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Faculteit Industriële Ingenieurswetenschappen

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Masterthesis

Characterization of polycyclic aromatic hydrocarbons exposure in Portugal by biomonitoring

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Scriptie ingediend tot het behalen van de graad van master in de industriële wetenschappen: chemie

Gezamenlijke opleiding UHasselt en KU Leuven

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UHASSELT

KU LEUVEN

Preface

Doing research while seeing other places around the world has always been my dream. Erasmus was thus the perfect opportunity to have my first glimpse of that life. However, going to an unfamiliar environment without knowing any people is not always easy. I always say that life begins at the end of your comfort zone, so I was up for the new experience. Nevertheless, overcoming the many obstacles during my journey abroad would never be possible without the support, advice and guidance of various people.

First of all, I would like to thank my promotor Prof. Dr. Simone Morais for all the support and advice. Due to her knowledge, I learned a lot about this subject and also how to do research. Further, I want to thank Dr. Marta Oliveira for her warm welcome in ISEP and all her guidance during my thesis work in the lab. She was always very friendly and helped me a lot to solve my problems. Because of her experience in this subject, she understood every obstacle I had. Both persons are one of the main reasons I loved my stay in Porto.

Second, I would like to express my gratitude to Prof. Dr. Leen Braeken for helping me organising my stay abroad and guiding me through my thesis.

Last, I would like to thank my family for being always there for me, giving me all the support I needed and motivating me to do this Erasmus.

Thank you all for making this Erasmus complete.

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List of abbreviations

A	Area of the peak
BMI	Body mass index
C	Concentration
HPLC	High-pressure liquid chromatography
IARC	International Agency for Research on Cancer
ISEP	Instituto Superior de Engenharia do Porto
LOD	Limit of detection
LOQ	Limit of quantification
OH-PAH	Hydroxylated polycyclic aromatic hydrocarbon
1-OH-Ace	1-hydroxyacenaphthene
1-OH-Naph	1-hydroxynaphthalene
1-OH-Phen	1-hydroxyphenanthrene
1-OH-Py	1-hydroxypyrene
2-OH-Flu	2-hydroxyfluorene
2-OH-Naph	2-hydroxynaphthalene
3-OH-B(a)P	3-hydroxybenzo[a]pyrene
PAHs	Polycyclic aromatic hydrocarbons
PM	Particulate matter
P_{25}	First quartile
P_{75}	Third quartile
R^2	Correlation coefficient
SD	Standard deviation
WHO	Word Health Organisation
US EPA	United States Environmental Protection Agency

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic pollutants, being some of them classified as (known/possible/probable) carcinogenic. Biomonitoring the exposure of vulnerable groups, such as children, to these compounds is thus highly relevant. Yet, European studies are scarce. PAHs exposure mostly occurs by food ingestion and air pollutants inhalation. PAHs are metabolised to hydroxylated forms (OH-PAHs) and excreted via biological fluids. Urine has several advantages to assess OH-PAHs since it is cheap and not invasive. Hence, the main goal of this study was to characterise the exposure of Portuguese children to six OH-PAHs by biomonitoring.

OH-PAHs were hydrolysed, extracted, preconcentrated and quantified by high-pressure liquid chromatography. The OH-PAH data were normalized by urinary creatinine levels and combined with information from questionnaires (age, gender and the distance from the place of residence to the industry) to explore possible associations.

Independently of the age or gender, 1-OH-Naph + 1-OH-Ace was the most abundant metabolite followed by 1-OH-Phen, 2-OH-Flu and 1-OH-Py. Overall, female children showed similar OH-PAH levels as males. However, significantly higher 1-OH-Naph + 1-OH-Ace values were present girls. This suggest that PAHs metabolization may be influenced by gender. Also, no relation between OH-PAH levels and age or distance from the industry was perceived. More research with a larger population is needed to comprehensively characterize children exposure to PAHs.

Abstract in Nederlands

Polycyclische aromatische koolwaterstoffen (PAK's) zijn vervuilende organische moleculen waarvan enkele geclassificeerd zijn als (erkend/mogelijk/waarschijnlijk) carcinogeen. De biomonitoring van de blootstelling van deze componenten aan kwetsbare groepen is dus van uiterst belang. Toch is er een tekort aan gepubliceerde studies in Europa. PAK's blootstelling treed meestal op bij voedselinname en het inademen van verontreinigde stoffen. Na opname worden PAK's gemetaboliseerd tot gehydroxyleerde vormen (OH-PAK's) en geëxtraheerd via biologische vloeistoffen. Urine heeft verschillende voordelen om OH-PAK's te evalueren aangezien het goedkoop en niet ingrijpend is. Vandaar dat deze masterproef via biomonitoring de blootstelling van zes soorten PAK's bij kinderen uit Portugal onderzoekt.

OH-PAK's werden gehydrolyseerd, geëxtraheerd, pre geconditioneerd en gekwantificeerd via hogedruk-vloeistof chromatografie. De OH-PAK data werd genormaliseerd doormiddel van urinaire creatinine niveaus. Vervolgens was in combinatie met informatie uit vragenlijsten (leeftijd, gender, afstand tussen woonplaats en industrie) gezocht naar mogelijke associaties.

1-OH-Naph + 1-OH-Ace waren ongeacht leeftijd of gender de meest prominente metabolieten, gevolgd door 1-OH-Phen, 2-OH-Flu en 1-OH-Py. Algemeen hadden meisjes gelijkaardige OH-PAK waardes als jongens. Er was echter een significant hogere 1-OH-Naph + 1-OH-Ace waarde in meisjes. Dit suggereert dat PAK metabolisatie mogelijks beïnvloed wordt door gender. Daarnaast is er geen relatie gevonden tussen OH)-PAK niveau en leeftijd of afstand tussen woonplaats en industrie. Meer onderzoek met een grotere populatie is nodig om de blootstelling van kinderen aan PAKs te karakteriseren.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic molecules consisting of more than one condensed aromatic ring. There are several types of PAHs, although sixteen of them are considered priority pollutants by the United States Environmental Protection Agency (US EPA) [1]. These sixteen priority PAHs are naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, fluoranthene, pyrene, anthracene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene and indeno[1, 2, 3-cd]pyrene.

Based on their source, these pollutants can be distributed in two groups. The first group is constituted by PAHs derived from petrogenic sources, such as naphthalene, fluorene, phenanthrene, dibenzothiophene and chrysene [2]. This group can be further separated into natural petrogenic PAHs, which originate from oil seepage and erosion, and anthropogenic petrogenic PAHs, which originate from acute petroleum spillages, urban runoff and waste treatment plants [3]. On the other hand, there are the PAHs created by pyrogenic sources, such as anthracene and benzo(a)pyrene [2]. Natural pyrogenic PAHs are caused by the incomplete combustion of wood and biomass. While the anthropogenic pyrogenic PAHs being the result of combustion during transport, power plant fuels, tobacco smoke and gas cooking, among others [3].

Depending on their molecular weight, PAHs can be distributed into the gas, liquid or particulate phase. Because PAHs can be present in different phases they have different ways to reach the human body, namely through ingestion, inhalation and skin contact [4]. When absorbed, PAHs can cause many negative health effects including cardiovascular disease e.g. increased blood pressure [5]-[6]-[7], respiratory disorders [7]-[8]-[9], diabetes [10], obesity [11] and oxidative stress [12]. Once PAHs reach the human cells, they are metabolised and can be detected in biological fluids, such as blood and urine among others.

Due to exposure from food, increase of air pollution and urban environments, biomonitoring exposure to PAHs is of the utmost importance to ensure the health and well-being for all citizens. The REQUIMTE-LAQV/Instituto Superior de Engenharia do Porto (ISEP) is an institution in Porto (Portugal) that has experience in biomonitoring. This master's thesis is conducted in this institution and characterizes the actual exposure of Portuguese children to these ubiquitous organic pollutants.

Until now, the biomonitoring and research of PAHs is too insufficient, especially research around vulnerable groups, such as pregnant women, children, and highly exposed labourers. First, there is a lack of data available on a global scale that focusses on these groups. Mainly Europe has a massive gap of evidence as to PAHs exposure. Second, there is a large quantity of sources that causes PAH exposure to these groups. Possible causes are the situation at home (e.g., food, smokers), the residence, the workplace, means of transport, etc. The consequence is that a significant research regarding all these PAH pollution sources is needed to assess the PAH problem in Europe. Last, research on this issue demands a tremendous amount of input of the population, considering the high number and variety of subjects needed to create representative results. Primarily obtaining data from preschool children is a challenge since support of the parents is needed.

The main goal of this project is to assess the exposure of preschool children of Portugal to PAHs. This objective is divided into several specific objectives, namely:

i) Review of the available data about PAH metabolites content and possible health effects in preschool children living in different areas around the world. The focus is set on the literature published from 2018

until April 2021 since a previous review about this topic was already performed by the REQUIMTE-LAQV/ISEP team [13].

ii) Determination of PAH metabolites in urine samples of Portuguese preschool children by previously validated chromatographic method [14].

iii) Characterization of the studied population group by analysing the respective questionnaires.

iv) Outline the pattern of variation of the determined urinary metabolites concentration and search for possible associations with data from questionnaires.

2. Literature study

Air pollution is becoming an increasingly environmental problem causing health issues, ecological problems, and climate changes. The pollutants that play a major role in this global problem are particulate matter (PM), ozone, carbon dioxide, methane, volatile organic compounds, such as PAHs, among others. Addressing the emissions of these components and their potential health risks is therefore of utmost importance. PAHs are non-polar organic persistent molecules consisting of only carbon and hydrogen atoms and structured in more than one condensed aromatic ring. Based on their source, PAHs can be distributed in two groups that overlap each other. The first group is constituted by PAHs derived from petrogenic sources, such as naphthalene, fluorene, phenanthrene, dibenzothiophene and chrysene [2]. This group can be further separated into natural petrogenic PAHs, which originate from oil seepage and erosion, and anthropogenic petrogenic PAHs, which originate from acute petroleum spillages, urban runoff and waste treatment plants [3]. On the other hand, there are PAHs emitted by pyrogenic sources, such as anthracene and benzo(a)pyrene, etc. [2]. Natural pyrogenic PAHs are caused by the incomplete combustion of wood and biomass while the anthropogenic pyrogenic PAHs are the result of combustion during transport, power plant fuels, tobacco smoke and gas cooking, among others [3]. However, it should be noted that PAHs are always emitted as a mixture of different compounds.

2.1. Properties

There are more than 100 PAHs with different properties due to different number of rings. Yet, sixteen of them are considered priority pollutants by US EPA [1]. Chemical structures and physical properties of these PAHs are respectively shown in figure 1 and table 1.

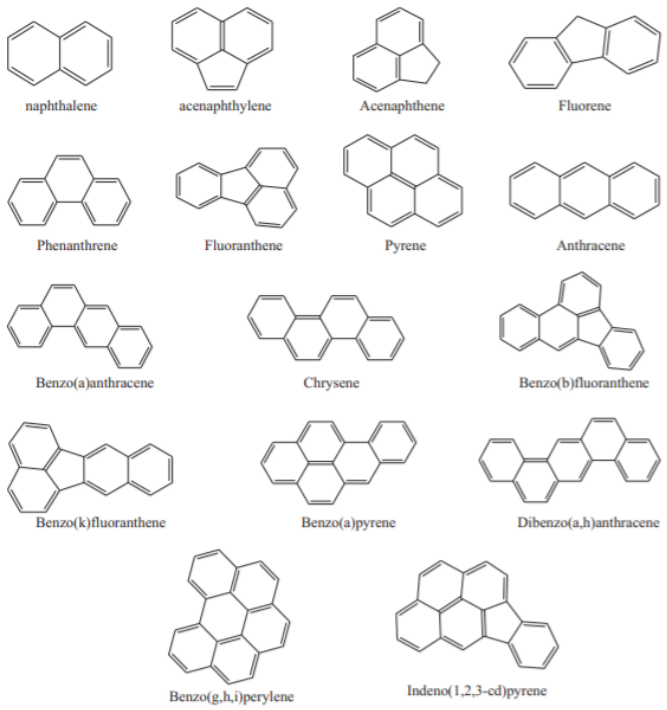

Figure 1: The molecular structures of US EPA priority PAHs [15].

Table 1: Chemical and physical properties of the 16 United States Environmental Protection Agency priority PAHs [1].

Compound	Molecular weight (g/mol)	Solubility (mg/L)	Melting point (°C)	Boiling point (°C)	Vapor pressure at 25°C (Pa)
Naphthalene	128.17	31	81	218	10.4
Acenaphthene	154.21	3.8	95	279	2.9×10^{-1}
Acenaphthylene	152.20	16.1	92-93	265	8.9×10^{-1}
Anthracene	178.23	0.045	216	342	8.0×10^{-4}
Phenanthrene	178.23	1.1	100	340	1.6×10^{-2}
Fluorene	166.22	1.9	115-116	295	8.0×10^{-2}
Fluoranthene	202.26	0.26	109	375	1.2×10^{-3}
Benzo(a)anthracene	228.29	0.011	161	400	2.8×10^{-5}
Chrysene	228.29	0.0015	254	448	8.4×10^{-5}
Pyrene	202.26	0.132	150	393	2.9×10^{-1}
Benzo(a)pyrene	252.32	0.0038	178	496	7.3×10^{-7}
Benzo(b)fluoranthene	252.32	0.0015	167	357	/
Benzo(k)fluoranthene	252.32	0.0008	216	480	1.3×10^{-7}
Dibenz(a,h)anthracene	278.35	0.0005	267	524	1.3×10^{-8} (20°C)
Benzo(g,h,i)perylene	276.34	0.00026	278	545	1.4×10^{-8}
Indeno[1,2,3-cd]pyrene	276.34	0.062	164	536	1.3×10^{-8} (20°C)

In the air, depending on their molecular weight, PAHs can be distributed into the gas and PM phases. Herewith, the high molecular weight PAHs, with more than four rings, dominate this PM phase. This is because of their lower volatility compared to the lower molecular weight PAHs [4].

2.2. PAHs metabolization

Since PAHs are ubiquitous, these pollutants have different ways to reach the human body, namely through ingestion, inhalation and dermal contact [4]. Regarding children, ingestion and inhalation are the main exposure routes but dermal absorption is also significant [16]-[17]-[18]. Once PAHs reach the human cells, they are metabolised by different enzymes. This metabolization occurs the most in the liver, followed by the lungs, the intestinal mucosa, skin and kidney [19]. The mechanism behind this process consists of two phases including various oxidative reactions. Herewith, complex compounds such as phenols, triols, quinines, dihydrodiols and tetrols are produced. The hydroxylated metabolites (OH-PAHs), also created in this step, may undergo a second metabolization step. Here, the OH-PAHs are conjugated with glutathione, glucuronide or sulphate [19]-[20]-[21]-[22]-[23]. Examples of some OH-PAHs are presented in figure 2.

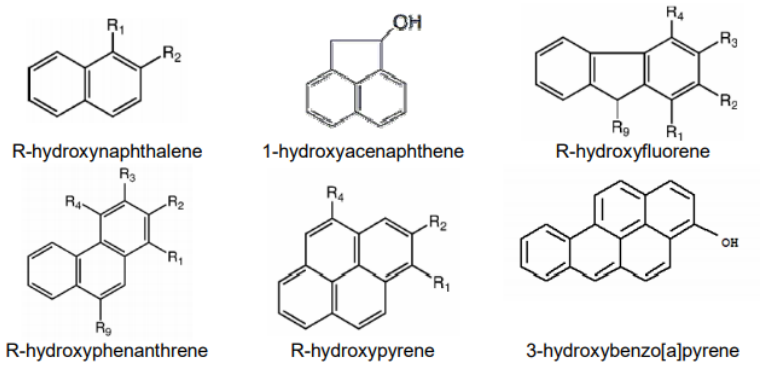

Figure 2: Hydroxylated PAH metabolites with the R groups indicating the positions of de hydroxy groups on the metabolites [24].

The main goal of the metabolization is to create hydrophilic molecules that can be excreted from the body via faeces, exhaled breath and/or biological fluids, such as bile, blood and urine among others [25]-[26]-[27]-[28]. 1-hydroxypyrene (1-OH-Py) is mostly found in urine due to its limited number of rings [29]-[30]-[31]-[32]. Moreover, 1-OH-Py is the metabolite of a very abundant PAH, which is present in the majority of the (environmental, food, biological, etc.) matrices. Tsai et al. [33] found that over 90% of the urinary excreted pyrene is in the form of 1-OH-Py. Further, naphthalene is mostly metabolised as 1-hydroxynaphthalene (1-OH-Naph) and 2-hydroxynaphthalene (2-OH-Naph) [34]. The major metabolites for acenaphthene, fluorene and phenanthrene are respectively 1-hydroxyacenaphthene (1-OH-Ace), 2-hydroxyfluorene (2-OH-Flu) and 1-hydroxyphenanthrene (1-OH-Phen). The main metabolite of benzo(a)pyrene (known as the biomarker of exposure to carcinogenic PAHs) is 3-hydroxybenzo[a]pyrene (3-OH-B(a)P).

2.3. Health effects

Because of the lipophilic behaviour of the PAHs, small quantities remain in the body fat and the liver. This phenomenon may result in bioaccumulation of the PAHs over time and can cause many negative health effects [35].

Cardiovascular diseases, such as increased blood pressure and heart variations, are examples of these health issues [6]-[7]. Cao et al. [5] observed that the risk of cardiovascular diseases is associated with the increased level of low molecular weight PAHs, namely naphthalene and phenanthrene being the main contributors for these health disorders.

Also respiratory disorders, such as asthma and lung wheezing, can be promoted by PAHs exposure [7]. For example, Liu et al. [8] reported that children, aged 13-19 years, who are exposed to higher concentrations of PAH are more likely to get asthma or asthma symptoms. More specifically, a positive association of asthma among boys aged 13-19 years who were exposed to 2-phenanthrene was found. In addition, girls aged 13-19 years that were exposed to 4-phenanthrene showed an increased risk for lung wheezing. Further, exposure to 1-pyrene increased the risk of asthma by 44.4% [7].

Nam et al. [10] proved that PAHs are also involved in the development of diabetes. Participants who experienced a higher exposure to 2-OH-Naph and 2-OH-Flu had significantly higher odds ratio for diabetes mellitus [10]. Moreover, a correlation between PAHs exposure and obesity in children is demonstrated by Bushnik et al. [11]. It turns out that a higher exposure of naphthalene has been associated with an increase of body mass index percentile for children aged 6-18 years. The higher naphthalene exposure has been related with increases in the waist circumference and the waist-to-height ratio for children aged 3-18 years. In addition, the waist-to-height ratio of children aged 3-11 years increases also when exposed to phenanthrene.

Furthermore, a recent study showed a significant increase of urinary 8-hydroxy-2-deoxyguanosine levels with the increase of exposure of total PAHs [12]. 8-hydroxy-2-deoxyguanosine is a biomarker to evaluate oxidative DNA damage in the subjects' bodies. Here, phenanthrene and pyrene are the major components associated with oxidative stress. Similarly, Cao et al. [5] also found a positive correlation between 8-hydroxy-2-deoxyguanosine and increased PAHs exposure.

To address the carcinogenic behaviour of PAHs, the International Agency for Research on Cancer (IARC) classified PAHs in three groups: carcinogenic (group 1), probable carcinogenic (group 2A) and possible carcinogenic (group 2B). The specific PAHs of each group are listed in table 2 [4].

Table 2: Classification of the 16 priority US EPA PAHs based on carcinogenic risk [36].

Carcinogenic (Group 1)	Probable carcinogenic (group 2A)	Possible carcinogenic (group 2B)
Benzo[a]pyrene	Dibenzo[a,l]pyrene	Naphthalene
	Dibenz[a,h]anthracene	Benz[a]anthracene
		Benzo[b]fluoranthene
		Benzo[j]fluoranthene
		Benzo[k]fluoranthene
		Chrysene
		Indeno[1,2,3-cd]pyrene

2.4. Characterization of children exposure

An option to assess one of the main PAH routes of exposure is air monitoring. Herewith, the concentrations of the gaseous and/or the particulate-bound PAHs in the air are determined, which then can serve as indicators of the inhaled PAHs. The downside of this method is that it does not capture the total PAHs exposure, i.e., those due to other routes. An alternative to measure these organic compounds is with the help of biomonitoring. Biomonitoring is defined as "the assessment of human exposure to an environmental chemical via the measurement of that chemical, its metabolite(s), or reaction product(s) (biomarkers) in human exhaled air, blood, urine, milk, saliva, adipose or other tissue in individuals taken separately but generally taken together to constitute a population" [26]-[37]-[38]-[39]. Biomarkers are defined as "any substance, structure, or process that can be measured within an organism, or its products, and influences or predicts the incidence of harmful effects or disease" [40]. As mentioned previously, when PAHs are metabolised, they are excreted in biological fluids such as urine. This matrix has several advantages since it is cheap, easy to work with and less invasive than others, such as blood. Despite these benefits, the biomonitoring of PAHs is only emerging. Especially research around vulnerable groups, such as pregnant women, children, and highly exposed labourers is clearly insufficient. First, there is a lack of data available on a global scale that focus on these groups; mainly Europe has a massive gap of evidence related with PAHs exposure. Second, there is a large quantity of sources that causes PAHs exposure to these groups. Possible causes are food, tobacco smoke, indoor air quality (home, workplace), means of transport, etc. The consequence is that significant research regarding these sources is needed. Third, research on this issue demands a tremendous amount of input of the population, considering the high number and variety of subjects needed to create representative results. Primarily obtaining data from preschool children is a challenge since support of the parents is needed. Last, apart from the chemical background, also medical and political insights are necessary to reduce the exposure to PAHs. Through this cooperation, the negative health effects on these vulnerable groups can be estimated in order to set a list of mitigation measures.

Children are more susceptible to PAHs exposure compared to adults. Reasons for this are: the not fully developed lungs and immune systems, the tendency to breathe through the mouth, the higher amount of inhaled air per body weight compared to adults, their behaviour (e.g. playing in the floor and hands to mouth activities) and their smaller peripheral airways resulting in proportionally larger airway obstruction caused by inflammation [41]. Several studies have characterized the levels of urinary PAH metabolites in children from several countries. Oliveira et al. [42] summarised 26 studies, between 2007 and 2017, regarding this topic. Additionally, appendix A shows the results of 20 studies executed between 2018 and 2021. Likewise, the previous review of Oliveira et al. [42], the majority of the data come from Asian studies (50%). Especially, China has done a lot of research surrounding this topic. The high amount of data from China may be due to its significantly higher levels of pollution when compared with most of the countries. Further, 25% of the data come from European studies, 20% from American

studies and only 5% of the data originates from Africa. Further, 4 of the 20 studies focussed on new-borns, while the rest focused on preschool children or adolescents. Thirteen studies normalized the metabolites concentrations by means of the urinary creatinine levels. Creatinine is a waste product originated from the high-energy molecule creatine phosphate in the muscle. Major advantages of creatinine is that their blood concentration and urinary levels remain constant in normal individuals [43]. Additionally, it is excreted from the human body at a constant rate. This makes it possible to reduce the effect of parameters (e.g. body temperature, physical exercise, individual fluid intake and ambient temperature) on the OH-PAHs concentration [44]. This allows a better comparison of levels between different subjects. Figure 3 shows the number of times a metabolite was measured in the urine of the children and reported in the literature from 2007-April 2021.

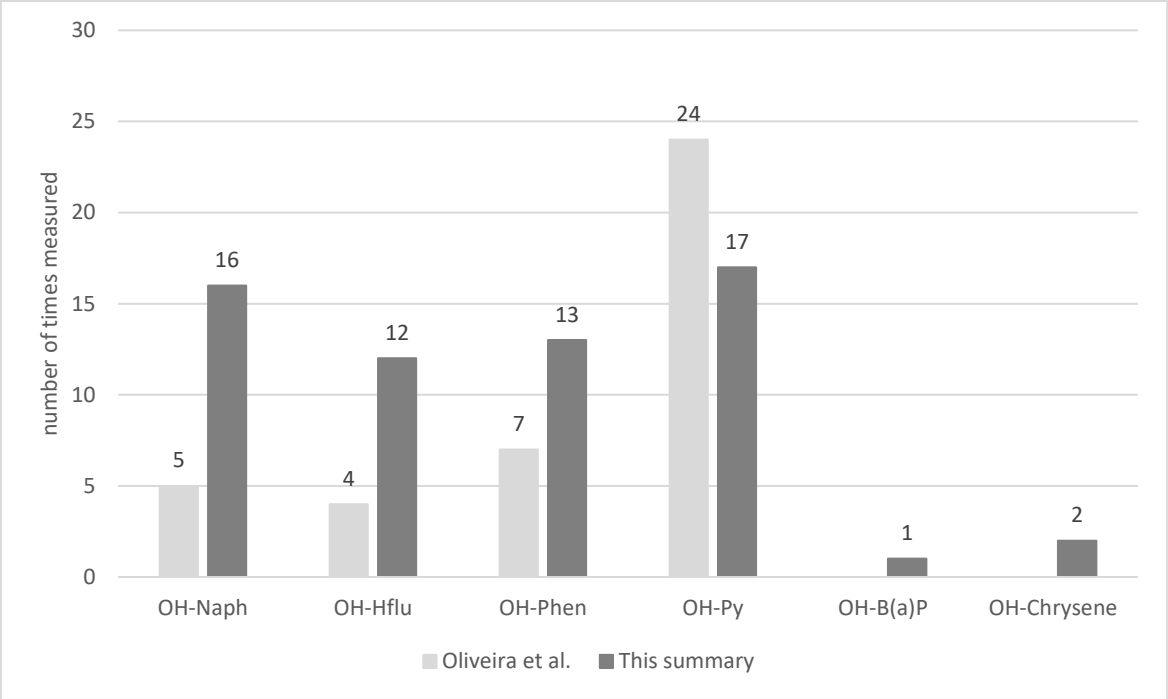

Figure 3: Number of times the different PAH metabolites were reported in children during 2007 to April 2021.

In the study from Oliveira et al. [42],1-OH-Py is by far the predominant measured PAH metabolite since it is considered the biomarker of exposure to PAHs. Until now, urinary OH-PAHs still do not possess reference standard guidelines. However, because 1-OH-Py is the most abundant metabolite in urine, Jongeneelen et al. [28] presented a reference value of 0.24 µmol/mol creatinine of 1-OH-Py for non-smokers and non-occupational exposed groups. Additionally, a no-biological effect value of 1.4 µmol/mol creatinine in exposed workers is proposed [28]. 3-OH-B(a)P is reported only in one study and thus the least detected metabolite [45], possibly because the low existing concentrations in air according to various studies [46]-[47]-[48]. Another possible reason is that metabolites of high molecular PAHs, such as 3-OH-B(a)P, are mostly excreted through the faeces according to Likhachev et al. [49]. However, the mechanism behind this process is still unclear.

The residence and living conditions of the subjects have a marked effect on their PAHs exposure. It appears that living near industrial sites causes an enhanced PAHs exposure. Zheng et al. [50] found a significantly higher level of urinary OH-PAHs and in particular OH-Flu in children living near an e-waste recycling company. Similarly, several studies concluded that children living in areas with a high amount of coal industries experience higher levels of exposure [12]-[51]. Likewise, Yu et al. [12] proved that children living in urban environments suffer higher exposure to PAHs. Green spaces in these urban

environments can be a solution to mitigate the exposure levels. Children living near green spaces showed a lower urinary 1-OH-Py level due to the fact that greenness act as a barrier and a sorbent for particulate and gaseous pollutants [47]. Kindergartens located in high traffic areas tend to result in higher exposure to PAHs. Even the mode of transport plays a massive roll in the exposure to PAHs. Children who walk to kindergartens are less exposed to PAHs compared to children that are transported in vehicles. Using the motorcycle, as mode of transport, has the biggest and worst impact on PAHs exposure [52].

However, the risk of PAHs exposure is also present inside the residence. The presence of smokers in the household may enhance the pyrene uptake by 90% [53]. This corresponds also with the findings of Lin et al. [54], which concluded that environmental tobacco smoke is the most indoor PAHs pollution source. Similarly, other studies found a correlation between tobacco smoke at home and PAHs exposure [22]-[52]-[55]-[56]-[57].

Cooking may be also a significant indoor PAHs source. A study conducted by Suter et al. [53] showed that cooking with solid biomass enhances the exposure to PAHs in comparison with households that are using cleaner fuels [58]. Similarly, other studies confirmed that the use of domestic fuel for heating or gas for cooking increases the concentrations of OH-Flu and OH-Phen [22]-[55].

When comparing the different age groups with each other, a negative age-dependency of the metabolites levels is also noticeable [55]. Chen et al. [59] found a significantly lower level of total urinary OH-PAHs in adults compared to children. However, when decreasing the age difference between two groups, no significant difference in urinary OH-PAHs is found. For example, Yu et al. [12] found no difference between the OH-PAHs content of 11-year-old children and younger age groups.

PAHs are a major threat for our population and the need for biomonitoring is becoming more prominent. Most studies that evaluate urinary PAH metabolites in children are conducted in Asia. Other continents suffer from a shortage of studies published in recent years. There is also a lack of research conducted on children younger than the age of six regarding the characterization of PAHs.

3. Materials and Methods

3.1. Sample collection

Urine samples form 53 children aged 2-13 years living in central Portugal were collected in the summer of 2018 and the winter of 2019. These samples were stored at -20°C in sterilized polycarbonate containers until analysis.

Validated questionnaires, adapted from Oliveira et al. [60] and the WHO [61], were filled out by the parents. Here, data were collected regarding gender, age, weight and height. Also, information regarding possible PAHs exposure factors was gathered. This included socio-demographic data, such as level of parental education, residence, the use of agrochemicals, the proximity to the industry, greenspaces, and animal farms. Additionally, the questionnaires contained the subjects eating habits, in particular the origin of the food and the place of consumption. A legal representant of each participant signed an informed consent form that was previously approved by the Ethics Committee of University School Vasco da Gama (Coimbra, Portugal).

3.2. OH-PAHs chromatographic analysis

3.2.1. Extraction

The extraction of the metabolites are described by Oliveira et al [60] and consist of two main phases: the reaction phase followed by the extraction phase. For the reaction phase, the samples were first defrosted at room temperature. The ideal reaction conditions were then prepared. First, the pH of 10.00 mL urine samples was adjusted to 5 with a 0.5 mol/L acetic acid buffer solution. Then, 80 µL enzyme β-glucuronidase/arylsulfatase (Helix pomatia, EC 3.2.1.31/EC3.1.6.1; 5.5/2.6 U/mL) purchased from Roche Diagnostics (Indianapolis, USA) was added to hydrolyse the urinary conjugated metabolites. To prevent the reaction of the metabolites with the surrounding oxygen, 20 µL of 1.0 g/L antioxidant tert-butylhydroquinone (TBHQ, purity > 98.0%; Sigma-Aldrich, Steinheim, Germany) were added followed by a 30 min purging step with a nitrogen flow. Afterwards, the samples were incubated for 2 hours at 37°C (Binder KBWF, Tuttlingen, German), with the absence of light and constant stirring at 120 rpm (Bunsen AO.400 orbital shaker, Madrid, Spain). After incubating, the hydrolysed samples were loaded into Sep-Pak® Light Plus C18 cartridges (55-105 µm, 125 Å, 360 mg; Waters; Sigma-Aldrich, Steinheim, Germany), which retained the metabolites. Before loading the samples, these cartridges were preconditioned with 5.00 mL of methanol and 10.00 mL of ultrapure water. After passing the samples through the cartridges, they were sequentially washed with 10.00 mL of ultrapure water, and 10.00 mL of methanol/water (20:80; v/v). Hereafter, the cartridges were dried for 15 min with a nitrogen flow. The retained metabolites were then eluted out of the cartridges with 20.00 mL of a 10:90 (v/v) methanol/ethyl acetate solution. Further, the extracts were evaporated till dryness at room temperature (Büchi R200 rotavapor and a Büchi Vac V-500 pump), redissolved in 500 µL methanol and filtered through a 0.22 µm PTFE syringe filter (Teknokroma, Barcelona, Spain) immediately before analysis.

3.2.2. Chromatographic analysis

The OH-PAHs in the redissolved extracts were analysed in triplicate in a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan). Herewith, the separation of the metabolites was accomplished by using a C18 column (CC 150/4 Nucleosil 100–5 C18 PAH, 150 × 4.0 mm; 5 µm particle size; Macherey–Nagel, Duren, Germany) at ambient temperature (20 ± 1 ºC). For the mobile phase, a mixture of filtered ultrapure water and methanol (1:1; v/v) was used. After 2 minutes, the water/methanol ratio takes 3 minutes to change linearly to a ratio of 3:7 (v/v). After staying 7 minutes constant at this ratio, it changes linearly to a 100% methanol flow where it stayed for 5 minutes. The linear increase of the concentration of methanol took 6 minutes. Hereafter, the ratio changed a last time linearly to 1:1 (v/v). Each injection consisted of 50 µL sample with a total runtime of 30 min at a flow rate of 1.0 mL/min. Additionally, a fluorescence detector is used to detect the metabolites at their optimum excitation/emission wavelength pair, which are shown in table 3.

Table 3: Excitation/emission wavelength pairs (nm) of the analysed metabolites.

PAH metabolite	Excitation/emission wavelength pair (nm)
1-OH-Naph	232/337
1-OH-Ace	232/337
2-OH-Flu	265/335
1-OH-Phen	263/363
1-OH-Py	242/388
3-OH-BaP	304/432

Furthermore, calibrations with 9 OH-PAHs standards in methanol at different concentrations were performed (table 4). Calibration curves were linearly fitted and used to calculate the limit of detection (LOD) and limit of quantification (LOQ) [62].

The metabolite concentrations were normalized by means of the urinary creatinine levels. Creatinine levels were determined by Jaffe's colorimetric method [43].

Table 4: Concentrations of the metabolites in the standards.

	OH-PAH concentration (µg/L)				
Name standard	1-OH-Naph + 1-OH-Ace	2-OH-Flu	1-OH-Phen	1-OH-Py	3-OH-BaP
P1	6.04	0.040	0.040	0.040	0.040
P2	8.08	0.060	0.060	0.060	0.060
P3	10.10	0.080	0.080	0.080	0.080
P4	20.20	0.100	0.100	0.100	0.100
P5	40.40	0.200	0.200	0.200	0.200
P6	60.60	0.300	0.300	0.300	0.300
P7	80.80	0.400	0.400	0.400	0.400
P8	101.00	0.500	0.500	0.500	0.500
P9	121.20	0.600	0.600	0.600	0.600

3.3. Statistical analysis

Results were statistically treated with Excel. Because normal distribution was not observed, urinary OH-PAH concentrations were expressed as median values and percentiles. The comparison of the median values was accomplished through the nonparametric Mann-Whitney U test if sample sizes were below 30 subjects, otherwise parametric t-test were used. Statistical significance was defined as $p \leq 0.05$.

4. Results and Discussion

4.1. Characteristics of the studied subjects

The data retrieved from questionnaires characterising the 53 studied subjects from Aveiro, Coimbra, Leiria and Porto are presented in table 5. The median age of the children was 6.7 years with a standard deviation of 2.5 years and a range of 2-13 years. These age groups are similar to the examined age groups mentioned in the literature [22]-[50]-[51]-[52]-[53]-[54]-[55]-[56]-[57]-[59]-[63]-[64]-[65]-[66]-[67]-[68]. Of these 53 children, 38% are in preschool. The rest of the children are attending the first and second grade of elementary school. Table 5 also shows that the examined group is almost equally divided in male (53%) and female (47%). Furthermore, 72% of the children showed a normal body mass index. However, a significant part of 24% exceeds the WHO overweight level [69]-[70]-[71]. Also notable is that an overwhelming amount, 39 of the 53 children, lives within a radius of 5 km to the industry. However, information regarding the type of industry was unavailable.

Table 5: Characterization of the studied subjects. n: number of examined subjects; SD: standard deviation; min: minimum; max: maximum.

Characteristic	**Children**
n	53
Age (mean ± SD; min-max)	6.7 ± 2.5 (2-13)
Educational level (n; %)	
Preschool (2-5)	20 (38%)
Elementary school (6-13)	33 (62%)
Gender (n; %)	
Male	28 (53%)
Female	25 (47%)
BMI (n; %)*	
Underweight	2 (4%)
Normal weight	33 (72%)
Overweight	11 (24%)
Proximity to the industry (n; %)	
≤ 5 km	39 (74%)
> 5 km	14 (26%)

* Not all participants indicated the necessary data to calculate BMI.

4.2. Urinary creatinine levels

Figure 4 shows the creatinine levels of the children organized by age category (preschool and elementary school children, figure 4.A) and by gender (figure 4.B). The WHO recommends that creatinine concentrations have to be between 0.3 g/L urine and 3 g/L urine [72]. Urine samples with too low concentrations of creatinine may be too diluted causing detection to be influenced by low levels of toxicants. In contrast, excessive creatinine levels may be related with dehydration which indicates alterations in kidney's secretion, excretion and reabsorption of the metabolites [73]. Only four samples were outside the WHO recommended values [72]. From figure 4.A, it is clear that there is a significant difference (t-test: $p < 0.05$) in creatinine median concentrations between preschool children (0.82 g/L urine) and elementary school (1.09 g/L urine) children. This result may indicate that creatinine-adjusted metabolite concentrations are age-dependent. Similarly, Barr et al. [73] found lower creatinine levels in

young children compared to adolescents. This is due to the lower lean muscle mass in younger children, which is responsible for the majority of the production of the creatinine [73]. This may explain why preschool children show lower creatinine levels than elementary school children. Hence, the use of urinary creatinine levels to adjust the OH-PAH concentrations is not consensual when comparing the exposure of different age groups. Also, female children show median concentration of 1.11 g/L urine, which is 0.15 g/L urine higher than male children. However, in this case, no significant difference was observed.

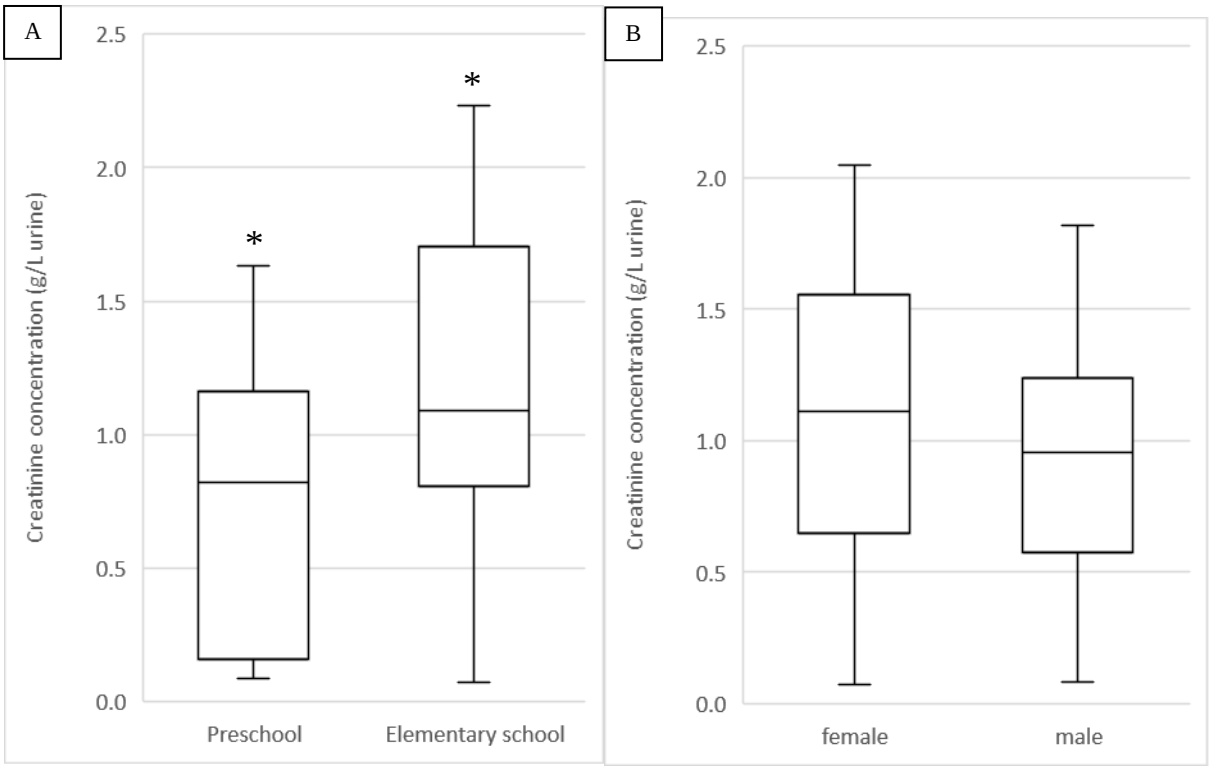

Figure 4: Boxplot of the urinary creatinine concentrations of preschool children and elementary school children (A) and female and male children (B). * Data are significantly different at $p < 0.05$ by the t-test.

4.3. Urinary OH-PAH levels

Table 6 shows the main figures of merit of the OH-PAHs (retention time, regression equations, LOD, LOQ, R^2). Calibration curves have all high linearity, with correlation coefficient of 0.999, except those of 2-OH-Flu and 1-OH-Phen (correlation coefficient of respectively 0.987 and 0.990). When comparing the LODs, 3-OH-BaP has the lowest detection limit, with a value of 0.01 µg/L, which is particularly interesting since it is the main metabolite of B(a)P, the biomarker of exposure to carcinogenic PAHs. This is followed by 1-OH-Py < 1-OH-Phen < 2-OH-Flu << 1-OH-Naph + 1-OH-Ace with LODs of respectively 0.02, 0.04, 0.05 and 3.05 µg/L.

Table 6: Calibration data of the determined OH-PAHs (A=area of the peak; C=concentration (µg/L)).

	1-OH-Naph + 1-OH-Ace	2-OH-Flu	1-OH-Phen	1-OH-Py	3-OH-B(a)P
Retention time (min)	5.88	7.80	9.58	14.40	21.49
Regression equation	A = 3719 × C– 23800	A = 765786 × C + 18682	A = 475315 × C – 833	A = 1155823 × C– 7706	A = 1462714 × C– 5421
LOD (µg/L)	3.05	0.05	0.04	0.02	0.01
LOQ (µg/L)	10.18	0.16	0.14	0.05	0.03
R^2	0.999	0.987	0.990	0.999	0.999

These calibration curves were applied to determine the urinary OH-PAH concentrations of the characterized children. A representative chromatogram of the mixed standard P9 with a concentration of 121.20 µg/L for 1-OH-Naph + 1-OH-Ace and 0.600 µg/L for 2-OH-Flu, 1-OH-Phen, 1-OH-Py and 3-OH-BaP is presented in figure 5. Here, the peaks of 1-OH-Naph + 1-OH-Ace, 2-OH-Flu, 1-OH-Phen, 1-OH-Py and 3-OH-B(a)P are clearly visible at a retention time of, respectively, 5.88, 7.80, 9.58, 14.40 and 21.49 min.

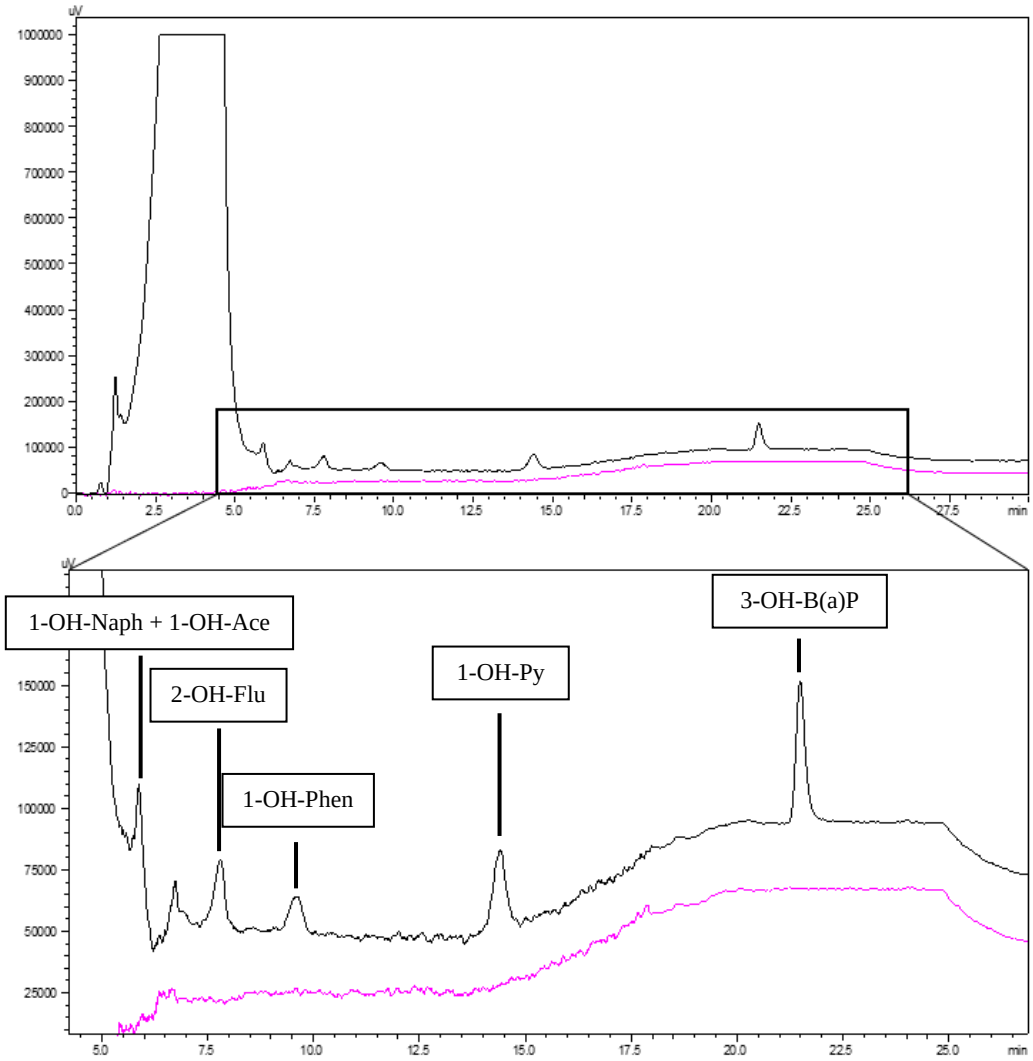

Figure 5: Chromatogram of mixed standard P9 (black) and the blank (purple) with 1-OH-Naph + 1-OH-Ace (121,20 µg/L), 2-OH-Flu (0.600 µg/L), 1-OH-Phen (0.600 µg/L), 1-OH-Py (0.600 µg/L) and 3-OH-B(a)P (0.600 µg/L) at retention times of respectively 5.88, 7.80, 9.58, 14.40 and 21.49 min.

The results of the overall urinary OH-PAH concentrations measured in the children's samples are shown in table 7. Here the median, mean, first and third quartiles ($P_{25} - P_{75}$), minimum (min) and maximum (max) ranges are presented. The attained median values are significantly different and lower than the mean values, due to the limited number of samples. Hence, the median concentrations of the metabolites are further used to compare the obtained results.

Table 7: Urinary OH-PAH concentrations (µg/g creatinine) measured in the children's samples. P_{25}: first quartile; P_{75}: third quartile; min: minimum; max: maximum.

OH-PAH	Median (µg/g creatinine)	Mean (µg/g creatinine)	(P_{25} – P_{75}) (µg/g creatinine)	Range (min – max) (µg/g creatinine)
1-OH-Naph + 1-OH-Ace	32.33	162.34	14.10 – 61.01	0.49 – 4627.18
2-OH-Flu	0.10	0.39	0.04 –0.33	0.01 – 5.30
1-OH-Phen	0.13	0.44	0.06 –0.30	0.01 – 9.66
1-OH-Py	0.08	0.13	0.03 – 0.14	0.002 – 1.42
Σ OH-PAH	69.56	325.63	31.69 – 119.02	2.29 – 9003.73

What stands out from table 7 is that the median value of 1-OH-Naph + 1-OH-Ace (32.33 µg/g creatinine) is significantly higher than the median values of the other metabolites. The median value of 1-OH-Naph + 1-OH-Ace is almost half of the median concentration of the total OH-PAHs (69.56 µg/g creatinine). Results of several previous studies reported levels of 0.11-3.838 µg/g creatinine [22]-[45]-[51]-[53]-[55]-[58]-[59]-[66]-[67]-[68] with 1-OH-Naph not being the most abundant metabolite. In addition, other studies [22]-[45]-[51]-[55]-[58]-[66]-[68] shown that the urinary concentration of 2-OH-Naph, the isomer of 1-OH-Naph which is not analysed in this study, was significantly higher than other metabolites. The presence of the metabolites of naphthalene may suggest that the children are more exposed to petrogenic sources such as erosion, oil seepage and acute petroleum spillages [2]-[3]. Murawski et al. stated that 1-OH-Naph may also originate from other naphthalene sources, such as of the insecticide carbaryl [55].

1-OH-Phen follows 1-OH-Naph + 1-OH-Ace as second highest concentration measured in the urine samples of the children (0.13 µg/g creatinine). A study conducted in China by Cheng et al. [59] found remarkably higher results, namely 1.50 µg/g creatinine, for children living in an agricultural county. However, children living near high exposed areas, such as areas with coke oven plants, showed a concentration of 2.60 µg/g creatinine. Also other studies performed in America, Czech Republic, Germany and Poland found results 1.6 – 6.5 times higher than the 1-OH-Phen levels in this study [22]-[45]-[51]-[55]-[66]. Manly the high western European cities showed superior levels of this metabolite [22]-[45]-[51]. Though, other European (Czech Republic, Germany, Spain) and American (USA) studies showed similar results with only a difference between 18 and 31% [45]-[55]-[56]-[68].

The concentrations of 2-OH-Flu (0.10 µg/g creatinine) obtained in this study are 1.4 – 9.4 times smaller than those of other studies conducted in Europe and America [22]-[51]-[55]-[68]-[66]. A study conducted in Czech Republic found similar results, with only a difference of 9% higher [45]. However, the examined subjects were new-born children. Additionally, Cheng et al. [59] reported urinary 2-OH-Flu concentrations one magnitude higher in Chinese children than those detected in Portuguese children, especially for children living near industrial sites.

The attained results of 1-OH-Py (0.08 µg/g creatinine) in the children's urine are similar as those of 2-OH-Flu and 1-OH-Phen. This value is 5.8 times lower than the reference value for non-smokers and non-occupational exposed groups (0.46 µg/g creatinine) presented by Jongeneelen et al. [28]. When comparing the urinary concentrations of 1-OH-Py with the values from literature, only two studies from Czech Republic reported lower values, namely 0.03 µg/g creatinine and 0.06 µg/g creatinine [45]-[51]. More polluted locations (including coal-industries, urbanisation, household air pollution) in countries, such as China, India, Kenia and Mexico found significant higher 1-OH-Py concentrations (0.8 µg/g creatinine – 5.98 µg/g creatinine) [53]-[58]-[59]-[67]. Reasons for the higher 1-OH-Py levels could be the use of wood as fuel to cook, cooking with solid biomass and the effect of tobacco smoke [53].

As expected and as already suggested in other works, 3-OH-B(a)P was not detected in the analysed samples [12]-[50]-[54]-[59]-[63]-[74]. This may indicate that the children are not exposed to the carcinogenic PAH benzo(a)pyrene or it could be that 3-OH-B(a)P is metabolised and mainly excreted

through other routes such as the faeces [49]. Yet, the process behind the metabolization of 3-OH-B(a)P is still unclear [49].

Figure 6 presents the median concentration of the five OH-PAHs detected in the 20 preschool children and the 33 elementary school children. When the different metabolites are compared with each other in the two school categories, it is clear that 1-OH-Naph + 1-OH-Ace is the most abundant metabolite for both preschool and elementary school children. This is followed by 2-OH-Flu > 1-OH-Phen > 1-OH-Py for preschool children and 1-OH-Phen > 2-OH-Flu > 1-OH-Py for elementary school children. Overall, the highest levels were always detected in the younger children independently of the target metabolite. Elementary school children show a smaller total urinary metabolites concentration (65.51 µg/g creatinine) than preschool children (86.78 µg/g creatinine) but this difference is not significant enough (t-test: $p > 0.05$).

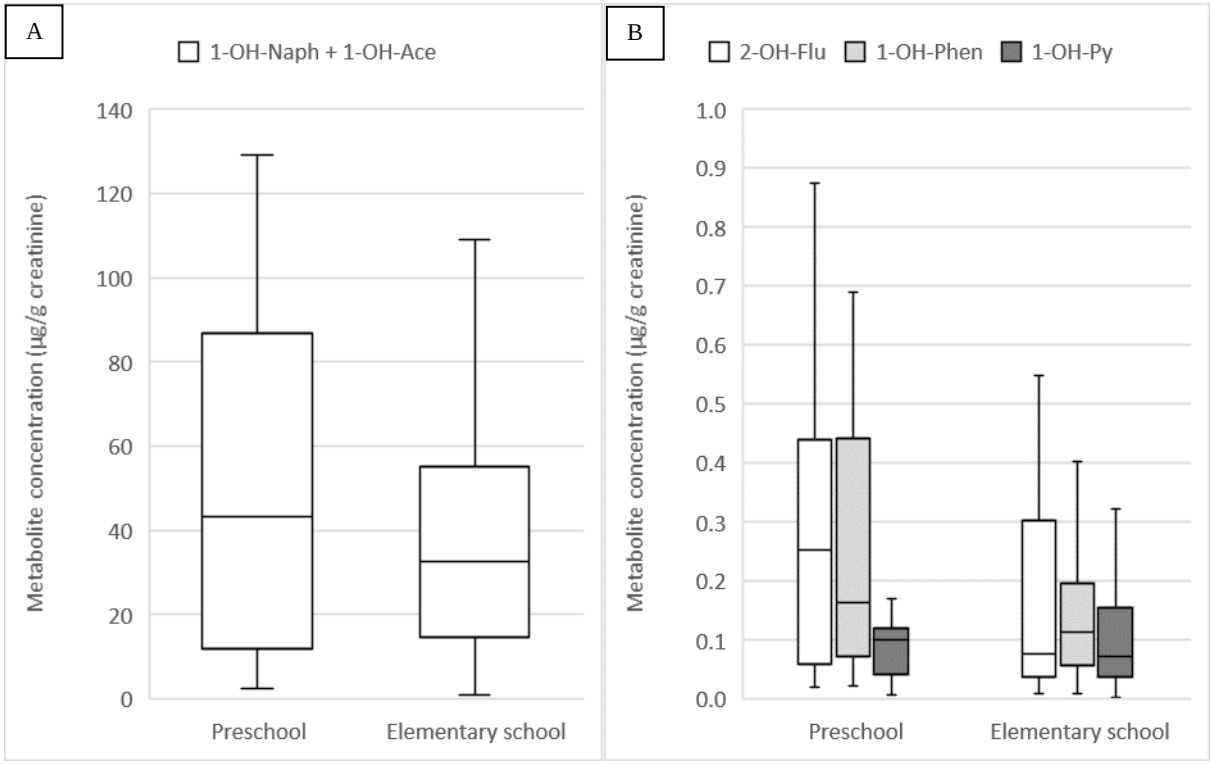

Figure 6: Boxplot of the urinary OH-PAH concentrations of preschool children and elementary school children: (A) 1-OH-Naph + 1-OH-Ace; (B) 2-OH-Flu, 1-OH-Phen and 1-OH-Py.

Moreover, it is clear that there are differences between the 1-OH-Naph + 1-OH-Ace concentrations of the preschool (43.27 µg/g creatinine) and elementary school (32.69 µg/g creatinine) children. Although, these differences are not significant (t-test: $p > 0.05$). The same can be said for 2-OH-Flu and 1-OH-Phen with preschool children exhibiting a median of 0.25 and 0.16 µg/g creatinine, while elementary school children displayed a value of 0.08 and 0.11 µg/g creatinine (3.1 and 1.5 times smaller), respectively. Similarly, no significant difference between the 1-OH-Py levels was found with a value of 0.10 and 0.07 µg/g creatinine in preschool and elementary school children, respectively. Fortunately, preschool and elementary school children do not exceed the reference value of 0.24 µmol/mol creatinine (0.46 µg/g creatinine) of 1-OH-Py for non-smokers and non-occupational exposed groups [28].

No age-dependency was found when comparing each individual metabolite concentration of the two age groups with each other. Similarly, Yu et al. [12] noticed no age-dependency, however the study was dedicated to smaller age groups (8-11 years) and data were not normalized with the urinary creatinine concentrations. An older study conducted in 2015 by Li et al. [75] observed significantly higher urinary OH-PAH values in children aged 6 years compared to children aged 3-5 years. Possible causes for this

positive age dependency may be the shift in muscle mass and metabolism of children around these ages. Yet, Chen et al. [59] found a negative age-dependency, but this was between children and adults [59]. Also, a survey performed by Murawski et al. [55] concluded that the metabolite concentrations of children between 3-5 years were double those of adolescents (aged 14 – 17 years). This may be promoted by lower creatinine concentrations in young children compared to adolescents [73], which is also the case in this study (figure 4) where preschool children show significantly (t-test: $p < 0.05$) lower creatinine concentrations (0.82 g/L urine) than elementary school children (1.09 g/L urine). Overall, 1-OH-Py is considered the biomarker of exposure to PAHs [42], and the other measured metabolite concentrations were higher in the preschool school children. Hence, it can be concluded that preschool school children are more exposed to PAHs than preschool children if no difference in the metabolization rate exists. However, no statistical correlation was observed.

Figure 7 presents the obtained data organized by gender (28 male and the 25 female children). 1-OH-Naph + 1-OH-Ace is the most abundant metabolite in both groups. Yet, in female subjects, this is followed by 1-OH-Phen > 2-OH-Flu > 1-OH-Py, while concentrations of male subjects show the following order: 1-OH-Phen > 2-OH-Flu = 1-OH-Py. In figure 7.A a clear significant difference (Mann-Whitney U test: $p < 0.05$, *) between the 1-OH-Naph + 1-OH-Ace concentration of the female subjects and the male subjects is visible. With a median value of 44.80 µg/g creatinine, the female children had a 1-OH-Naph + 1-OH-Ace value of 2.1 times higher than the male subjects. Lin et al. [54] examined the urinary 2-OH-Naph concentrations in 3 year old Taiwanese children and found a higher 2-OH-Naph concentration in male subjects. However, the focus was only set on 2-OH-Naph. Besides, no normalization with the creatinine levels of the children was performed. Additionally, no significant difference was found between the two genders. Conversely, this study found significant higher 1-OH-Naph + 1-OH-Ace concentrations in girls, which might indicate that OH-PAH levels are influenced by gender. Furthermore, in figure 7.B both the median concentrations of 2-OH-Flu and 1-OH-Py are higher in the male subjects. In fact, 2-OH-Flu concentrations for male and female children are respectively 0.11 and 0.08 µg/g creatinine, while for 1-OH-Py, they are 0.11 and 0.07 µg/g creatinine. Yet, nor the urinary concentrations of 2-OH-Flu or 1-OH-Py show a significant difference between the two groups (Mann-Whitney U test: $p > 0.05$). Both the female and male subjects do not exceed the reference value of 1-OH-Py for non-smokers and non-occupational exposed groups [28]-[42]. Poursafa et al. [65], who conducted a study on Iranian children aged 5-7 years, reported an urinary OH-Py concentration for boys and girls of respectively 0.241 µg/L and 0.279 µg/L, but these results were not normalized by the creatinine levels. In contrast with the values of 2-OH-Flu and 1-OH-Py, the urinary 1-OH-Phen concentration in the male subjects (0.12 µg/g creatinine) is almost identical to the female 1-OH-Phen concentration (0.13 µg/g creatinine).

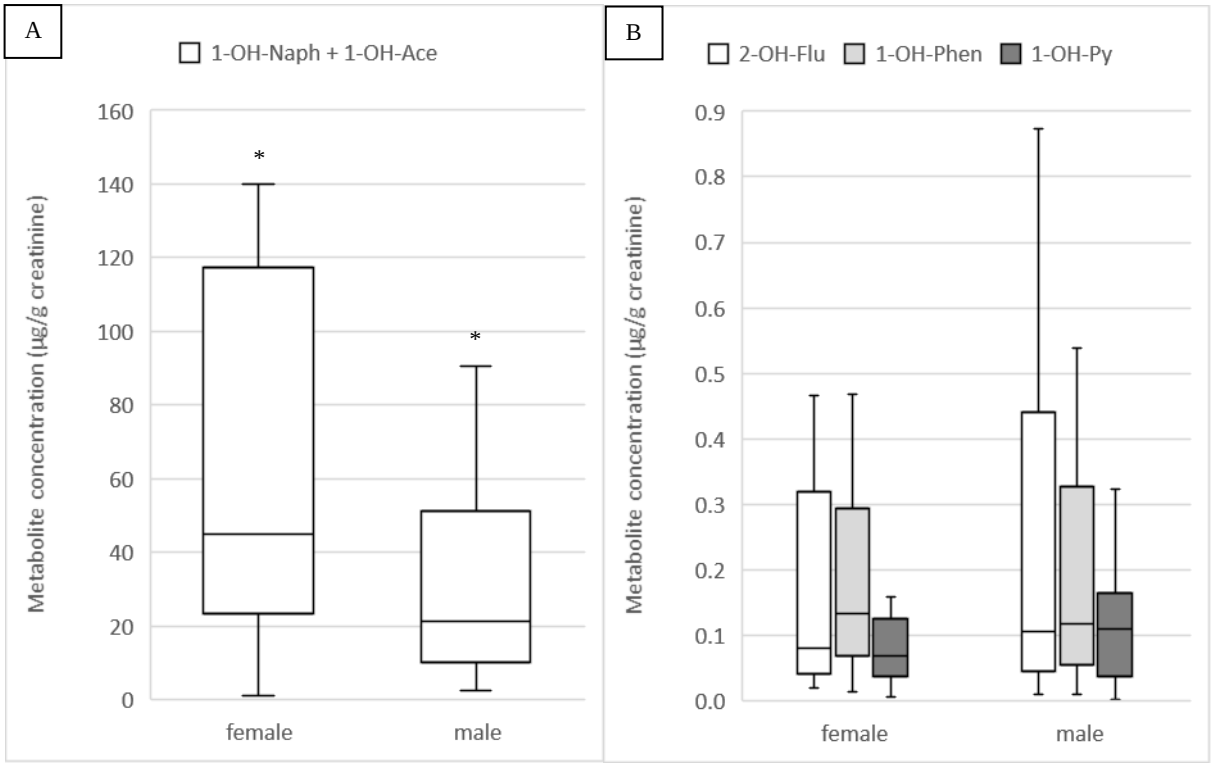

Figure 7: Boxplot of the OH-PAH concentration of male and female children: (A) 1-OH-Naph + 1-OH-Ace concentrations of these two groups; (B) 2-OH-Flu, 1-OH-Phen and 1-OH-Py concentrations. * Data are significantly different at $p < 0.05$ Mann-Whitney U test.

The median concentration of the five detected metabolites in the 39 children that live within a radius of 5 km to the industry and 14 children living far away are displayed in figure 8. For both groups, the order that describes the most abundant metabolites is 1-OH-Naph + 1-OH-Ace >> 1-OH-Phen > 2-OH-Flu > 1-OH-Py. When the concentration of these metabolites of the two groups are compared to each other, no significant differences are perceived. More specifically, children living in a radius of 5 km to the industry have a 1-OH-Naph + 1-OH-Ace concentration of 34.73 µg/g creatinine, while the other subjects 39.50 µg/g creatinine. Likewise, children living near the industry have a 1-OH-Py value of 0.08 µg/g creatinine, which is 11% lower than the children living outside a radius of 5 km, although this difference is not significant (Mann-Whitney U test: $p > 0.05$). Further, equal 1-OH-Phen concentrations are observed between the two groups (0.13 µg/g creatinine *vs.* 0.12 µg/g creatinine for children living near and further away from the industry, respectively). In contrast with other studies [12]-[50]-[51], metabolites showed a similar concentration for the children living near and outside industrial sites. Thorsen et al. [2] indicated that PAHs consisting of 3-5 aromatic rings are mainly emitted by pyrogenic sources, such as the combustion during transport. Also most industrial sites are known petrogenic sources that emit PAHs such as naphthalene, fluorene [2]. However, the concentrations in the samples of the children are generally low and similar within the different geographical areas. More information about the types of industries would be valuable to understand this profile of variation.

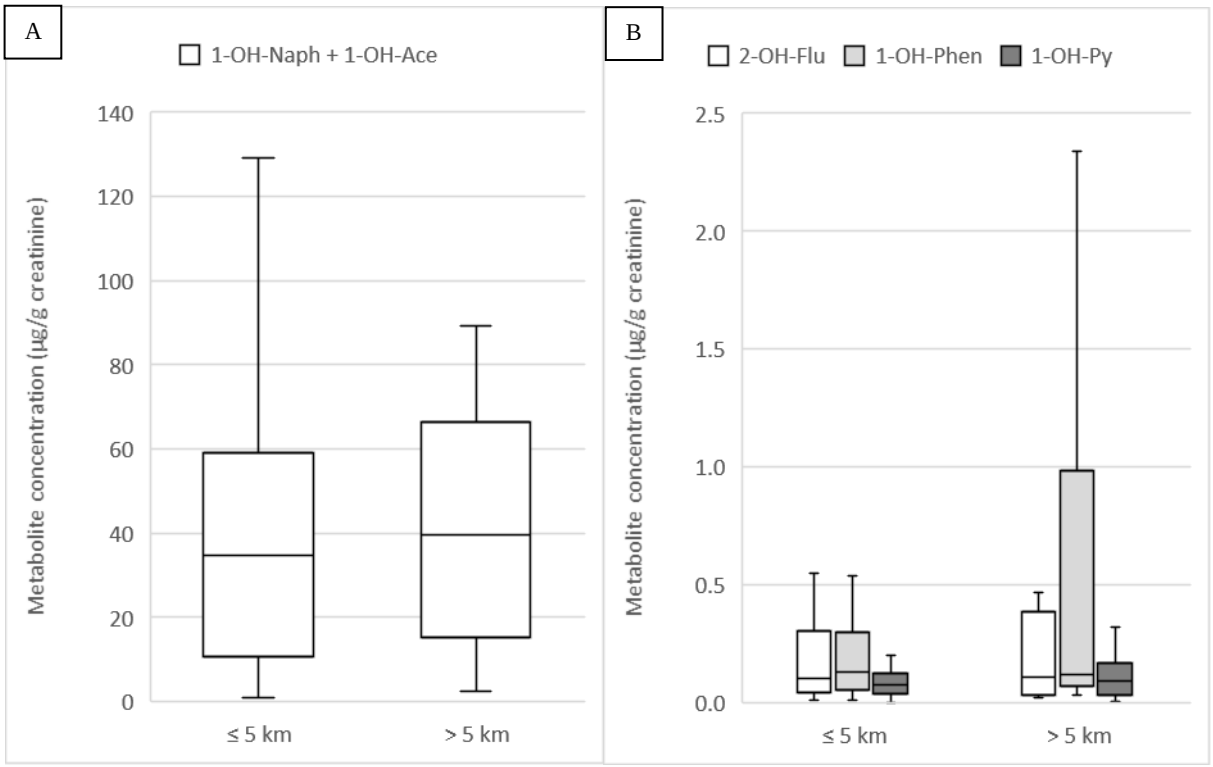

Figure 8: Boxplot of the OH-PAH concentration children living within a radius of 5 km to the industry and children living outside a radius of 5 km to the industry: (A) 1-OH-Naph + 1-OH-Ace concentrations of these two age groups; (B) 2-OH-Flu, 1-OH-Phen and 1-OH-Py concentrations.

5. Conclusion

This study assessed the exposure of children living in the centre of Portugal to PAHs by means of biomonitoring. Five metabolites, including 1-OH-Naph, 1-OH-Ace, 2-OH-Flu, 1-OH-Phen and 1-OH-Py were detected in the urine of the examined subjects; 3-OH-B(a)P was not detected. Overall, the metabolite concentrations were similar to the levels retrieved from other European children. More specifically 1-OH-Naph and 1-OH-Ace were the predominant metabolites, with median concentrations two order of magnitudes higher than the other metabolites. This may indicate that the children are more exposed to petrogenic sources. Generally, the highest levels were constantly identified in younger children independently of the target compound, although significantly differences were not detected. Also, no significant differences in age, gender and living near industry were found for 1-OH-Py, 2-OH-Flu and 1-OH-Phen. However, a significantly higher 1-OH-Naph and 1-OH-Ace concentration was perceived in female children, which suggests that metabolization rate may be influenced by the gender. Further research regarding metabolised PAHs and unmetabolized PAHs in urine and faeces are needed to comprehensively characterize the total PAHs exposure in children. Additionally, a larger population examined for a longer period of time and characterized with more extensive questionnaires (that include information about the mode of transport, exposure to tobacco smoke and full description of the diet) would be crucial to identify the main sources and propose possible mitigation actions.

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Appendices

Appendix A: Overview of urinary PAH metabolite concentrations reported in children between 2018 and April 2021.

Table I: Urinary PAH metabolite concentrations (median and range, µg/g creatinine except when indicated otherwise) reported in children (n = number of enrolled participants) in the last years (2018-2021)

Continent	Country	Place	n	Age (years)	Note	Metabolites							
						OH-Naph	OH-HFlu	OH-Phen	OH-Py	OH-B(a)P	OH-Chrysene	Σ OH-PAHs	
Asia	China	Guiyu	105	3-7	E-waste recycling town	**1-OH-Naph** 4.020 (2.820-6.120) µg/L Urine	**2-OH-HFlu** 1.020 (0.822-1.214) µg/L Urine	**1-OH-HPhen** 0.980 (0.912-1.072) µg/L Urine				6.322 (5.104-9.092) µg/L Urine	[50]
		Haojiang	98	3-7	Reference area	**1-OH-Naph** 3.040 (2.360-4.725) µg/L Urine	**2-OH-HFlu** 0.853 (0.703-0.928) µg/L Urine	**1-OH-HPhen** 0.994 (0.918-1.155) µg/L Urine				5.789 (4.675-7.129) µg/L Urine	
		Shenzhen	166	8-11	Important industrial centre	**1-OH-Naph** 0.86 ± 1.29 µg/L Urine **2-OH-Naph** 1.95 ± 2.14 µg/L Urine	**2-OH-HFlu** 0.58 ± 0.39 µg/L Urine	**1-OH-HPhen** 0.29 ± 0.23 µg/L Urine **2-OH-HPhen** 0.25 ± 0.17 µg/L Urine **4-OH-HPhen** 0.10 ± 0.13 µg/L Urine **9-OH-HPhen** 0.10 ± 0.09 µg/L Urine	**1-OH-HPy** 0.44 ± 0.40 µg/L Urine			4.57 ± 3.55 µg/L Urine	[12]
					Suburban regions							4.05 µg/L Urine	
					Urban regions							5.29 µg/L Urine	
		Guangzhou	50	8-9	3 days 3th day: highest PAH air pollution	**1-OH-Naph** Day 1: 1.894 µg/L Urine Day 2: 2.452 µg/L Urine Day 3: 2.728 µg/L Urine **2-OH-Naph** Day 1: 4.737	**2-OH-HFlu and 3-OH-HFlu** Day 1: 0.306 µg/L Urine Day 2: 0.484 µg/L Urine Day 3: 0.435 µg/L Urine	**1-OH-HPhen and 9-OH-HPhen** Day 1: 0.185 µg/L Urine Day 2: 0.257 µg/L Urine Day 3: 0.290 µg/L Urine	**1-OH-HPy** Day 1: 0.133 µg/L Urine Day 2: 0.164 µg/L Urine Day 3: 0.168 µg/L Urine				[63]

					µg/L Urine Day 2: 6.584 µg/L Urine Day 3: 7.414 µg/L Urine		**2-OH-HPhen and 3-OH-HPhen** Day 1: 0.243 µg/L Urine Day 2: 0.329 µg/L Urine Day 3: 0.292 µg/L Urine **4-OH-HPhen** Day 1: 0.0442 µg/L Urine Day 2: 0.0806 µg/L Urine Day 3: 0.0735 µg/L Urine				
	Qingdao	38	< 1							10.23 (6.44- 16.25)	[74]
	Investigated area	72	6-12	5 coke oven plants, 18 controlled-exhaust emission sites, 30 fugitive exhaust emission sites, 8 directly discharged waste water sites	**1-OH-Naph** 3.74 ± 9.62 **2-OH-Naph** 2.75 ± 11.9	**2-OH-HFlu** 5.53 ± 10.88	**1-OH-HPhen** 2.60 ± 3.41 **2-OH-HPhen** 0.89 ± 1.20 **3-OH-HPhen** 2.05 ± 1.85 **4-OH-HPhen** 0.23 ± 0.29	**1-OH-HPy** 1.46 ± 2.18	**6–OH–Chrysene** 0.01 ± 0.02	22.57 ± 29.96	[59]
	Control area	149		agricultural county	**1-OH-Naph** 1.28 ± 1.70 **2-OH-Naph** 1.57 ± 4.99	**2-OH-HFlu** 4.25 ± 6.19	**1-OH-HPhen** 1.50 ± 1.31 **2-OH-HPhen** 0.66 ± 0.78 **3-OH-HPhen** 1.43 ± 1.03 **4-OH-HPhen** 0.15 ± 0.26	**1-OH-HPy** 0.93 ± 1.55	**6–OH–Chrysene** 0.01 ± 0.03	13.41 ± 14.45	
Taiwan		187	3	Male	**2-OH-Naph** 4.89 µg/L Urine						[54]
		266		female	**2-OH-Naph** 2.51 µg/L Urine						
India	Villupuram and Nagapattinam districts	800	< 1	Communities depended on solid biomass fuel for cooking	**2-OH-Naph** 23.08 (3.51-102.8)			**1-OH-HPy** 4.22 (0.73-17.61)			[58]
Iran	Sabzevar city	200	5-7	Located next to crowded highway				0.257 µg/L Urine			[52]

					walking				0.131 µg/L Urine		
					Motorcycle				0.775 µg/L Urine		
					Car				0.296 µg/L Urine		
					Bus				0.560 µg/L Urine		
			200	5-7	Boys				0.241 µg/L Urine		[64]
					girls				0.279 µg/L Urine		
		Isfahan	56	7-13	Normal weight + no cardiometabolic risk factor	**1-OH-Naph** 0.7554 µg/L Urine **2-OH-Naph** 0.7330 µg/L Urine		**9-OH-HPhen** 0.06827 µg/L Urine	**1-OH-HPy** 0.03915 µg/L Urine	1.6148 µg/L Urine	[65]
			47	9-13	Normal weight + at least one cardiometabolic risk factor	**1-OH-Naph** 0.8003 µg/L Urine **2-OH-Naph** 1.0811 µg/L Urine		**9-OH-HPhen** 0.07998 µg/L Urine	**1-OH-HPy** 0.06385 µg/L Urine	2.03930 µg/L Urine	
			36	7-13	Excess weight + no cardiometabolic risk factor	**1-OH-Naph** 0.8182 µg/L Urine **2-OH-Naph** 1.0809 µg/L Urine		**9-OH-HPhen** 0.09797 µg/L Urine	**1-OH-HPy** 0.05513 µg/L Urine	2.1329 µg/L Urine	
			47	7-15	Excess weight + at least one cardiometabolic risk factor	**1-OH-Naph** 0.8777 µg/L Urine **2-OH-Naph** 1.3242 µg/L Urine		**9-OH-HPhen** 0.08990 µg/L Urine	**1-OH-HPy** 0.08094 µg/L Urine	2.356 µg/L Urine	
Europe	Czech Republic	Ceske Budejovice and Most	330	New born		**1-OH-Naph** 0.24 (0.03-13.31) **2-OH-Naph** 6.09 (0.46-41.12)	**2-OH-HFlu** 0.17 (0.03-1.06)	**1-OH-HPhen** 0.45 (0.01-4.86) **2-OH-HPhen** 0.21 (0.01-3.08) **3-OH-HPhen** 0.05 (0.01-0.57) **4-OH-HPhen** 0.03 (0.01-0.30)	**1-OH-HPy** 0.06 (0.02-0.94)	8.48 (0.46-47.62)	[51]

							9-OH-HPhen 0.37 (0.01-3.36)					
	Ceske Budejovice district	87	New born	Ambient air in Karvina had higher PAH concentrations	**1-OH-Naph** 0.11 ± 0.18 **2-OH-Naph** 2.95 ± 2.38	**2-OH-HFlu** 0.11 ± 0.10	**1-OH-HPhen** 0.16 ± 0.16 **2-OH-HPhen** 0.11 ± 0.11 **3-OH-HPhen** 0.02 ± 0.02 **4-OH-HPhen** 0.06 ± 0.13 **9-OH-HPhen** 0.36 ± 0.69	**1-OH-HPy** 0.03 ± 0.04	**3-OH-B(a)P** 0.45	**6–OH–Chrysene** 0.01	3.88 ± 2.90	[45]
	Karvina district	55			**1-OH-Naph** 0.70 ± 0.56 **2-OH-Naph** 5.37 ± 4.44	**2-OH-HFlu** 0.26 ± 0.16	**1-OH-HPhen** 0.84 ± 0.67 **2-OH-HPhen** 0.42 ± 0.23 **3-OH-HPhen** 0.08 ± 0.06 **4-OH-HPhen** 0.17 ± 0.48 **9-OH-HPhen** 1.74 ± 2.29	**1-OH-HPy** 0.13 ± 0.11	**3-OH-B(a)P** 0.45	**6–OH–Chrysene** 0.01	9.71 ± 5.60	
Germany		516	3-17		**1-OH-Naph** 0.688 (0.617-0.765) **2-OH-Naph** 3.706 (3.429-4.005)	**2-OH-HFlu** 0.338 (0.304-0.376)	**1-OH-HPhen** 0.122 (0.114-0.130) **2-OH-HPhen** 0.075 (0.070-0.080) **3-OH-HPhen** 0.115 (0.109-0.122) **4-OH-HPhen** 0.040 (0.036-0.043) **9-OH-HPhen** 0.050 (0.046-0.055)	**1-OH-HPy** 0.087 (0.082-0.092)				[55]
		99	3-5		**1-OH-Naph** 1.086 **2-OH-Naph** 5.736		**1-OH-HPhen** 0.208 **2-OH-HPhen** 0.108 **3-OH-HPhen** 0.175 **4-OH-HPhen** 0.080 **9-OH-HPhen** 0.105	**1-OH-HPy** 0.125				

			200	6-11		**1-OH-Naph** 0.814 **2-OH-Naph** 3.573		**1-OH-HPhen** 0.135 **2-OH-HPhen** 0.080 **3-OH-HPhen** 0.127 **4-OH-HPhen** 0.042 **9-OH-HPhen** 0.053	**1-OH-HPy** 0.083		
	Poland	Krakow	218	3	One of the most polluted cities in Poland	**1-OH-Naph** 3.838 (3.413-4.315) **2-OH-Naph** 8.449 (7.652-9.33)	**2-OH-HFlu** 0.942 (0.879-1.01) **3-OH-HFlu** 0.366 (0.335-0.4) **9-OH-HFlu** 0.998 (0.923-1.079)	**1-OH-HPhen** 0.639 (0.597-0.683) **2-OH-HPhen** 0.169 (0.159-0.181) **3-OH-HPhen** 0.403 (0.374-0.434) **4-OH-HPhen** 0.104 (0.096-0.112)	**1-OH-HPy** 0.357 (0.331-0.385)	17.626 (16.192-19.186)	[22]
	Spain	Valencian Region	566	5-12		**1-OH-Naph** 0.4 ± 1.4 **2-OH-Naph** 11 ± 24	0.24 ± 0.89	**1-OH-HPhen** 0.10 ± 0.28 **2-OH-HPhen** 0.04 ± 0.08 **3-OH-HPhen** 0.09 ± 0.15 **4-OH-HPhen** 0.02 ± 0.05 **9-OH-HPhen** 0.06 ± 0.10	**1-OH-HPy** 0.10 ± 0.10	0.088 ± 0.166 µmol/g creatinine	[68]
America	USA		2097	6-11	50 states + districts of Colombia	**1-OH-Naph** 1.202 (1.077-1.341) µg/L Urine **2-OH-Naph** 2.816 (2.642-3.002) µg/L Urine	**2-OH-HFlu** 0.178 (0.167-0.190) µg/L Urine **3-OH-HFlu** 0.077 (0.070-0.084) µg/L Urine **9-OH-HFlu** 0.174 (0.160-0.189) µg/L Urine	**1-OH-HPhen** 0.099 (0.092-0.107) **2-OH-HPhen** 0.043 (0.040-0.047) **3-OH-HPhen** 0.076 (0.070-0.082)	**1-OH-HPy** 0.127 (0.116-0.140)		[56]

			2642	12-19		**1-OH-Naph** 2.213 (1.993-2.458) µg/L Urine **2-OH-Naph** 4.804 (4.394-5.253) µg/L Urine	**2-OH-HFlu** 0.313 (0.283-0.345) µg/L Urine **3-OH-HFlu** 0.135 (0.121-0.151) µg/L Urine **9-OH-HFlu** 0.279 (0.258-0.302) µg/L Urine	**1-OH-HPhen** 0.136 **2-OH-HPhen** 0.062(0.056-0.070) **3-OH-HPhen** 0.100 (0.094-0.107)	**1-OH-HPy** 0.156 (0.142-0.171)		
		Northern California	99	6-7	PAH and BMI relation	2.150 (1.830-2.530)	0.586 (0.532-0.646)	0.255 (0.232-0.281)	0.104 (0.0927-0.118)	0.0197 (0.0171-0.0227) mol/g creatinine	[66]
			305	>= 7		2.050 (1.850-2.270)	0.496 (0.469-0.526)	0.228 (0.215-0.242)	0.0951 (0.0885-0.102)	0.0191 (0.0175-0.0209) mol/g creatinine	
		San Francisco Bay	431	7.3 (6.4-8.1)	Girls	**1-OH-Naph** 1.510 (1.320-1.710) µg/L Urine **2-OH-Naph** 1.570 (1.420-1.740) µg/L Urine	**2-OH-HFlu** 0.140 (0.130-0.150) µg/L Urine **3-OH-HFlu** 0.0618 (0.0571-0.0668) µg/L Urine **9-OH-HFlu** 0.172 (0.160-0.186) µg/L Urine	**1-OH-HPhen** 0.0755 (0.0701-0.0814) µg/L Urine **2-OH-HPhen** 0.0317 (0.0296-0.0341) µg/L Urine **3-OH-HPhen** 0.0664 (0.0616-0.0716) µg/L Urine	**1-OH-HPy** 0.0742 (0.0689-0.0800) µg/L Urine		[57]
	Mexico	Urban area (low vehicular traffic	20	6-12	San Luis Potosí state				0.10 ± 0.08		[67]
		Urban area (high vehicular traffic)	25						0.17 ± 0.10		
		Municipal landfill	30						0.19 ± 0.15		
		Brickyard Industry	30						0.96 ± 0.29		
		Biomass combustion	30						5.98 ± 2.41		
Africa	Kenia	Nairobi	32	6	**HIV infected** Type of cooking fuel:						[53]

		-Wood	5.0
		-Propane	1.0
		-Charcoal	1.2
		-Paraffin	1.2
		Smoker in household:	
		-Yes	1.2
		-No	1.2
		Cooks inside living area:	
		-Yes	1.0
		-No	1.7
		Non-electric lamp for lighting:	
		-Yes	0.8
		-No	1.5
		Garbage burned nearby with smoke entering kitchen:	
		-Yes	1.4
		-No	1.0
	43	**HIV uninfected**	
		Type of cooking fuel:	
		-Wood	
		-Propane	0.8
		-Charcoal	1.4
		-Paraffin	1.2
		Smoker in household:	
		-Yes	1.9
		-No	1.0
		Cooks inside living area:	
		-Yes	1.2
		-No	0.8
		Non-electric lamp for lighting:	
		-Yes	1.20
		-No	1.02
		Garbage burned nearby with smoke entering kitchen:	
		-Yes	1.2
		-No	1.2