 120%. Furthermore, z-scores indicating the trueness were between -1.5 and 2.9. An overview of the validation results is shown in Table 13.

Table 13. A summary of the validation results for the PAH4 compounds in studied food matrices.

Validation parameter	Fish*	Meat*	Vegetable oil*	Butter and margarine	Bread	Cereal and muesli
Validation level (µg/kg)	2.5, 5.0 and 7.5	2.5, 5.0 and 7.5	1.0, 2.0 and 3.0	2.0	0.9 and 2.5	0.9 and 2.5
Ruggedness	Validations were performed by different analysts and on different days					
Specificity	The mean ion ratio of the validation samples was acceptable within the specific tolerance range calculated in each matrix for PAH4 compounds. The retention time of the analyte in a standard solution corresponded to that in a sample within tolerance ±2.5%.					
Selectivity (*p*-value)	0.3–1.0	0.2–0.6	0.5–0.7	0.2–0.5	0.2–0.4	0.3–0.8
Linearity	Residuals mostly within ±20% and all residuals within ±30%					
Repeatability (%RSD_r)	1.7–19	0.8–17	4.4–23	3.4–14	5.8–33	9.8–24
$HorRat_r$ (repeatability)	0.4–0.7	0.2–0.5	0.2–0.9	0.2–0.5	0.3–0.6	0.3–0.4
Within-laboratory reproducibility (%RSD_R)	6.9–16	5.0–13	6.3–24	5.6–14	13–28	12–18
$HorRat_R$ (within- laboratory reproducibility)	0.2–0.6	0.2–0.6	0.4–0.9	0.3–0.8	0.1–0.7	0.1–0.6
Recovery (%), ISTD included	95–115	100–114	88–103	95–120	76–104	86–114
Limit of detection (µg/kg)	0.3	0.3	0.3	0.3	0.3	0.3
Limit of quantification (µg/kg)	0.8	0.8	0.8	0.8	0.9	0.9
Trueness (z-score)	-1.2–0.6 (2009, 2014, 2015, 2020)	-1.0–2.0 (2011, 2013, 2014)	-1.5–2.9 (2009, 2010, 2012, 2013, 2017)	-	-	-
Measurement uncertainty (%) at specified spiking level	5.0 µg/kg		2.0 µg/kg		2.5 µg/kg	
	16–27, PAH4 sum 44	10–22, PAH4 sum 38	13–48, PAH4 sum 63		17–28, PAH4 sum 41	

*Accredited according to ISO 17025 (ISO 2005)

5.1.2 Detection of PAH4 in foods

In general, mean and median BaP and PAH4 lower-bound concentrations were comparatively low and below the LOD in fats and oils, bread, cereal and muesli, whereas in fish and meat products, the mean PAH4 levels were higher (Table 14). Particularly, median values proved to be zero in other foods except PAH4 in fish. By contrast to these low median values, fish and meat results showed a large variation in concentrations up to 40 µg/kg and 200 µg/kg of BaP and PAH4, respectively. Furthermore, the division between small and larger fish species indicated small fish having clearly higher median PAH4 content. All small fish products were analysed with the skin, whereas the large fish representing fillets, pieces or whole fish were determined with or without the skin. It was noteworthy that pork samples contained elevated BaP and PAH4 levels compared to other meat products.

Table 14. Lower bound concentrations (µg/kg) for BaP and PAH4 in foods.

Food group	Mean (µg/kg)		Median (µg/kg)		Standard deviation (µg/kg)		Relative standard deviation (%)		Maximum (µg/kg)	
	BaP	PAH4	BaP	PAH4	BaP	PAH4	BaP	PAH4	BaP	PAH4
Fish (n=83)	0.6	4.6	0	0.9	1.4	7.7	220	170	8.2	44
Meat (n=79)	1.6	8.9	0	0	1.3	27	83	300	40	200
Fat and oil (n=17)	0	0.2	0	0	0	0.4	-	190	0	1.1
Bread (n=20)	0.1	0.1	0	0	0.3	0.3	447	447	1.4	1.4
Cereal and muesli (n=10)	0	0	0	0	0	0	-	-	0	0

n=number of samples

5.2 Impact of the smoking factors on the PAH4 levels

Traditionally smoked fish and meat samples were utilised to investigate the link between the smoking factors and the formed PAH4 levels (Table 15). The comparison between direct and indirect techniques in smoked fish and meat samples demonstrated that the concentrations of BaP and PAH4 were higher in those samples processed using the direct technique. A clear difference was observed in meat samples, where direct smoking produced remarkably higher BaP and PAH4 levels compared to indirect smoking. Moreover, direct smoking was applied in all four meat products, which were found to be noncompliant with the prevailing regulation.

In addition to smoking technique, smoking time was considered another critical factor. It was hypothesised that the longer the smoking time, the more

PAHs will be formed, which proved to be true in smoked meat products. However, the shorter smoking time (<5 h) produced higher BaP and PAH4 concentrations in fish products. This was especially the case with the PAH4, leading to in-depth investigations. Smoked salmon fillet products accounted for more than 70% of smoked fish samples, which were mostly warm smoked with a smoking time of less than five hours. Compared to cold-smoked fillets, warm smoking resulted in higher BaP and PAH4 levels. Moreover, small fish species contained higher PAH4 levels than larger fish and all of them were smoked within five hours. The other studied factors demonstrated clearly that the non-optimised smoke generation temperature and the shorter distance (<5 metres) between the food and the smoke source produced higher BaP and PAH4 levels in both fish and meat samples.

Table 15. Selected smoking factors and their effects on the formed BaP and PAH4 levels (µg/kg).

Food group	**Smoking technique**		**Smoking time**		**Optimisation of the smoke generation temperature (400-600 °C)**		**Smoking distance**	
Fish	BaP	PAH4	BaP	PAH4	BaP	PAH4	BaP	PAH4
	Direct (n=35)		<5 hours (n=54)		Optimised (n=18)		<5 metres (n=60)	
Mean (µg/kg)	0.6	4.6	0.6	4.6	0.1	1.0	0.5	4.2
Median (µg/kg)	0	2.5	0	1.1	0	0	0	0.9
Standard deviation (µg/kg)	1.1	6.5	1.2	7.0	0.3	2.1	1.1	6.7
	Indirect (n=45)		>5 hours (n=18)		Non-optimised (n=17)		>5 metres (n=9)	
Mean (µg/kg)	0.4	3.3	0.1	1.3	0.8	6.9	0.5	4.2
Median (µg/kg)	0	0.3	0	0	0.3	4.6	0	0.9
Standard deviation (µg/kg)	1.0	6.0	0.5	3.2	1.0	6.9	1.1	6.7
Meat	BaP	PAH4	BaP	PAH4	BaP	PAH4	BaP	PAH4
	Direct (n=23)		<5 hours (n=22)		Optimised (n=15)		<5 metres (n=38)	
Mean (µg/kg)	5.3	29	0.4	3.4	0.5	4.7	2.8	16
Median (µg/kg)	1.6	13	0	0	0	0.8	0	3.1
Standard deviation (µg/kg)	10	45	0.9	7.7	1.2	9.4	8.0	37
	Indirect (n=39)		>5 hours (n=22)		Non-optimised (n=11)		>5 metres (n=5)	
Mean (µg/kg)	0.1	1.0	4.3	25	4.8	26	0	0.2
Median (µg/kg)	0	0	0	7.8	0	2	0	0
Standard deviation (µg/kg)	0.4	1.9	10	47	12	59	0	0.4

5.3 Screening of the mutagenicity of processed foods

Mutagenic activity of the selected foods was tested by the Ames test and the detailed results are shown in Table 16. Based on the obtained results, no mutagenic potential was observed in any of the meat samples in either *Salmonella* TA 100 or TA 98 strains with or without the metabolic activation (S9 mix). The amount of the revertants produced in the meat extracts was less than two-fold the DMSO control value in all tested food products. In contrast, at least twice the number of revertants were observed in each of the three batches of hot smoked Baltic herring compared to the corresponding DMSO control in both *Salmonella* strains. The observed mutagenic responses in Baltic herring samples were statistically significant (p<0.05).

Table 16. The mutagenic activity on *Salmonella* TA 100 and TA 98 strains with or without metabolic activation (S9 mix) and the corresponding BaP and PAH4 concentrations (µg/kg). Water and dimethyl sulfoxide served as negative controls

Sample	Batch	Mutagenicity mean±SD (revt/g)				Concentration±MU (µg/kg)	
		+S9 (TA100)	-S9 (TA100)	+S9 (TA98)	-S9 (TA98)	BaP	PAH4
Water		151±2.3	74±10.4	24±0.2	15±1.4	-	-
DMSO		160±10.7	81±5.6	25±2.2	13±0.0	-	-
Smoked ham	1	201±9.1	128±10.5	34±1.5	21±0.8	<0.3	0
	2	174±12.5	106±9.8	32±0.8	19±2.2	<0.3	0
	3	189±7.9	115±8.4	37±4.1	15±0.8	<0.3	0
Honey-roasted chicken	1	247±11.0	98±8.6	25±2.1	20±1.0	<0.3	0
	2	198±15.0	124±15.2	41±6.4	19±4.8	<0.3	0
	3	258±9.3	104±9.4	34±4.0	19±2.1	<0.8	0
Grilled turkey	1	297±19.8	132±8.1	45±0.0	14±1.8	<0.3	1.6±0.6
	2	225±0.0	132±3.2	39±3.4	17±2.8	<0.3	0
	3	188±10.1	130±10.8	27±1.5	16±2.4	<0.3	0
Pepper salami	1	241±14.6	120±12.0	32±4.9	21±3.0	<0.3	0.9±0.3
	2	168±8.7	123±8.2	30±0.6	18±2.1	<0.3	0
	3	200±4.9	104±4.1	34±3.2	16±2.1	<0.3	0
Cold-smoked beef	1	209±5.1	132±0.0	39±5.0	14±1.5	<0.3	0
	2	188±8.7	124±12.4	28±0.0	20±4.2	<0.8	0
	3	188±0.0	108±8.4	29±2.1	15±3.1	<0.3	0
Sauna-smoked ham	1	268±11.2	140±12.4	42±1.2	13±0.0	<0.3	0
	2	158±3.8	132±14.5	28±1.2	15±2.4	<0.3	0
Hot-smoked Baltic herring	1	392±12.0*	201±16.2*	51±4.7*	40±5.4*	4.7±0.8	18±7.9
	2	478±41.23*	224±21.4*	64±4.9*	46±4.2*	8.2±1.5	44±19
	3	401±22.8*	214±18.0*	55±2.0*	40±4.8*	1.0±0.2	8.7±3.8

*The statistically significant mutagenic response (p<0.05). The number of revertants was at least twice as high as the revertants in DMSO control.

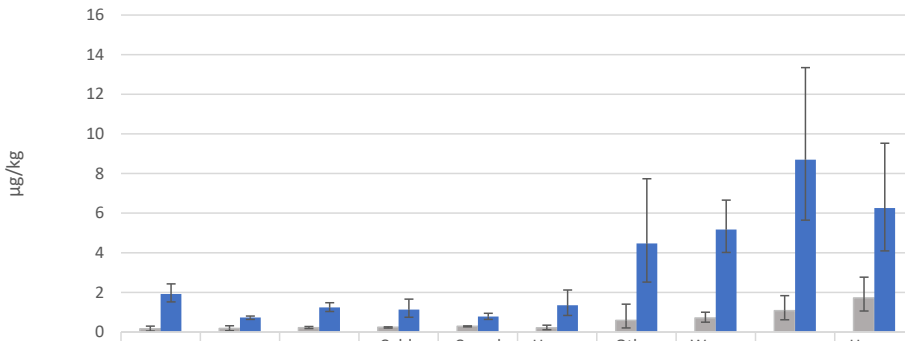
5.4 Comparison between the mutagenicity and chemical PAH4 results

Chemical analyses were conducted to compare whether the selected processed foods tested for mutagenicity contained PAH4 concentrations. The results in Table 16 demonstrated mutagenically inactive meat samples having generally low BaP and PAH4 levels below the LOD. Furthermore, all Baltic herring samples generating statistically significant mutagenic response also contained quantitative BaP and PAH4 levels, even though the concentrations between these lots varied.

5.5 Finnish children's dietary exposure to PAH4

5.5.1 Modelled PAH4 concentrations and children's consumption of PAH related foods

The obtained PAH4 concentrations from literature and analyses were modelled showing the highest estimated BaP and PAH4 levels in warm smoked fish, smoked ham and home-grilled meat (Figure 7). The lowest estimated BaP levels were obtained in bread and sausage, whereas the lowest PAH4 levels were in cereal and muesli as well as in bread. Considering the daily consumption of foods, bread was clearly the most consumed foodstuff (Figure 8). Although the consumption rate of fat and oil was the second highest, the consumption rate was considerably lower than that of bread. Among the high consumers (97.5th percentile), the bread consumption exceeded 5.0 g/kg bw/day while sausage and fat and oil were consumed approximately 2.0 g/kg bw/day.



	Sausage	Bread	Fat and oil	Cold-smoked fish	Cereal and muesli	Home-grilled sausage	Other smoked meat	Warm-smoked fish	Smoked ham	Home-grilled meat
BaP mean	0,16	0,18	0,22	0,24	0,29	0,21	0,59	0,71	1,08	1,72
PAH4 mean	1,92	0,73	1,24	1,13	0,78	1,35	4,47	5,17	8,7	6,26

Figure 7. The modelled mean and 95% credible interval for the mean BaP and PAH4 concentrations (µg/kg) in foodstuff. Credible interval refers to the uncertainty of the mean concentration.

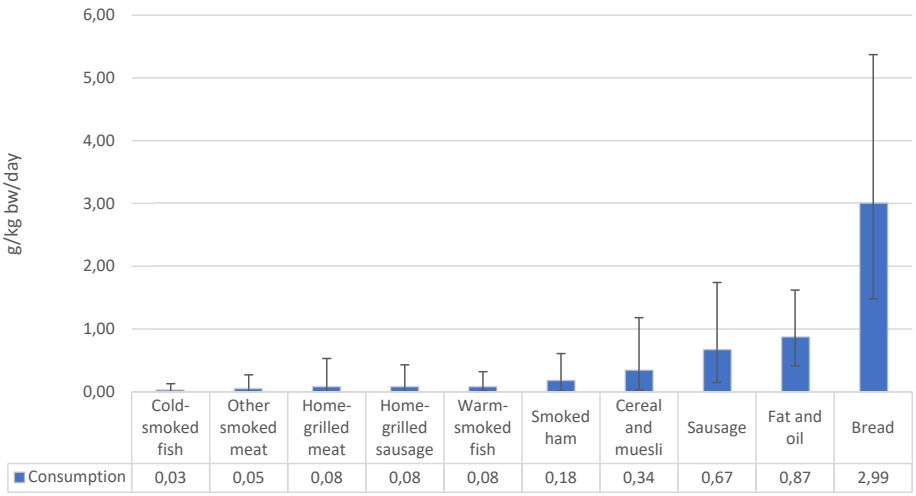
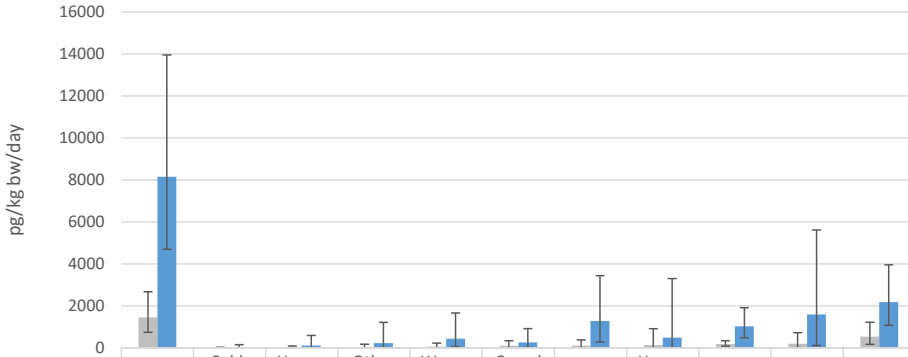


Figure 8. Modelled mean food consumption (g/kg bw/day) with 2.5th and 97.5th percentiles. *Consumption* refers to the mean use and its long-term variation between individuals (uncertainty included).

5.5.2 Children's total dietary exposure to PAH4

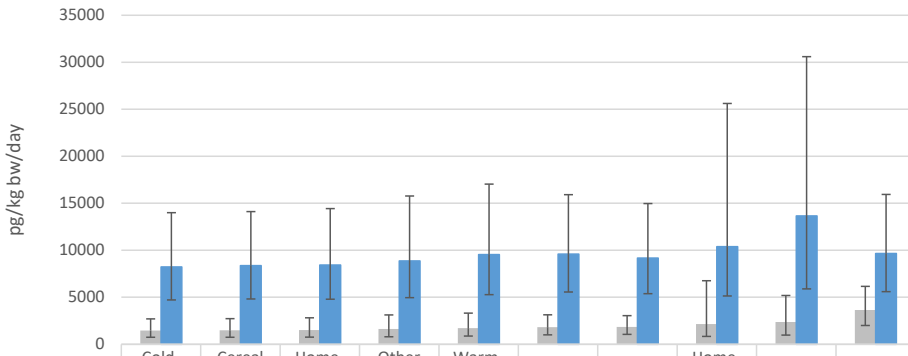
Estimates of the daily dietary exposures to children were calculated, based on the determined levels and literature data of PAH4 in daily food and the daily mean food consumption. The total mean dietary exposure was to BaP 1,500 pg/kg bw/day and to PAH4 8,100 pg/kg bw/day (Figure 9). Furthermore, the corresponding 97.5th percentiles of the total exposures to BaP and PAH4 were 2,700 pg/kg bw/day and 14,000 pg/kg bw/day, respectively. The major contributors to the BaP and PAH4 exposure were bread, smoked ham, fat and oils and sausage, whereas cold-smoked fish contributed the least to the total dietary exposure.

For highly exposed children, the total chronic exposures were estimated in a scenario where a modelled 97.5th percentile concentration was used for the specified foodstuff and the mean concentrations for the other foods (Figure 10). As a result, the highest total chronic exposure to BaP was estimated, when the 97.5th percentile concentration was applied for bread and the mean concentrations for other foods. Moreover, using the 97.5th percentile concentration for smoked ham, the highest total chronic exposure to PAH4 was assessed.



	Total	Cold-smoked fish	Home-grilled sausage	Other smoked meat	Warm-smoked fish	Cereal and muesli	Sausage	Home-grilled meat	Fat and oil	Smoked ham	Bread
BaP mean	1453	7	18	29	60	98	107	135	182	198	535
PAH4 mean	8145	33	114	226	438	262	1285	488	1023	1591	2180

Figure 9. Contributions of different foods to BaP and PAH4 exposure and the total dietary exposure (modelled mean, 2.5th and 97.5th percentile) expressed in pg/kg bw/day. *Credible interval* refers to the variation of the chronic exposure in Finnish children (uncertainty included).



	Cold-smoked fish	Cereal and muesli	Home-grilled sausage	Other smoked meat	Warm-smoked fish	Sausage	Fat and oil	Home-grilled meat	Smoked ham	Bread
BaP mean	1461	1477	1508	1622	1717	1814	1844	2140	2366	3644
PAH4 mean	8222	8361	8422	8862	9542	9587	9167	10378	13653	9655

Figure 10. The modelled mean, 2.5th and 97.5th percentile for the total chronic exposure to BaP and PAH4 grouped according to food groups (pg/kg bw/day). The 97.5th percentile concentration was used for the defined food item and the mean concentrations for the other foods.

5.5.3 Risk characterisation of children's exposure to PAH4

In a scenario where the mean concentration value for all foods was applied, the total MOE was considered to be the MOE of the total exposure and not the sum of the MOEs. As a result, the calculated total MOEs were 482,000 for BaP and 42,000 for PAH4 (Table 17). The safety margin of 10,000 was exceeded in each food

group and the lowest MOEs were estimated in bread. Another scenario for highly exposed children (97.5th percentile concentration) demonstrated lower MOEs in comparison to the scenario of the mean concentrations, in which all MOEs are still above 10,000 (Table 18). When the 97.5th percentile concentration was used for bread and the mean concentration was used for the other foods, the lowest MOE was observed.

Table 17. The margins of exposure to BaP and PAH4 by food groups in the scenario in which the mean concentration values for all foods were used. The MOE values were obtained using mean, 97.5th and 2.5th percentile exposures.

Food	**MOE (BaP), x1000**	**MOE (PAH4), x1000**
Bread	1 308 (573–4 142)	156 (86–317)
Smoked ham	3 535 (968–50 000)	214 (61–2 957)
Fat and oil	3 846 (2 059–8 046)	332 (178–698)
Home-grilled meat	5 185 (764–700 000)	697 (103–68 000)
Sausage	6 542 (1 828–70 000)	265 (99–1 206)
Cereal and muesli	7 143 (2 053–100 000)	1 298 (370–17 895)
Warm-smoked fish	11 667 (3 057–116 667)	776 (204–6 939)
Other smoked meat	24 138 (4 000–700 000)	1 504 (279–68 000)
Home-grilled sausage	38 889 (7 527–700 000)	2 982 (572–85 000)
Cold-smoked fish	100 000 (21 875–700 000)	10 303 (2 179–340 000)
Total	**482 (263–945)**	**42 (24–72)**

Table 18. The margins of exposure to BaP and PAH4 by food groups in the scenario, in which the 97.5th percentile concentration was used for the specified foodstuff and the mean concentration value for the other foods. The MOE values were obtained using mean, 97.5th and 2.5th percentile exposures.

Food	**MOE (BaP), x1000**	**MOE (PAH4), x1000**
Bread	192 (114–349)	25 (11–58)
Smoked ham	296 (135–719)	33 (13–66)
Home-grilled meat	327 (104–837)	35 (21–61)
Fat and oil	380 (230–662)	38 (22–69)
Sausage	386 (224–697)	35 (21–61)
Cereal and muesli	408 (211–805)	36 (20–64)
Warm-smoked fish	432 (224–879)	37 (23–63)
Other smoked meat	464 (249–923)	38 (22–69)
Home-grilled sausage	474 (257–932)	40 (24–71)
Cold-smoked fish	479 (259–943)	41 (24–71)

6 DISCUSSION

6.1 Evaluation of the method performance

High-quality analytical data is essential in order to provide a realistic picture of the PAH levels present in foods. The method performance values for ruggedness, specificity, selectivity, repeatability, within-laboratory reproducibility, recovery, LOD, LOQ, linearity and trueness acceptably met the legislative requirements and recommendations set for validation (EC 2002a, 2011b). Since the recovery was calculated by using ISTDs, it does not represent the recovery of the method as a yield. Instead, ISTDs compensate for any possible variation during the entire process of sample preparation and quantitative determination. Despite their use, the recoveries showed some variation and were mostly over 100%, particularly in the case of CHR. High recoveries may be explained by possible PAH contamination in florisil used as a stationary phase in a PLE system or somewhere else (Veyrand et al. 2007). Besides that, PAH isomers such as CHR and TPH as well as BbF and benzo[k,j]fluoranthenes can co-elute and cause an enhancement effect. Peak overlapping hampered the proper quantification in some cases and the condition of the analytical column played a major role in peak separation. Moreover, response variations in matrices could be due to ionisation source contamination with matrix compounds that were not retained in the column (Belo et al. 2017). Considering all the validated matrices, no matrix effect (p>0.05) was observed in any of them.

Whether the method is applicable for official control analyses, each PAH4 compound shall fulfil the criterion of the maximum expanded MU set in EU legislation (EC 2011b). Since the MU is a combination of random and systematic error comprising the results of within-laboratory reproducibility and trueness, both of them were assessed. Most of the calculated expanded MUs of the individual PAH4 were within this criterion. Only a few PT results in the past were questionable (z-score>±2), thereby affecting the expanded MU of fat and oil. However, the trueness was monitored in a number of PTs over the years with the satisfactory performance in various matrices, including fat and oil. Furthermore, based on the MU equation, an LOD of 0.3 µg/kg has a greater effect on the maximum expanded MU than the lower LOD value would have. Thus, it can be concluded that the method applied based on our validation results is suitable for PAH analyses in a wide range of food matrices.

Despite the high reliability of our method, an apparent limitation is the laborious and time-consuming sample preparation for all the studied foodstuffs except the oil and fat samples. Although the automated ASE® system was utilised for solid samples, the preparation of the ASE® cylinders took some time. Recently, Surma et al. (2018) succeeded in analysing high diversity of dried and smoked fruits

using the modified QuEChERS method and GC-MS. Their publication considers not only PAHs, but also 3-monochloropropane-1,2-diol (3-MCPD) and acrylamide. There are currently a few studies that employ the QuEChERS method for PAH analysis, all of which use GC-MS detection (Surma et al. 2014; Duedahl-Olesen et al. 2020; Slámová et al. 2020). QuEChERS is considered a promising method used to improve efficiency and thus increases the number of PAH analyses.

6.2 Occurrence of PAH4 in foods

Overall, our low PAH4 levels in fats and oils are well in accordance with findings reported by other studies (Larsson et al. 1987; Fromberg et al. 2007; Abramsson-Zetterberg et al. 2014). For instance, in rapeseed oil, the reported BaP levels were between 0.3-1.3 µg/kg (Larsson et al. 1987) and even lower BaP levels below 0.2 to 0.3 µg/kg were indicated by another study (Fromberg et al. 2007). Similar low mean concentrations of 0.1 µg/kg BaP and 0.6 µg/kg PAH4 were detected in fats (Abramsson-Zetterberg et al. 2014). On the other hand, previous Finnish studies (Hopia et al. 1986; Hietaniemi et al. 1999) demonstrated higher BaP levels up to 2.2 µg/kg in margarines compared to our results. Moreover, in another study, the determined concentrations of BaP and PAH4 in rapeseed oil were 11 and 84 µg/kg, respectively. A possible cause for these high concentrations can be an atmospheric pollution or contaminated soil in Iran (Yousefi et al. 2018). In addition, diesel exhaust fumes derived from harvesters are a notable source of contamination (Bertoz et al. 2021). It appears that the oil contamination is originated from both the environment and the process. Since the literature data consists mainly of PAHs in olive oil, it seems that the other vegetables oils and fats are also worth studying.

Besides certain vegetable oils, PAH amounts in cereals have not been extensively studied despite the request by EC to monitor PAHs in cereals (EC 2011a). Generally, PAHs are not generated to any notable extent during bread baking (Dennis et al. 1991; Rostampour et al. 2017; Rozentale et al. 2017). The recent study by Rozentale et al. (2017) was consistent with our results, demonstrating a low mean BaP level between 0.061 and 0.084 µg/kg and PAH4 levels in the range 0.61-0.71 µg/kg in cereals and different types of bread. Likewise, in a Swedish market basket study BaP and PAH4 concentrations in cereal products were 0.03 and 0.14 µg/kg, respectively (Abramsson-Zetterberg et al. 2014). However, since the LOQ of our method was 0.9 µg/kg, most of the trace concentrations detected in the latest studies would have been excluded. Therefore, given this fact not only in cereal products but in other matrices as well, our results can be underestimated from that perspective. Further method improvements leading to lower LOD and LOQ levels would increase the number of PAH4 analytes detected and provide a better picture of PAH4 occurrence. Furthermore, comparing our results with some older

literature data should be treated with caution, as not all methods of analysis were capable of guaranteeing the separation and identification of each PAH4 compound. Other limitations in the result interpretation of the monitoring samples are related to the manufacturing process of the studied products, which is unknown. The number of different cereal-based products is large and thus the production process and the PAH content may vary.

Of the analysed food matrices, smoked fish and meat products contained the highest BaP and PAH4 levels. Particularly in meat samples, the results revealed a large variation in concentrations up to 200 µg/kg of PAH4. The comparison to other traditionally smoked meat studies shows that elevated PAH4 levels were detected similarly in smoked meat samples up to 628 µg/kg, where those of darker surface colour were selected for analyses (Rozentale, Zacs, et al. 2018) or in products that were "sauna" smoked (Wretling et al. 2010). Despite most of our determined BaP and PAH4 concentrations remained below MLs, four meat products were noncompliant with the prevailing legislation. Control measures were taken in all these cases based on our results. Authorities enforced these operators to develop their smoking procedures in order to produce safe products. The follow-up samples were collected thereafter by the authorities to ensure that the products were compliant with the legislation prior to the placement of these products on the market. In part, the elevated PAH4 levels can be explained by the risk-based sampling strategy, in which only traditionally smoked fish and meat products were chosen for analyses. Moreover, differences were found in PAH4 levels, both between pork and other meat products and between small and larger fish species. Median PAH4 concentrations of both pork and small fish samples (e.g. Baltic herring and vendace) were higher than in other products. The previous Finnish study revealed that, in line with our results, the BaP concentrations were the highest in pork (13 µg/kg), whereas the BaP level in Baltic herring was relatively low (0.7 µg/kg). It is noteworthy that in that study, the number of fish and meat samples was negligible, for instance containing only a single Baltic herring sample (Hietaniemi et al. 1999). In addition, the results from the Swedish study indicated the highest meat and fish BaP levels in pork and in herring (Wretling et al. 2010). Herring is heavily smoked fish due to its lower surface-volume ratio compared to larger fish species (Lawrence and Weber 1984; Duedahl-Olesen et al. 2010). Fish skin or meat casing reduces PAH contamination by preventing them from migrating to fish or meat adipose tissue (Duedahl-Olesen et al. 2010; Fasano et al. 2016). It has been assumed that only a few consumers eat the fish skin (Duedahl-Olesen et al. 2010). However, according to the information provided in our study, small fish species were eaten with the skin. Whether a consumer eats fish with skin may increase the PAH exposure, as skin contains more PAHs compared to other parts of the fish. Depending on the country and the region, different fish species prevail, as marine species (cod, sprat, Baltic herring) differ from freshwater

species (vendace, whitefish). In addition, consumed meat products differ from country to country. In Finland, our study was the first to investigate the PAH4 levels in smoked foods. Valuable information on the PAH4 amounts in typical Finnish smoked fish and meat products and potential risk products were provided.

6.3 Relation of the smoking factors to formed PAH4 levels

Based on our results, the differences in smoking processes resulted in highly varying PAH4 concentrations in the final fish or meat product. There was high variability among the applied smoking procedures due to differences in equipment and smoking practices in Finland. Smokehouses are typically small- to medium-sized enterprises (SMEs), many of which only operate during summertime. By identifying the critical smoking factors associated with the formation of PAHs, it is possible to control PAH levels in foods (CAC 2009).

Fish and meat smoking with traditional direct and indirect techniques is widely employed in Finland. Although traditional smoking has been defined in our study to encompass both direct and indirect smoking techniques, it is often defined as comprising only direct techniques (Duedahl-Olesen et al. 2010). Our results clearly demonstrate that the selected smoking technique affects the PAH4 levels generated. The comparison between the direct and indirect technique indicated indirect smoking leading to lower BaP and PAH4 concentrations in fish and meat products. A particularly large difference between the techniques was observed in meat samples. This is consistent with the observations in previous studies (Duedahl-Olesen et al. 2010; Wretling et al. 2010). It is noteworthy that 100% of our directly smoked fish products and 82% of the directly smoked meat products complied with the legislation. Therefore, based on our results, direct smoking is safe to use when correctly controlled. Challenges may still occur, as the combustion temperature is typically very high and challenging to control, and the smoke is produced from an open fire located in the same chamber as the product. By contrast, the indirect method allows us to adjust the amount of smoke under controlled conditions and wash the smoke from particles prior to contact with the food, resulting in notably reduced PAH contamination in the product (EC 2002b; Ciecierska and Obiedziński 2007; CAC 2009).

Considering the impact of the smoking time on the PAH4 levels, our results were not consistent. In meat products, the shorter smoking time produced lower BaP and PAH4 concentrations while in fish products, the shorter smoking time generated surprisingly higher amounts of BaP and especially PAH4. In several studies, the linkage between shorter smoking time and lower PAH levels in smoked fish and meat has been confirmed (Varlet, Serot, Monteau et al. 2007; Duedahl-Olesen et al. 2010; Essumang et al. 2013; Racovita et al. 2020). Therefore, further

investigations of fish samples were required to explain this rather contradictory result. A possible reason may be that all small fish species were smoked within five hours, which contained relatively high PAH amounts compared to larger fish species. Small fish appear to be more heavily smoked due to their smaller surface-volume ratio compared to larger fish species (Duedahl-Olesen et al. 2010). In addition, smoking processes can be divided into three groups based on smoke chamber temperature as follows: cold smoking (18-25 °C), semi-warm smoking (30–40 °C) and warm (or hot) smoking (70-90 °C) (Alimentarius 2009). PAH levels are known to increase continuously in a smoking temperature range of 55-95 °C (Racovita et al. 2020). This explains that since most of the warm-smoked salmon samples were smoked in a shorter smoking time, they also contained higher PAH levels than the corresponding cold-smoked fish products. Our finding was in line with a previous study (Duedahl-Olesen et al. 2010). However, the smoke chamber temperatures of the cold-smoked or warm-smoked products were not asked for and therefore they may vary from the reported literature values. Most likely there are also other factors that can explain our unusual result. Besides the studied smoking variables, several factors such as the position of food material in the smokehouse (Guillén et al. 2011; Pöhlmann et al. 2013b), the wood type (Varlet, Serot, Knockaert et al. 2007; Essumang et al. 2013; Racovita et al. 2020), the type of casing, the product size and the fat content of the product may have affected the formed PAH levels (Duedahl-Olesen et al. 2010; Pöhlmann et al. 2013b). Overall, it is important to emphasise that the smoking process should be performed in as short a time as possible without jeopardising the microbiological safety and shelf life (CAC 2009).

Alongside the other critical parameters, the optimisation of the smoke formation temperature plays a critical role in the PAH formation. As stated by Stołyhwo et al. (2005), the temperature of the smoke formation is the most important parameter that has an impact on PAH levels. Adverse health effects related to smoked foods may be due to carcinogenic components of wood smoke (Stołyhwo 2005). The smoke composition depends on the production temperature, which is regulated to reduce PAHs (CAC 2009). Based on our results, it is clear that by optimising the smoke formation temperature between 400 and 600 °C, PAH4 levels were lower compared to non-optimised processes, especially in meat samples. Generally, there is a linear increase in the levels of PAHs in smoke from 400 to 1000 °C (Jägerstad and Skog 2005). In the non-optimised process, the smoke generation temperature fluctuates typically in the range of 500-800 °C and can be adjusted by the air supply (Pöhlmann et al. 2013a). However, it is important to correctly interpret our results and, in this context, the direct smoking technique affects the outcome. Approximately half of the smoking processes were non-optimised, which strongly refers to the direct smoking where the smoke formation temperature is challenging to control. From the perspective of the manufacturer, it is evident that the indirect

technique enables the optimisation easier, and our results support the conclusion that the smoke generation temperature below 600 °C may remarkably decrease the forming PAH levels (Ledesma et al. 2016). In addition, a filter can be used to remove particulate material, such as PAHs, from the smoke (CAC 2009). On the other hand, the smoke temperatures below 400 °C generate very low levels of PAHs but also fewer of those compounds responsible for the organoleptic characteristics of the final product. The optimisation should thus achieve a flavoured smoke while reducing PAHs (Varlet, Serot, Knockaert et al. 2007).

As expected, the distance over five metres between the food and the smoke source resulted in lower BaP and PAH4 levels in fish as well as meat samples. PAHs are known to be bound to smoke particles and thus a longer distance between the food and the smoke source may decrease the PAHs in the final product (EC 2002c; CAC 2009). To the best of our knowledge, no studies were found regarding the optimal length between the food and smoke source on PAHs. As with the optimisation of the smoke generation temperature, the smoking technique had a strong impact on our results. Since all the products smoked with direct technique were located in the same chamber as the smoke source, the distance was naturally less than five metres. It can be stated that higher PAH4 levels are likely to be formed at shorter distance.

Our extensive study confirmed that each of the selected smoking factors had an impact on the formed PAH4 levels in fish and meat products. It should be emphasised that this work was carried out under real-life smoking conditions in smokehouses instead of laboratory conditions. Therefore, our data provided a realistic basis for establishing good science-based practices for the smoking process. Based on our findings, to achieve safe, traditionally smoked fish and meat products, indirect smoking, a smoking time of less than five hours, optimised smoke generation temperature in the range of 400-600 °C and a distance of more than five metres are advisable. However, it is noteworthy that by studying one factor, the effect of other factors was present in the background. The high variability in our results showed that the smoking process in Finland seems to be either managed or not. Certainly, big differences in size and technological equipment of the smokehouses affect the variability. Since this project, authorities have established guidance to manufacturers and thus the situation is likely to be different today. Moreover, liquid smoke may be used more commonly than earlier as it is known to contain reduced levels of PAHs (Gomaa et al. 1993; Šimko 2005; Varlet, Serot, Monteau et al. 2007). Our guidance was especially intended for SMEs to provide current knowledge in order to manage the risks and thus prevent and decrease PAH4 levels in traditionally smoked fish and meat products. In certain countries, such as Finland or Sweden, the consumption of the traditionally smoked fish and meat can be higher compared to other countries, which can possibly lead to higher PAH exposure (Wretling et al. 2010). Therefore, the data on the Finnish

smoking processes and the association with the PAH4 contents in the final fish and meat products are required.

Some limitations hampered the interpretation of the results. Firstly, not all data regarding the smoking process utilised were available. The obtained results showed high variation, especially in smoked meat products, and a few very high BaP and PAH4 values influenced the mean and median values of the whole data set. However, it was decided to keep all the results in the calculations in order to summarise the situation in Finland. Based on these results, risk-based studies should be continued. Monitoring the current situation in Finland would clarify whether the measures on smoking processes have contributed to decreased PAH4 levels. The focus could be on those traditionally smoked foods which showed high PAH4 levels in our study and other affecting factors on smoking could be studied concurrently. On the other hand, the formation of PAH4 in home-cooked foods is a vast and difficult to cover, although most of the PAH containing food is prepared at home. The legislative limits do not apply to home-cooked foods but publishing guidance on safe cooking practices would certainly help to protect consumer health.

6.4 Evaluation of the mutagenic potential of processed foods

Testing whether our daily foods show mutagenic activity is a complex task. The detection of mutations in bacterial or other tests is a sign that a chemical may be carcinogenic in animals (NRCC 1982). Depending on the *in vitro* mutagenicity test, numerous factors may affect the outcome. Some Ames strains, such as TA 97, TA 100 and TA 102, are more susceptible to oxidative stress, leading to possible positive findings (Kirkland et al. 2014). In addition, the sensitivity of the TA 98 strain to food mutagens differs from the TA 100 strain and further the assay conditions and sample matrix may influence the sensitivity (Omoruyi and Pohjanvirta 2014). These must be considered because the statistically significant mutagenic responses observed in three lots of smoked Baltic herring used TA 100 and TA 98 strains. Certainly, food smoking can cause mutagenic compounds, but Baltic herring can also be exposed to man-made environmental chemicals, which are often detected at levels that are not directly toxic to fish, but the exposure causes adverse effects (Hamilton et al. 2016). Since no complementary biological assays were included in our study, we must treat our findings with caution.

The lack of the relevant studies hindered the comparison of our results to other studies, but a few were found. Contrary to our findings, chicken and beef steak broiled at a high temperature as well as hamburgers showed a wide range of

mutagenicity towards *Salmonella* strain TA 1538 (Bjeldanes et al. 1982). However, histidine is present in meat products and its possible leaching may not have been taken into account. Moreover, different Salmonella strains are optimised to detect different types of mutagens. Another study associated with the mutagenic activity by the Ames test was performed in Finnish heat-treated meat, fish and poultry products (Tikkanen 1991). Most of the food products were mutagenic in *Salmonella* TA 98, of which fried, grilled, broiled and restaurant foods showed higher mutagenic activity compared to ready-made food products. However, the variation between the samples was high. It was indicated that the higher cooking temperature may lead to higher mutagenicity. In addition, the potential histidine leaching to the plates and/or the limitations applying the *Salmonella* TA 98 strain alone may have affected the outcome.

Another Ames test study on Finnish processed foods and ready-to-eat snacks led to findings that 40% of the selected processed Finnish meat and 27% of the fish products were mutagenic in the *Salmonella* TA 100 and TA 98 strains with or without metabolic activation, followed by methylcellulose overlay and treat-and-wash assays. The observed mutagenic activity varied a lot between the lots (Omoruyi and Pohjanvirta 2014). Compared to our findings, a higher number of statistically significant mutagenic products were observed in their study in all three assays with the TA 100 strain with and without the metabolic activation, including cold-smoked beef, grilled turkey and smoked chicken. However, those mutagenic products were further tested by the comet assay, which indicated no mutagenic potential in the products. No particular reason for these contradictory findings was found, but there are substantial differences between the comet assay measuring DNA strand breaks and the Ames test, where the *Salmonella* strains are sensitive to base-pair substitutions (e.g. TA 100 and TA 104) and frame-shift mutations (e.g. TA 98) (Omoruyi and Pohjanvirta 2014).

The Ames test suffers from limitations related to biological samples and proteins, peptides or amino acids (e.g. histidine), which may interfere with enabling the bacteria to grow and generate a false-positive result (Nylund and Einistö 1992; Thompson et al. 2005; Khandoudi et al. 2009). As no test method alone can prove confirmation for mutagenicity, parallel tests are recommended for evaluation (Thompson et al. 2005; Kumaravel et al. 2009). Other biological tests alongside the Ames test would likely have improved the interpretation of our results. For instance, the treat-and-wash assay washes the bacteria free of the test substance prior to plating out on plates and the methylcellulose overlay method is modified from the Ames test by replacing agar with methylcellulose in order to stabilise the test compound, preventing precipitation and amino acid release (Thompson et al. 2005). Furthermore, the comet assay is regarded as highly sensitive in quantitation for DNA damage, i.e. a wide variety of compounds with unknown mutagenic potential as well as a low level of mutagenic activity by

known mutagenic compounds can be detected (Kawaguchi et al. 2010). Despite the lack of complementary biological assays, chemical PAH analyses indicated interestingly that all our negative samples in the Ames test contained low PAH4 levels below LOQ or LOD in chemical analyses. Similarly, higher PAH4 levels above LOQ were determined in those samples detected as statistically significant mutagens. Since only PAHs were analysed, we do not know what other possible mutagens were present in our samples. More information and understanding are thus required regarding the association between the potential mutagenicity and the chemical contamination. As it is known, a high number of potential mutagens can occur in foods. Some of them are clearly identified (e.g. aflatoxins) and suspected food mutagens (e.g. N-nitrosamines and heterocyclic amines). Those inherently occurring mutagens, such as mycotoxins, are originated from microbes, plants and animals. The content of the possible mutagens in different foods varies and complex mixtures are difficult to evaluate. Foods can include both mutagens and substances, which can enhance or inhibit the mutagenic activity of other compounds. Considering, for example, antioxidants or some types of fibres, they are likely to reduce cancer risk (Goldman and Shields 2003).

6.5 Children's dietary risk assessment to PAH4

It is well known that a chemical with mutagenic potential may be considered a suspected carcinogen and especially in the case of PAHs, the correlation between mutagenicity and carcinogenicity appears to be high (NRCC 1982). The daily exposure to BaP, as well as to other PAHs, progressively increases with decreasing age. This holds true for both the mean and the high exposure (EC 2002). Therefore, the science-based assessment of the dietary exposure is a crucial factor for quantifying risks and assessing whether PAHs pose a risk to children. Moreover, the foods contributing most to the exposure can be identified and further actions may be planned by risk managers.

In our study, the applied PAH4 concentration data on foods were modelled in order to estimate children's exposure. To our knowledge, this is the first study regarding Finnish children's dietary exposure to PAHs. Besides PAH4 levels, food consumption impacts the exposure. Our results indicated bread as the most consumed food by children, followed by fat and oils while Falcó et al. (2003) reported that the highest daily consumption volumes for children were in milk, cereals and fruits. Despite the fact that cereal-based food products typically contain low PAH levels, they can be regarded as a considerable source of PAH exposure due to their high consumption volumes (Ciecierska and Obiedziński 2013; Abramsson-Zetterberg et al. 2014). This is consistent with our findings concluding that bread was the major contributor to PAH4 exposure for children.

Similarly, cereals were found to be major sources of children's BaP exposure in the Spanish study (Falcó et al. 2003). Based on the EFSA's evaluation for consumers, cereals, cereal products, seafood and seafood products contributed most to the dietary exposure of PAHs (EFSA 2008).

On the other hand, our results indicated that despite the consumption of smoked ham having been relatively low, its contribution to PAH4 exposure was high. This was due to relatively high PAH4 levels detected in smoked meat products. Since our sampling strategy in smoked fish and meat products was targeted to traditionally smoked products, these samples were likely to contain higher PAH4 levels, resulting in the overestimation of the exposure. In addition, our research was limited by many food groups in children's diets (e.g. dairy products, vegetables, fruits, chocolate and cocoa), which were not included in our estimates and will inevitably affect the assessment of the total dietary exposure. According to the previous Finnish study, meat and cereal products (including bread) contributed most to human PAH exposure (Hietaniemi et al. 1999). However, comparison with the major contributors to children's exposure showed differences between the studies. Meat was the major contributor to BaP exposure in Martorell's study (2010), whereas other studies appointed oils (Veyrand et al. 2013) and egg-based products (Cirillo et al. 2010) as primary dietary sources to BaP exposure. Moreover, concerning the food groups contributing most to the total chronic exposure to BaP and PAH4, it was found similarly to our own findings, that bread and smoked ham had the greatest effect, respectively (COT 2002; Cirillo et al. 2010; Veyrand et al. 2013).

Regarding our total mean dietary exposure estimates to BaP and PAH4, they appeared to be consistent with the previous studies with relatively high variation between the countries. The reported children's total mean dietary exposures to BaP in France, the United Kingdom, Italy and Spain were between 300 and 6,000 pg/kg bw/day (COT 2002; Falcó et al. 2003; Cirillo et al. 2010; Martorell et al. 2010; Veyrand et al. 2013). Even greater variation between studies was observed in children's dietary exposure to PAH4 (2,300-47,000 pg/kg bw/day) (Cirillo et al. 2010; Veyrand et al. 2013). The distinction in exposure estimates between studies is likely to stem from a number of reasons. The differences in food culture and diet, study design (e.g. market basket study, total diet study), age of the children and the methodology of the exposure estimation may explain the variation (Domingo and Nadal 2015). In addition to the mean dietary exposure, children's estimated total chronic exposures to BaP and PAH4 were assessed and were relatively low compared to findings of other studies, although there again appeared to be a great variation from one study to another. The reported estimates of exposure to BaP were between 600 and 39,000 and those for exposure to PAH4 were between 4,700-336,000 pg/kg bw/day (COT 2002; Cirillo et al. 2010; Veyrand et al. 2013).

Despite the fact that EFSA has no data on children's exposure to PAHs, EFSA has estimated the median total dietary exposures for an average EU consumer. The estimated rate of exposure to BaP was 3,900 pg/kg bw/day and to PAH4, 19,500 pg/kg bw/day. Interestingly, the equivalent exposure values for Finnish consumers to BaP and PAH4 were lower, 3,100 and 16,300 pg/kg bw/day, respectively (EFSA 2008). However, it is noteworthy that the Finnish data used by EFSA was limited (n=65) (EFSA 2008) and the estimates may have been different if there were more data. Possible reasons can be speculated as to why the consumer's dietary exposure is lower in Finland than in the EU. First of all, environmental PAH contamination is likely higher in some other parts of the EU, which can have an impact on PAH exposure (EC 2002c). In addition, different dietary habits can explain the result. In Finland, home grilling and smoking are popular food preparation methods, especially in the summertime. The consumption of home-grilled and home-smoked foods can lead to an increase in human exposure, even a considerable one, when consumed heavily (EFSA 2008; Rose et al. 2015), although challenges in exposure estimation may occur due to differences in grilling regularities and practices, meat types and heat sources (Duedahl-Olesen and Ionas 2021). Some literature data on grilled meat and sausage were included in our study, but more research would be required. Since the exposure assessment is tailored to a specific country and population, the comparison of the dietary exposures between studies is often complicated. Therefore, the best benefit can be achieved through the use of the national data on food consumption and food concentrations consumed by the subpopulation under that study.

To compare the potential risks between substances or distinguish between different risk levels is not possible with the ALARA principle. Consequently, in order to overcome the potential limitations of ALARA, the MOE approach has been recommended by SC (EFSA 2005). Based on our results, the calculated total mean MOEs to BaP and PAH4 clearly exceeded the MOE reference value of 10,000 and thus the exposure to PAHs was estimated not to pose any risk to 3-6 year old children. The lack of relevant studies complicated the comparison as only two other MOE studies concerning children's exposure to PAH4 were found. Compared to our study, the total median MOEs of BaP and PAH4 were lower in an Italian study but still exceeded the reference value (Cirillo et al. 2010), whereas in the French study the total mean MOE of PAH4 was higher than our estimate (Veyrand et al. 2013). Furthermore, taking into account our calculated MOEs for highly exposed children, values were lower than those MOEs calculated using the mean values, but all MOEs were still above 10,000. This indicates a higher dietary risk to PAH4 for highly exposed children and was consistent with the findings from the French study, in which the MOE of PAH4 for highly exposed children clearly exceeded the reference value (Veyrand et al. 2013). By contrast, reported median MOEs of BaP and PAH4 for highly exposed children appeared to be clearly below

10,000 in an Italian study, indicating a potential health concern for children and need for risk management actions. However, due to their reported limitations, such as a short monitoring period of children's 24-hour food collection and possible incorrect diary notations, results have to be interpreted with caution (Cirillo et al. 2010). EFSA has also calculated the mean MOEs of BaP and PAH4 for high consumers to be close to or less than 10,000, demonstrating a potential concern for consumer health and a possible urge for risk management measures, whereas the corresponding mean MOEs of BaP and PAH4 for consumers were clearly above the reference value (EFSA 2008). In addition to the fact that bread was a major contributor to children's PAH4 exposure, the lowest MOEs of BaP and PAH4 were assessed in bread according to our scenarios.

Although the MOEs obtained did not pose a risk to children's exposure even to high consumers, our exposure estimates have certain limitations that undermine the generalisability of the results. For instance, the participants were selected via pre-agreed day care centres instead of a random sampling of children. The studied day cares were located in southern and western Finland, and therefore the food consumption data were not representative of the whole of Finland. Another limitation concerns the interpretation of the findings, as the PAH4 levels in smoked fish and meat products are probably lower today than during our studies. The implemented sampling was targeted and since our EVO control project in 2012, the awareness of the critical factors concerning the smoking process has grown and food authorities have given guidance to manufacturers on how to mitigate PAHs in smoked foods. Consequently, smoking processes have been developed, and the current PAH4 exposure may be lower than reported. In addition, humans are exposed to complex chemical mixtures rather than individual compounds. As risk assessment of single chemicals is usually conducted, potential combined effects are not taken into account. Due to a huge number of possible chemical combinations, the major challenges in the risk assessment are a lack of toxicity data on mixtures and a valid exposure model for that purpose (Ramesh et al. 2004; FAO/WHO 2009). This can be considered an important issue for future research.

7 CONCLUSIONS

The applied GC-MS/MS method was validated for fish, meat, oil and fat, bread, cereal and muesli. Validation performance values met legislative requirements for the most part, with the exception of the expanded MU, which exceeded the criterion intended for official control analyses (EC 2011b).

However, taking into account the acceptable validation data and numerous PTs performed on a regular basis with satisfied results, our method can be considered suitable for PAH analyses in studied matrices.

Following successful validation, the quantitative analyses were performed, of which the majority of the individual PAH4 concentrations were detected below the respective LOD. The comprehensive survey of the occurrence of the BaP and PAH4 levels in Finnish traditionally smoked fish and meat products was carried out in 2012, covering the whole of Finland. In general, the detected mean BaP and PAH4 concentrations were relatively low. Nevertheless, it is noteworthy that the large variation in PAH4 levels was observed, especially among meat samples, resulting in four non-compliant meat products. It seemed that the smoking process is either controlled or not, and scientific guidance is required to overcome these challenges in the food smoking process. Based on our findings, indirect smoking, smoking for less than five hours, optimised smoke generation temperature from 400 to 600 °C, and distance more than five metres between the food and the smoke source are recommended to mitigate the amounts of PAH4 in smoked fish and meat products. Overall, this study strengthens the idea that by applying the principle of PAH prevention and mitigation, safer smoked products can be produced and further reduce the dietary exposure to PAHs.

The mutagenic potential of Finnish fish and meat products was tested by the Ames test. The statistically significant mutagenic response was observed in each of the three Baltic herring lot. Similarly, the chemical PAH4 analyses clearly showed higher BaP and PAH4 concentrations in smoked Baltic herring than in other samples tested. The meat products tested were not mutagenically active in *Salmonella* TA 100 and TA 98 strains with or without the metabolic activation and the corresponding individual PAH4 levels were mostly below the LOQ or the LOD. Despite the possible limitations related to the Ames test, these results are valuable in light of identifying potentially mutagenic food products on the market. The evidence from this study highlights the importance of comparing the outcome of the screening mutagenicity test with the quantitative chemical analyses. In addition, other mutagenicity tests would definitely complement and increase the reliability of the outcome. Since there are lack of data concerning the potential mutagenicity of food products, this would be a fruitful area for further work.

Based on our information, this is the first study of Finnish children's dietary exposure to PAH4. Children's total mean dietary exposure was estimated to be 1,500 pg/kg bw/day to BaP and 8,100 pg/kg bw/day to PAH4. Moreover, the principal contributors to BaP and PAH4 exposure appeared to be bread, smoked ham, fat and oil as well as sausage. Although the bread contained low PAH4 concentrations, the amount of its consumption is high. The calculated MOEs for children were 482,000 for BaP and 42,000 for PAH4, indicating no health risk to Finnish children between three and six years. Furthermore, the MOEs for highly exposed children to BaP and PAH4 were lower than those determined using the mean values, even though all values were still above 10,000. Due to growing awareness of the effects of smoking factors on the PAH levels and the developmental efforts by FBOs, the PAH4 amounts are presumably lower today.

Our findings provide insights for future research. Additional risk-based PAH4 analyses, combined with the food mutagenicity studies, would broaden the understanding of the potential PAH risks to human health. On the other hand, diverse home cooking practices and their impacts on PAH4 levels would complement the picture of the sources of dietary exposure and the data would also be valuable when investigating dietary exposure in Finland's adult population.

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