



Facultade de Ciencias
UNIVERSIDADE DA CORUÑA

Chemistry Degree

Final dissertation report

*Oral bioavailability of Polycyclic Aromatic Hydrocarbons in
atmospheric particulate matter*

*Biodisponibilidade oral de Hidrocarburos Aromáticos Policíclicos
en material particulado atmosférico*

*Biodispoñibilidade oral de Hidrocarburos Aromáticos
Policíclicos en materia particulada atmosférica*

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Academic year: 2018-2019 - Call: July.



UNIVERSIDADE DA CORUÑA

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That the work described in the present report, titled “Oral bioavailability of PAHs in particulate matter” was carried out under their guidance by Ms. Paula Gómez Meijide in the Faculty of Science laboratories and, once concluded, authorize its presentation as a *Final Degree Dissertation Report*.

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Acknowledgements.

First of all, to the Applied Analytical Chemistry Department for allowing me to develop this study with them, specially to my advisors, Jorge and Puri, for teaching me with patience, giving me always support and, most important, confidence.

To Joel, for being there day after day available at any time for anything I needed, for teaching me things every day and for making the laboratory work much more pleasant. I am going to miss all the good times, that were not few, and of course complaining together when an equipment did not work.

To Vero, Carmen and Fany, for making me feel at home from the first day and making coffee time, the most expected moment in the morning, because for me, you are the pillar of this laboratory. For shearing every day a little bit of your life and making me laugh, because I have learnt that everything in this life has its "chupi" part. In particular, express my gratitude to Fany for the morning conversations and also for all the things you have done for me during these months.

To all the people with whom I have coincided in the lab, particularly to the teachers, because despite being a student, they have been treated me as a colleague.

To my "Cenas Química", you are the best of these years, because of the millions of sheared moments, the lost hours in the cafeteria, all the nights giving war, and without doubt, thank you Grobas for all your notes, none of this would have been possible without you. Because despite being such different people, I can say that I have made great friends these years.

To my friends and workmates for supporting me.

And finally, to my family, my parents, my grandparents and Miguel, for having always been there, because you taught me to fight in difficult moments and also that everything is possible with work, and without you nothing would make sense.

Thank you.

Abbreviations.

- Ace: acenaphthene.
- Ant: anthracene.
- BaA: benzo(a)anthracene.
- BaP: benzo(a)pyrene.
- BbF: benzo(b+j)fluoranthene.
- BC: Black Carbon.
- BeP: benzo(e)pyrene.
- BghiP: benzo(ghi)perylene.
- BkF: benzo(k)fluoranthene
- COV: Organic Volatile Compounds.
- Chry: chrysene
- DBahA: dibenzo(a,h)anthracene.
- EC: Elementary Carbon.
- Fl: fluorene.
- Ft: fluoranthene.
- GC: Gas Chromatograph.
- HAPs: Hidrocarburos Aromáticos Policíclicos.
- HPLC: High Performance Liquid Chromatography.
- HPLC-FD: High Performance Liquid Chromatography coupled with Fluorescence Detection.
- IP: indene(1,2,3-cd)pyrene.
- LOD: Limit of Detection.
- LOQ: Limit of Quantification.
- MASE: Membrane-Assisted Solvent Extraction.
- MWCO: Molecular Weight Cut-off.
- Naph: naphthalene.
- OC: Organic Carbon.
- PAHs: Polycyclic Aromatic Hydrocarbons.
- Phe: phenanthrene.
- PHWE: Pressurized Hot Water Extraction.
- PIPES: 1,4-Piperazinediethanesulfonic acid sodium salt.
- PM: Particulate Matter.
- PP: polypropylene.

- PTV-GC-MS/MS: Programmed Temperature Vaporization-Gas Chromatography-Ion Trap tandem Mass spectrometry detection.
- Pyr: pyrene.
- SWE: Subcritical Water Extraction.
- TSP: Total Suspended Particulate.
- USEPA: United States Environmental Protection Agency.
- VALLME: Vortex Assisted Liquid-Liquid Microextraction.

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1.1 Abstract.

The transport of deposited particles in lung during breathing to the gastrointestinal tract is one of the clearance mechanism that may occur into the respiratory system. In the context of human health-risk assessment, oral bioavailability refers to the pollutant fraction that diffuses across the gastrointestinal tract and reach the systemic circulation (blood).

The main aim of this research is the development of a novel *in-vitro* testing to assess the oral bioavailability of polycyclic aromatic hydrocarbons (PAHs) in PM₁₀ samples at urban site of A Coruña, and the accurately toxicity prediction of PAHs in PM₁₀ using bioavailable concentrations. *In-vitro* oral bioavailability test involves the use of pepsin solution for simulated gastric digestion (37 °C, 150 rpm, 120 min) and pancreatin/bile salts solution for simulated intestinal digestion (37 °C, 150 rpm, 120 min). During simulated intestinal digestion, a dialysis membrane of 10 kDa MWCO filled PIPES (1,4-Piperazinediethanesulfonic acid sodium salt) solution (pH 7.5) was used to simulate cell walls of the intestine.

Total PAHs concentrations of the samples were necessary to determine in order to obtain bioavailable percentages, by using an already developed new green analytical method. Low oral bioavailability ratios (< 1 %) were found for several PAHs such fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(e)pyrene and benzo(k)fluoranthene.

Key words: PM₁₀, oral bioavailability, PAHs, dyazability, risk assessment.

1.2 Resumen.

El transporte de las partículas depositadas en los pulmones durante la respiración al tracto intestinal es uno de los mecanismos de despeje que tienen lugar en el sistema respiratorio. En el contexto humano de la evaluación del riesgo de la salud, la biodisponibilidad oral hace referencia a la fracción de contaminante que se difunde a través del tracto gastrointestinal y llega al sistema circulatorio.

El principal objetivo de esta investigación es el desarrollo de un nuevo método *in-vitro* para evaluar la biodisponibilidad oral de hidrocarburos aromáticos policíclicos (HAPs) en muestras de PM₁₀ procedentes de una zona urbana de la ciudad de A Coruña, y de la predicción apropiada de la toxicidad de los HAPs en PM₁₀ utilizando las concentraciones biodisponibles. El estudio de biodisponibilidad oral *in-vitro* implica el uso de pepsina para realizar la simulación gástrica (37 °C, 150 rpm, 120 min) y de pancreatina y sales biliares para la simulación de la digestión intestinal (37 °C, 150 rpm, 120 min). Durante la simulación de la digestión intestinal, una membrana de diálisis de 10kDa MWCO que contiene una disolución de PIPES (1,4-Piperazinediethanesulfonic acid sodium salt) a pH 7,53 se emplea para simular las paredes celulares del intestino.

Las concentraciones totales de HAPs de las muestras se determinaron para obtener los porcentajes de biodisponibilidad utilizando un método de análisis verde ya desarrollado. Se obtuvieron bajos porcentajes de biodisponibilidad (< 1 %) para algunos HAPs, como para el fluoranteno, pireno, benzo(a)antraceno, criseno, benzo(e)pireno y benzo(k)fluoranteno.

Palabras clave: PM₁₀, biodisponibilidad oral, HAPs, dializados, evaluación de riesgo.

1.3 Resumo.

O transporte das partículas depositadas nos pulmóns durante a respiración ó tracto intestinal é uno dos mecanismos de despeixe que teñen lugar no sistema respiratorio. No contexto humano da avaliación do risco da saúde, a biodispoñibilidade oral fai referencia á fracción de contaminante que se difunde a través do tracto gastrointestinal e chega ó sistema circulatorio.

O principal obxectivo desta investigación é o desenvolvemento dun novo método *in-vitro* para a avaliación da biodispoñibilidade oral de hidrocarburos aromáticos policíclicos (HAPs) en mostras de PM₁₀ procedentes dunha zona urbana da cidade de A Coruña, e da predicción apropiada da toxicidade dos HAPs en PM₁₀ utilizando as concentracións biodispoñibles. O estudo de biodispoñibilidade oral *in-vitro* implica o uso de pepsina para realizar a simulación gástrica (37 °C, 150 rpm, 120 min) e de pancreatina e sales biliares para a simulación da dixestión intestinal (37 °C, 150 rpm, 120 min). Durante a simulación da dixestión intestinal, unha membrana de diálise de 10kDa MWCO que contén unha disolución de PIPES (1,4-Piperazinediethanesulfonic acid sodium salt) a pH 7,53 empréase para simular as paredes celulares do intestino.

As concentracións totales de HAPs das mostras foron determinadas para obter as porcentaxes de biodispoñibilidade utilizando un método de análise verde xa desenvolvido. Baixas porcentaxes de biodispoñibilidade (< 1 %) foron obtidas para algúns HAPs, como para o fluoranteno, pireno, benzo(a)antraceno, criseno, benzo(e)pireno e benzo(k)fluoranteno.

Palabras clave: PM₁₀, biodispoñibilidade oral, HAPs, dializados, avaliación de risco.

2. Introduction.

2.1 Particulate matter.

Particulate Matter (PM) is the combination of solid and liquid materials (excluding pure water) suspended in the atmosphere that has different nature, composition, properties, effects, number, shape and size, which can fluctuate between $0.001 - 100 \mu\text{m}^3$. It is considered a complex system in continuous evolution due to the big amount of chemical species that constitute the particulate matter (López-Mahía, 2016).

PM can be classified independently according to their mechanism of formation, their emission sources and their size. The suspended particles can be primary or secondary pollutants. If they are directly emitted to the atmosphere and maintain their initial chemical structure, they are called primary. On the other hand, secondary particles are originated in the atmosphere as a result of chemical reactions with other substances called gas precursors. The most important gas precursors presented in the atmosphere are SO_2 , NO_x and Organic Volatile Compounds (COV).

According to their emission sources, it is possible to differentiate the natural and the anthropogenic ones. In natural sources it should be pointed out the mineral fraction originated by the natural floor emissions, a part from the volcanic ones, and the particles generated from the sea's surface, called marine aerosol. Nevertheless, the anthropogenic sources catch the attention and interest because they are the ones which occur close to the population, in fact, they are generated in urban and industrial areas. Traffic emissions constitute the main source of primary particles, although it is important to consider the wide variety of industrial activities, specially the combustion of coal and another ones such as the foundry of metals like copper or zinc, the production of cement and pottery. Furthermore, there are domestic actions which contribute with the anthropogenic emissions (MeteoGalicia y Laboratorio de Medio Ambiente de Galicia, 2017).

As it was mentioned before, particulate matter encompasses an extensive range of sizes arising from their diameter, including: coarse particles or $\text{PM}_{2.5-10}$ (with a diameter between $2.5 - 10 \mu\text{m}$), fine particles or $\text{PM}_{2.5}$ (with a diameter less than or equal to $2.5 \mu\text{m}$), ultrafine particles or $\text{PM}_{0.1}$ (with a diameter less than or equal to the $0.1 \mu\text{m}$) and nanoparticles or $\text{PM}_{0.05}$ (with a diameter less than or equal to $0.05 \mu\text{m}$) (Hester *et al.*, 2016).

In addition, it is important to consider that the composition of the particulate matter is a wide ranging, being the mineral matter and the marine spray the ones with a highest influence. But it is also necessary to take into account the compounds derived from sulphur, carbon and nitrogen:

- Mineral matter:

They are mainly primary particles that come from the wind action over the land surface, being predominant in arid areas. Their chemical composition fluctuate depending on their section, and mainly exhibit quartz, calcium and magnesium carbonates, feldspars, aluminosilicates, sulphates, phosphates and metallic oxides. This group provides the majority fraction in terms of mass of particulate matter.

- Marine sprays:

They are primary natural particles derived from oceans and seas, which are composed of marine salt (NaCl), apart from sodium and magnesium chlorides and sulphurs. It is the group with the second greatest influence in terms of emissions.

- Compounds derived from sulphur:

Their principal component is sulphur and they have a biogenic origin, but also an anthropogenic one, coming from the fuels burning. Inorganic compounds are included, and organic ones of secondary origins are included too.

- Compounds derived from nitrogen:

They are the ones whose main component is nitrogen, and their main emission sources are, on the one hand naturals, such as the land, biomass combustion and microorganism's emissions, and on the other hand, anthropogenics, like farming and stockbreeding.

- Carbon compounds:

The group includes the elementary carbon (EC), also named as black carbon (BC) and the organic carbon (OC). The black carbon is directly emitted to the atmosphere from the incomplete combustion of coal, gas or biomass, whereas the organic carbon can be primary or being formed different precursors (López-Mahía, 2016).

2.2 Legislation.

Until 1972, there was no environmental regulation related to the atmospheric particles. It was then when appeared the first one, the Law 38/1972 (December, 1972) of Protection of the atmospheric ambient which considered the Total Suspended Particulate (TSP) as a quality air parameter (García-Gaico, 2013). However, the current legislation about quality and evaluation of the air was born with de Directive 96/62/CE, knowing as the Mother Directive, which adopted a general approach and fixed some criterions, objectives and evaluation techniques.

In the following years, the Mother Directive was supplemented with four extra directives, named the Daughter Directives, developing different regulation for different air pollutants (*Marco Legal de la Calidad del Aire*, 2019):

- The Directive 1999/30/CE or 1st Daughter Directive (April, 1999) related to limit values of SO₂, NO₂, NO and lead air particles.
- The Directive 2000/69/CE or 2nd Daughter Directive (November, 2000) related to the limit values of benzene and CO in the air.
- The Directive 2002/3/CE or 3rd Daughter Directive (February, 2002) related to the ozone in the air.
- The Directive 2004/107/CE or 4th Daughter Directive (December, 2004) related to arsenic, cadmium, mercury, nickel and aromatic polycyclic hydrocarbons in the air.

Nowadays, the European regulation of air quality comes from the following Directives (*Normativa europea*, 2019)

- The Directive 2004/107/CE of the European Parliament and the Council (December, 2004) also known as the 4th Daughter Directive, is the only one of the Original Frame that is still in effect.
- The Directive 2008/50/CE of the European Parliament and the Council (May, 2008) related to the environmental air quality and an European clean atmosphere, which was approved with the aim of reducing the atmospheric pollution and maintaining a good air quality. It also added regulation for new pollutants such as the PM_{2.5} and some rules and guidelines from the World Health Organization (WHO).
- The Decision 2011/850/UE (December, 2015) addressed the information interchange and notification of the air quality in all the countries of the European Union.
- The Directive 2015/148/CE (August, 2015) modified some appendix of 2004/107/CE and 2008/50/CE Directives, where were established particular reference methods, data validations and the location of certain measuring stations.
- The Directive 2016/2284/UE of the European Parliament and the Council (December, 2016) related to the reduction of national emissions of certain atmospheric pollutants, forcing to all the components of the European Union not to exceed annually in 2020 some fixed values of sulphur dioxide, nitrogen oxides, organic volatile compounds and ammonia.

At the national level, the legislation in force is the next one (Ministerio de Agricultura, Pesca y Alimentación, 2017):

- The Law 34/2007 (November, 2007) of the air quality and protection of the atmosphere, that update the legal bases for developments related to the evaluation and management of the air quality in Spain, and whose main aim is to achieve optimal levels of air quality in order to avoid, prevent and reduce the risks and negative effects on the human's health and the environment.
- The Royal Decree Law 102/2011 (January, 2011) related to the improvement of the air quality, fixing limit values for different air pollutants.

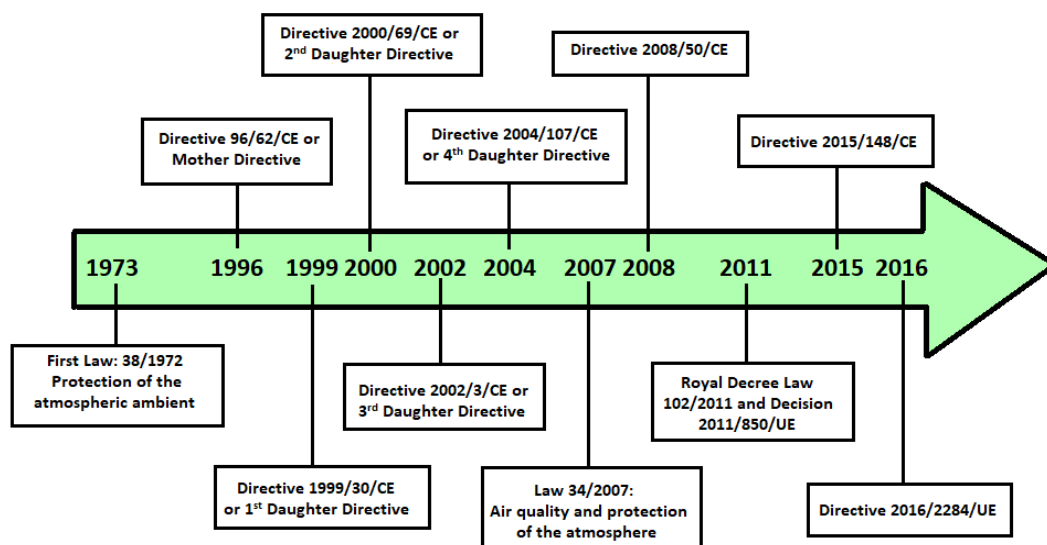


Figure 1. Legislation diagram.

Previously to the evaluation of the air quality, the territory of Spain is divided into some areas based on homogeneity criteria of emission and concentration of pollutants. In each area is carried out the evaluation and management of the air quality by using some measuring stations. In these places are controlled the fixed parameters in the Royal Decree Law 102/2011, with limit and target values (*Tables 1 and 2*) (Ministerio de Agricultura, Pesca y Alimentación, 2017).

Pollutant	Average of the time measured	Limit value	Maximum number of surpasses	Application year
SO ₂	1 hour	350 µg/m ³	< 24 hours/year	2005
	24 hours	125 µg/m ³	< 3 days/year	
NO ₂	1 hour	200 µg/m ³	< 15 hours/year	2010
	1 year	40 µg/m ³		
PM ₁₀	24 hours	50 µg/m ³	< 35 days/year	2005
	1 year	40 µg/m ³		
PM _{2.5}	1 year	25 µg/m ³		2015
Pb	1 year	25 µg/m ³		2015
C ₆ H ₆	1 year	5 µg/m ³		2010

Table 1. Limit values of some pollutants.

Pollutant	Average of the time measured	Target value	Maximum number of surpases	Application year
O ₃	Daily maximum of 8hours mobile average	120 µg/m ³	< 25 days/year (in an average of 3 years)	2010
As	1 year	6 ng/m ³		2013
Cd	1 year	5 ng/m ³		2013
Ni	1 year	20 ng/m ³		2013
B(a)p	1 year	1 ng/m ³		2013

Table 2. Target values of some pollutants.

2.3 Health effects associated to particulate matter.

In the last decade, hundreds of scientific publications associated to the particulate matter exposure have been published, providing toxicological and epidemiological effects. Although the particulate matter affects to the whole population, the groups that have developed a high sensitive are the elderly ones besides the unborn babies and very young children (World Health Organization, 2010).

The particulate matter exposure is considered the reason of the increment of hospital admissions, emergency room visits, respiratory symptoms, exacerbation of respiratory and cardiovascular diseases, premature mortality and a decrease lung function.

As a result of these studies, it is assessed than at least 3% of cardiopulmonary and 5% of lung cancer deaths are related with particulate matter. Moreover, it is possible to relate that the increment of µg/m³ in PM₁₀ levels produces the increment of the number of deaths in a 0.6% (Kim, Kabir and Kabir, 2015).

2.4 Risk assessment: bioaccessibility and bioavailability.

The potential health risk of some pollutants that is present in particulate matter arises from the inhalation of these particles during breathing, followed by the deposition and diffusion of the particles into the respiratory tract. In order to improve risk assessment, the concentrations of the pollutants have to be considered, but it is also important to consider how they are assimilated by the people exposed (Mukhtar and Limbeck, 2013).

Despite the fact that the effect of particulate matter exposure is related with the physical characteristics of each person, the particle's size is directly proportional to their potential of causing health problems owing to they can penetrate into the human's body. The particles with a highest impact on human health effects are those with less than 10 µm of diameter, and, the smaller is the particle, deeper they go through the respiratory tract (Kim, Kabir and Kabir, 2015).

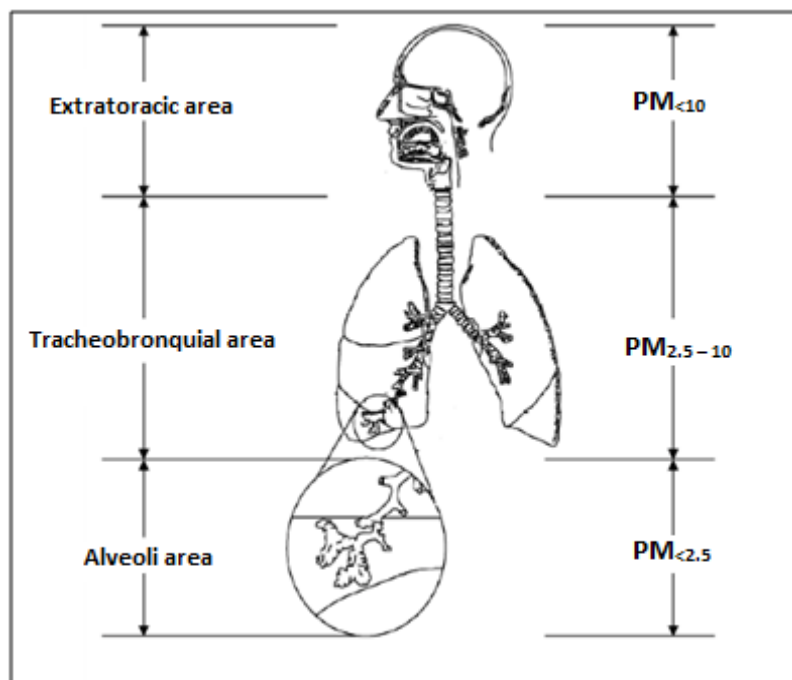


Figure 2. Diagram of the penetration of the particulate matter according to their size.

The followed pathway of the particulate matter (Figure 2) goes from the nasal passages until the alveoli, being able to reach the depth of the lungs because of their huge penetrability. First of all, when the air is introduced into the nose or the mouth and goes to the throat, it is directly filtered because the body reacts in order to eliminate these strange particles using process like sneezes and cough (López-Mahía, 2016).

Once the particles are inside the extrathoracic or nasopharyngeal region, there is an equilibrium among them and the body temperature and humidity, being the large particles restricted (Kastury, Smith and Juhasz, 2017). After that, they penetrate into the respiratory tract, remaining retained the particles between 5 and 10 μm in the tracheobronchial tree, while those between 1 and 5 μm can be stretched until bronchioles and alveolis. These are the specifical ones which can affect to the gaseous interchange that takes place into the lungs, being able to penetrate inside and get to the bloodstream (López-Mahía, 2016).

Nowadays, published researches study toxicity taking into account the bioaccessibility and bioavailability terms. Considering these two expressions, once the particles are deposited into the respiratory tract, the clearance of them can take place by one of these three mechanisms: absorption of the dissolved fraction by blood (inhalation bioaccessibility/bioavailability), transport to the gastrointestinal tract (oral bioaccessibility/bioavailability) and transport to the lymph node. Depending on the mechanism that the particles follow, it is possible to distinguish between oral and inhalation bioaccessibility and bioavailability (Kastury, Smith and Juhasz, 2017).

Inhalation bioaccessibility is defined as the fraction of toxicants dissolved in simulated tracheobronchial fluids that is potentially available to cross the air-blood barrier and to reach blood circulation, whereas inhalation bioavailability is the concentration of pollutants that cross the air-blood barrier and reach the blood circulation.

The first step to oral bioavailability assessment is the study of oral bioaccessibility, which is associated with the pollutant fraction soluble in the gastrointestinal tract that is available for absorption (Ruby *et al.*, 1999). Thereby oral bioavailability can be defined, being related to the pollutant fraction which goes through the gastrointestinal tract and reaches blood circulation.

Notwithstanding the evaluation of human data provides the most relevant information about particulate matter toxicity, human exposure studies can be easily variable. That is why nowadays, the bioaccessibility and bioavailability studies are done using different exposure methods:

- *In-vivo* experiments:

Bioaccessibility and bioavailability can be evaluated by *in-vivo* models using animals such as rats, hamsters and swines. They are supposed to be anatomically, physiologically and metabolically similar to humans. Nevertheless, they are expensive and time-consuming experiments, and there are some ethical implications that try to avoid harmful effects. Furthermore, the obtained data is limited and there is inaccuracy when it is extrapolated to humans (Turner, 2011).

- *In-vitro* exposure to cultured cells:

Animal or human cell lines are exposed to pollutant solutions in order to measure the bioaccessible/bioavailable fraction. Its main advantage over *in-vivo* experiments are the ease of operation and the fewer bureaucratic requirements regarding ethic committees. Frequently, these kinds of experiments are performed using lung epithelial and alveolar monocultures due to it is where the particulate matter deposition occurs. However, there are more than forty types of cells in the respiratory system, therefore, it is not a representative approach (Kastury, Smith and Juhasz, 2017). Considering oral bioavailability, cell studies have been developed in conjunction with *in-vitro* digestion techniques where gastric and intestinal digestion are simulated using commercially available digestive enzymes, being the final absorption process assessed using Caco-2 cell lines (Glahn *et al.*, 1998). Caco-2 cells are a human intestinal cell model that comes from a colon carcinoma which experiments a spontaneous differentiation in culture and display similar characteristics to enterocytes. Despite of their colonic origin, Caco-2 cells show the same morphology and functionality as small intestinal cells (Braga *et al.*, 2018).

- In-vitro experiments:

It is the best approximation to the evaluation of the particulate matter toxicity. Synthetic gastrointestinal and pulmonary fluids are used and human's body conditions like pH, temperature, agitation and chemical composition are simulated to estimate the concentration of target compounds. They are cheaper experiments with a greater ease of control, have a good reproducibility and high precision and accuracy, being able to be validated using reference materials (Moreda-Piñeiro, Moreda-Piñeiro and Bermejo-Barrera, 2017).

2.5 Background.

The terms bioavailability and bioaccessibility were born in England. In the 80's, food contamination established an important concern due to the bad soil conditions. For this reason, metals were the first analytes that were studied in these kinds of investigations. In 1981, the oral bioavailability of iron from meals was determined (Miller *et al.*, 1981), providing an essential contribution to the calculation of the bioavailable fraction using *in-vitro* methods.

After that, another oral metal bioavailabilities were analyzed, such as the determination of iron in food (Luten *et al.*, 1996), lead in forest soil (Turpeinen, Salminen and Kairesalo, 2000) and cadmium in sediments (DeWitt *et al.*, 2005).

Bioaccessibility has been also determined in a lot of researches, having better progresses. For instance, the application area has been quickly increased, being included apart from metals in food and soils, many organic compounds and other sample matrix that constitute a significant health risk. It was applied to the oral bioaccessible fraction quantification of metals in dusts (Turner, 2011), phthalate esters in indoor/outdoor dusts (Wang, Wu, *et al.*, 2013) and chlorinated organophosphate flame retardants in indoor dust (Quintana *et al.*, 2017) besides many others. Moreover, the inhalation bioaccessible fraction has been also studied, for example in polychlorinated biphenyls (PCBs) contained in indoor and outdoor dusts (Wang, Huang, *et al.*, 2013) and in trace metals in particulate matter (Mukhtar and Limbeck, 2013).

There are also comparative experiments, like the *in-vivo/in-vitro* assessment of oral bioaccessibility and bioavailability of arsenic, selenium and mercury species in food samples (Moreda-Piñeiro *et al.*, 2011) and limitations of inhalation bioaccessibility and bioavailability reviews of metals in particulate matter and dusts (Kastury, Smith and Juhasz, 2017).

Nonetheless, it is possible to see that there are a lot of studies based on the bioaccessibility of different analytes in different fields, but it is not very easy to find this diversity when discussing about bioavailability. Until a few years ago, the bioavailability studies focused their interest on metals;

however, in recent years they have been extended to some organic compounds such as polycyclic aromatic hydrocarbons, which is our analyte of interest.

2.6 Polycyclic Aromatic Hydrocarbons.

Polycyclic Aromatic Hydrocarbons (PAHs) are an ubiquitous group of organic compounds composed only of carbon and oxygen which form, at least, two fused aromatic benzene rings and additional ones with less than six carbons (Figure 3) (Callén *et al.*, 2013). They are characterized by being lipophilic, having high melting and boiling points, low vapour pressure and low water solubility (Jackson, 2017).

PAHs can be distinguished depending on their molecular weight: if they have less than three fused rings are named as low molecular weight and appear normally in the vapour phase of the atmosphere, whereas if they have more than four, are high molecular weight PAHs which are largely bound to particles. The ones with four fused rings are distributed between the particulate and the atmosphere phases depending on the atmosphere temperature. Those particles bounded to PAHs are very hazardous to human's health (World Health Organization, 2010).

They are a widespread group of pollutants that come from the incomplete combustion of organic materials which are originated by two different sources, naturals and anthropogenics. Volcanic eruptions and forest fires are the main natural sources of PAHs; however, the most important ones are anthropogenics. The main anthropogenic fraction comes from the partial combustion of coal, diesel, wood, vegetation and the elimination of fumes from manufacturing industries. In addition, there are daily life sources like the environmental tobacco smoked and the wood-stove (Yebra-Pimentel *et al.*, 2015). The sources of these pollutants vary based on if we are analyzing outdoor air, where traffic emissions have a greatest contribution; or indoor air, where tobacco, cooking and heating have a bigger impact on the total concentration (World Health Organization, 2010).

Due to these characteristics, many organizations such as the United States Environmental Protection Agency (USEPA) have listed 16 PAHs (Figure 3), being some of them considered probable human carcinogens and mutagenics as priority pollutants, in particular, the benzo(a)pyrene (BaP), that has been used as a marker of all the PAHs for many years because it is the most powerful one, and also the most regulated. The benzo(a)anthracene, chrysene and dibenzo(a,h)anthracene have to be taken into account too as human and animal carcinogens (Ravindra and Sokhi, 2008).

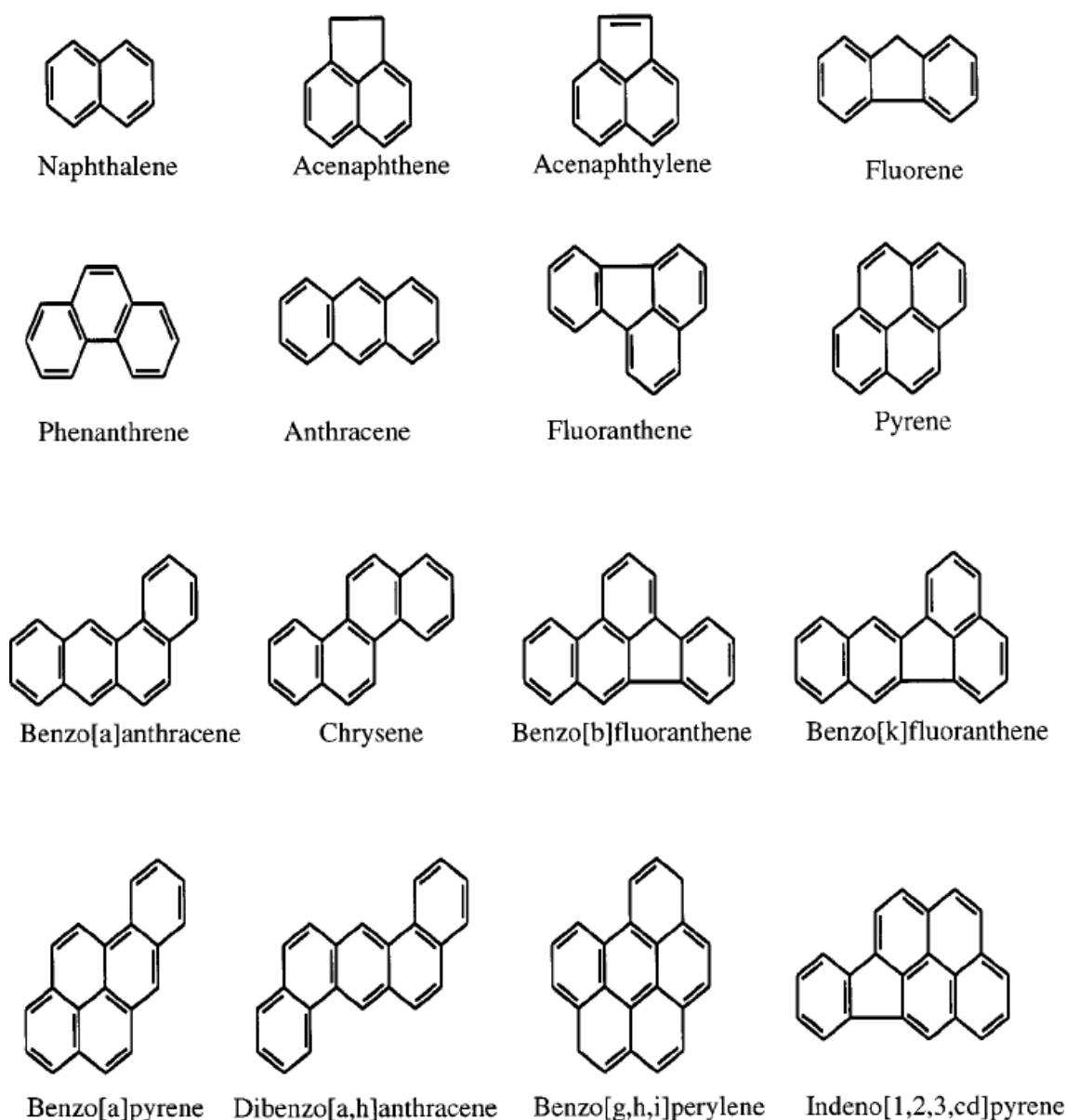


Figure 3: Structure of the 16 PAHs listed by the USEPA

2.7 Bioaccessibility and bioavailability of polycyclic aromatic hydrocarbons.

Since bioaccessibility and bioavailability appeared as new concepts, they are current issues in environmental chemistry, and are the focus of many analytical researches. The studies of PAHs started being in soils, and, over the years, it was spread out to food issues. Nowadays, the first studies related to particulate matter have just been appeared, and are the contemporary centre of interest. Some actual examples are shown below.

The oral bioaccessibility of polycyclic aromatic hydrocarbons has been estimated in seafood, which varies depending on the foodstuff and the cooked: it was obtained a 59.29% in dried seaweed, a

84,2 % of phenanthrene in steamed clams, and a 22,0 % of benzo(k)fluoranthene in raw clams, whereas in steamed clams, the bioaccessible fraction is the 55,5 % (Helena *et al.*, 2018).

There are also soil samples studies, for example in different areas of in Nigeria, where the bioaccessibility percentages of one of them are shown in *Table 3* (Adetunde *et al.*, 2018).

Compound	Oral bioaccessible fraction (%)
Naphthalene	14
Anthracene	1
Benzo(a)pyrene	17
Dibenzo(a,h)anthracene	0.9

Table 3. Oral bioaccessible fraction of PAHs in Nigerian soil.

In addition, there are some studies about inhalation bioaccessibility in PM_{2.5}, where the bioaccessibility of PAHs range from 3,21 % in the benzo(c)fluoranthene to 44,2 % in acenaphthylene (Li *et al.*, 2019).

Despite these examples, the number of studies related to oral bioaccessibility and bioavailability of PAHs is very limited being currently necessary to deepen this line of research.

3. Objective.

The particulate matter is formed by different compounds, being many of them hazardous to human's health. There is regulation related to the atmospheric concentration of some compounds in order to protect environmental conditions, but it is not enough. For this reason, it is necessary to extend the risk assessment not only to the determination of total concentration of pollutants, but also to the bioaccessible and bioavailable fractions.

This final dissertation report is focused on the achievement on the total concentration and the bioavailable fraction of polycyclic aromatic hydrocarbons by using an in vitro approach of particulate matter with a diameter equal or less than 10 μm . The aim is to calculate the relation between the bioavailable and the total concentration of these compounds owing to know what amount of the total PAHs concentration is capable to reach the bloodstream.

4. Experimental procedure.

4.1 Instruments and equipments.

- Electronic analytical balance ADN, EA-180A model, with an accuracy of ± 0.0001 g (Thebarton SA, Australia).
- GRAM balance, SV-510ix model, with an accuracy of 0.001 g (Barcelona, Spain).
- PHmeter, CRISON microPH 2001.
- Centrifuge, Eppendorf 5804 (Hamburg, Germany).
- Accelerated Solvent Extractor Dionex ASE 200 system (Sunnyvale CA, USA), equipped with stainless-steel extraction cells of 11 cm and cellulose filters (D18, 1.983 cm of diameter) also from Dionex.
- Incubator, Boxcult incubator and agitator Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain).
- High-volume sampler, Digital DH-80 (Digital Elektronik AG, Hegnau, Switzerland).
- PM₁₀ size selective inlet, Graseby-Andersen Gibsonville (TISCH Environmental INC Cleves, Ohio, EEUU).
- Vortex, IKA-VIBRAX-VXR Drogalleja (Galicia, Spain).
- High Performance Liquid Chromatography (HPLC) system, Waters 2695 Alliance with automatic injection, which is directly connected to two detectors, a photodiode array detector (Waters, 996) and fluorescence detector (Waters 2475, multi λ fluorescence detector) (Milford, MA, USA).
- Gas Chromatography (GC), Thermo-Finnigan (Waltham, MA, USA) Trace GC chromatograph equipped with a GC PAL autosampler (CTC-Analytics, Zwingen, Switzerland), Programmed Temperature Vaporizing (PTV) injector and coupled to an ion trap mass spectrometer (Polaris Q).
- Automatic micropipettes, Eppendorf Research, of variable volumes: 500-5000, 100-1000, 20-200, 10-100 and 2-20 μ L.

4.2 Consumable material.

- Volumetric glass material, class A.
- 100 mL amber glass flask.
- Tweezers.
- Steel punching tool (Selecta, Barcelona, Spain).
- Disposable glass pipettes.

- Dialysis membranes, Cellu Sep® H1 high grade regenerated cellulose tubular membranes (molecular weight cutoff 10 kDa, 50 cm x 25.5 mm), Membrane Filtration Products Inc (Seguin, TX, EEUU).
- Quartz fiber filteres, PM10 QF20 quartz fiber filters 27 cm×15 cm, (Schleicher&Schuell, D-Dassel, Germany).

4.3 Chemical reagents.

- Milli-Q water with a $18 \text{ M}\Omega\text{cm}^{-1}$ resistivity and obtained by using Milli-Q purification system, Millipore Co (Bedford, MA, USA).
- PAH calibration mix of certified reference material, 10 $\mu\text{g}/\text{mL}$ each component in acetonitrile, containing naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(ghi)perylene and indeno(1,2,3-C,D)pyrene. TraceCERT®, Supelco, Sigma Aldrich (Bellefonte, PA, USA).
- Deuterated PAH surrogate benzo(e)pyrene-d12 and anthracene-d10, 100 $\text{ng}/\mu\text{L}$ in cyclohexane, Dr. Ehrenstorfer GmbH (Augsburg, Germany).
- PAH calibration mix, 2000 $\mu\text{g}/\text{mL}$ in methylene chloride containing acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-CD)pyrene, naphthalene, phenanthrene and pyrene, Restek Corporation (Bellefonte, PA, USA).
- Deuterated- labelled PAH surrogate cocktail, 200 $\mu\text{g}/\text{mL}$ in 50% methylene chloride (D2, 99.9%) and 50% methanol (D2, 99%) containing acenaphthylene-d8, benzo(a)pyrene-d12, benzo(g,h,i)perylene-d12, fluoranthene-d10, naphthalene-d8, phenanthrene-d10 and pyrene-d10, Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).
- Deuterated PAH surrogate benzo(e)pyrene-d12, 100 $\mu\text{g}/\text{mL}$ in cyclohexane and chrysene-d12 10 $\mu\text{g}/\text{mL}$ in cyclohexane, Dr. Ehrenstorfer GmbH (Augsburg, Germany).
- Internal standards: anthracene-d10, 10000 $\mu\text{g}/\text{mL}$ in cyclohexane and dibenzo(a,h)anthracene-d14, 10 $\mu\text{g}/\text{mL}$ in cyclohexane, Dr. Ehrenstorfer GmbH (Augsburg, Germany).
- Disodium salt of the 1,4-piperazinediethanesulfonic acid (PIPES), Sigma Aldrich (USA).
- Hydrochloric acid 36.5–38%, Baker (Phillipsburg, PA USA).
- Sodium bicarbonate (NaHCO_3), Sigma Aldrich (USA).
- Sodium hydroxide (NaOH), Sigma Aldrich (USA).

- Pepsin from porcine gastric mucosa, Sigma Aldrich (China).
- Pancreatin from porcine, Sigma Aldrich (USA).
- Bile salts, Sigma Aldrich (USA).
- Acetonitrile (ACN), gradient grade for liquid chromatography, Merk (Darmstadt, Germany).
- Hexane, n-hexane SupaSolv®, Merk (Darmstadt, Germany).
- Methanol (MeOH), gradient quality, Romil (Cambridge, UK).

4.4 Cleaning procedures-

As we are working with trace analysis, it is essential to maintain the cleanliness during the total steps of the analytical procedure (sample collection, transport, storage, preparation and analysis) because each of them is a source of sample contamination. In order to do that, all the ware and glassware have to be washed with ultrapure water and kept for 48 hours into an alkaline bath owing to eliminate the remaining organic components. Finally, it is rinsed many times with ultrapure water again before being used.

4.5 Sample collection.

The sample collection was done using a discontinuous sampler system in order to collect the atmospheric particulate matter using a filtration technique, which is the most common sample collection.

The particulate matter collection and characterization by filtration requires two steps: first the collection of a representative sample of particulate matter, where the particles are transferred from their dispersive state in the air, to a compact one in a filter; and secondly, the analysis of particles by using gravimetric, microscopic or other analytical techniques.

A high-volume sampler, which was the one used in this study, is composed by a size selective inlet, which allows us to restrict the diameter of the particles (there are inlets of PM₁₀, PM_{2.5} and PM₁), an aspiration system, a sensor to control the flow, in such a way that the variations in the aspiration flux are balanced out with the regulation of the flow intensity. There is also a device that controls the aspiration time and a filter support. During its operation, the air goes across the inlets and it is accelerated through some nozzles that direct it to an impacting surface where remain detained, considering that, in order to avoid the bounce of the particles, the surface is recoat with a layer of grease or silicone. At this point, the particles with a larger diameter than the inlet have been retained and the ones with a lower size, continue until the filter deposition.

In this case, the sample collection was done using the high-volume air sampler Digitel automatic high-volume sampler DHA-8 (*Figure 4*), which is capable of breathing in a range of volumes between 20 – 100 m³/h. The Digitel automatic high-volume sampler DHA-80 contains, apart from the necessary components, a cassette filter loading system for 15 samples and the advantage of having programmable sample times and filters changing, being easy to manage (Couling, 2019).



Figure 4. External and internal vision of the DHA-8 high-volume sampler.

The analysis of the particles was performed using gravimetric analysis, where the operation is to divide the increase in the filter weight after sampling by the particulate matter volume sampled. The mass determination of the particles was done according to the European Legislation, enacted by the Law UNE-EN 12341 (1998), being the filters weighted before and after the sampling. Before each weighting, they have to be conditioned at 20 ± 1 °C and at a relative humidity of 45 - 50% for 48 hours. Furthermore, it is necessary to consider that all the filters have suffered an exhaustive control during their production, have been subdued to 12 hours of calcinations at 400 °C owing to eliminate the remaining organic material, and once they were ready to be used, they have been storage individually in a freezer at -18 °C.

The analysed samples come from a high-volume sampler located in the neighbourhood of San Diego, in A Coruña. It is an urban area which is highly influenced by traffic emissions and domestic activities. In addition, it is next to the sea, being the salt presence very important, more specifically close to the port, where hundreds of unloadings occur every day.

The analyzed samples are monthly samples of 2015 (excluding May because there were no filters due to sampler problems) each of one formed by circular portions of each day of the month, so that they are composite samples. The circular portions were obtained using a steel punching tool of 1.6 cm of diameter, so each sample is formed by thirty portions of 2.01 cm².

4.6 Bioavailable fraction of PAHs in particulate matter.

The bioavailable fraction of PAHs was determined with an *in-vitro* digestion procedure, followed by a preconcentration step using a Vortex Assisted Liquid-Liquid Microextraction (VALLME) and a High Performance Liquid Chromatography coupled with Fluorescence Detection (HPLC-FD) analysis.

4.6.1 Extraction of the bioavailable fraction: *in-vitro* digestion procedure.

The current bioavailability studies are based on the *in-vitro* method for the estimation of available iron in food that was done by Miller in 1981 (Miller *et al.*, 1981). The proposed digestion procedure was developed by the Analytical Chemistry Department of the A Coruña University and the Analytical Chemistry, Nutrition and Bromatology Department of the Santiago de Compostela University (Moreda-Piñeiro *et al.*, 2012) (Figure 5).

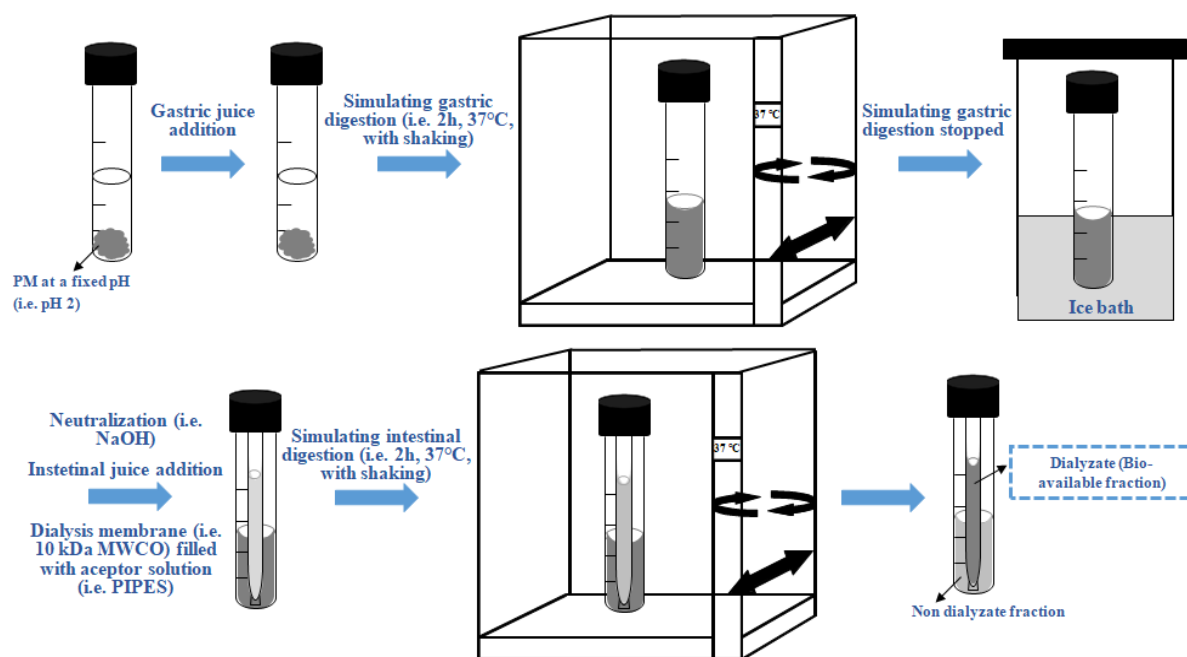


Figure 5. Flux diagram of the digestion procedure.

Thirty portions of PM₁₀ corresponding to a month were placed in a 100 mL amber glass flask and 20 mL of ultra-pure water were added. After fifteen minutes, pH was adjusted at 2 using small volumes of hydrochloric acid 6M. Then, a freshly 6.0% gastric solution was prepared just before use with pepsin

in a solution of hydrochloric acid 6 M. Approximately 0.15 g of this solution were added to the flask and the simulated gastric digestion took place heating at 37 °C in an incubator coupled to an orbital-horizontal shaking at 150 rpm during 120 minutes. Once the time has passed, the flasks were placed in an ice-water bath to stop the enzymatic digestion.

While the flask was tempered, the intestinal solution was prepared with 4.0% pancreatin and 2.5% bile salts dissolved in 0.1M sodium hydrogen carbonate. 5 mL of this solution were added to the flasks and the pH was adjusted at 7.5 using NaOH. At that time, a dialysis membrane of 10 KDa Molecular Weight Cut-off (MWCO), which is a pore size measure referring to the lowest molecular weight of a solute which is retained by the membrane at least in a 90 %, that were previously cleaned with ultra-pure water, was introduced in the flask, filled with 20 mL of a PIPES solution 0.15N and a pH of 7.53 and closed with plastic tweezers. The flask was placed again in an incubator at 37 °C and shaken during 120 minutes at 150 rpm in an orbital-horizontal shaking. At this moment the intestinal digestion occurred, and, as a consequence of that, took place the intestinal absorption process simulation using the semipermeable dialysis membrane and PIPES as the acceptor solution.

Finally, while the intestinal digestion had ended, the membrane was removed and the content was transferred to a centrifuge vial measuring the exact PIPES volume, because it is there where the bioavailable fraction of PAHs was. Blanks were performed followed exactly the same digestion procedure in order to control possible contamination.

4.6.2 Preconcentration of the bioavailable fraction.

The bioavailable fraction of PAHs was analyzed using a High Performance Liquid Chromatography (HPLC) system, so it was necessary to have the analytes in a suitable solvent for the liquid chromatography injection, an organic one, acetonitrile in this case.

Once the bioavailable fraction was in the centrifuge vial, the first step was the addition of surrogates. A surrogate is a pure compound with a small difference from the analyte which has the same chemical properties as the analyte but providing a different analytical signal. They are added in a known concentration before an analytical treatment, so they are used to determine the extraction efficiency and to correct the experimental errors. In this case, 15 µL of anthracene-d10 50 µg^L⁻¹ were added, and also 350 µL of hexane.

The follow step was the Vortex Assisted Liquid-Liquid Microextraction (VALLME). A liquid-phase microextraction is a miniaturization of the traditional liquid-liquid extraction, but where the extracting solvent is limited to a few microliters for the extraction of the target analytes, causing a preconcentration and enhancing the final analysis signal. Here, it was assisted by vortex, which

originated an empty center by mechanical energy, influencing positively in the mass transference. This technique provides an effective and mild mixing, increasing the contact surface and causing an improvement in the extraction recovery. Moreover, the amount of solvent needed is drastically reduced, being this technique considered as a green analytical method (Yamini, Rezazadeh and Seidi, 2019).

The Vortex was used for 5 minutes, followed by 10 minutes of centrifugation at 3500 rpm. At this point, both phases were clearly distinguishable, the lower was the PIPES, and the upper one, the 350 μL of hexane with the PAHs. The next step was the most complex, removing the organic solvent using a glass pipette without taking anything of the aqueous fraction (*Figure 6*). Finally, the organic phase was transferred to an amber vial, 150 μL of ACN were added and the hexane was evaporated with a nitrogen flow. The extracts were immediately analyzed in order to avoid the loss of analytes and then, stored in the freezer in case they were necessary to be used again.

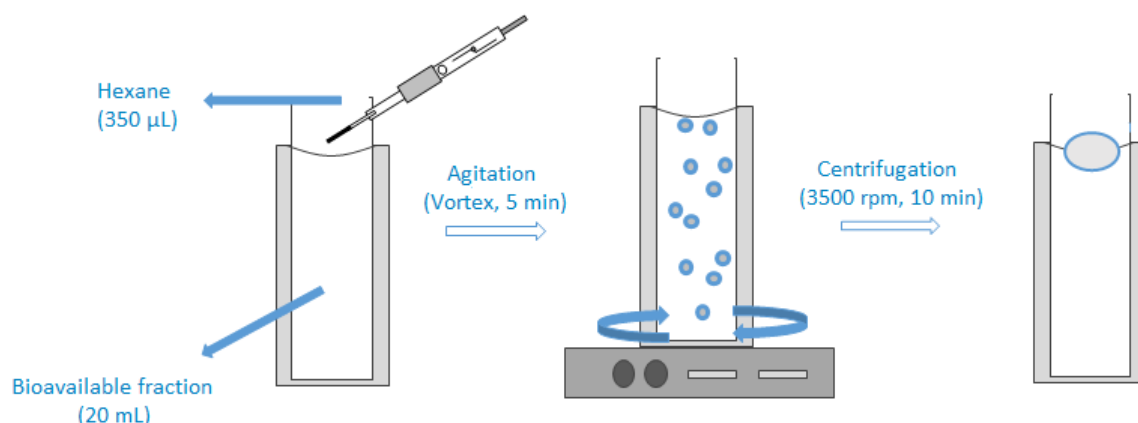


Figure 6. VALLME flux diagram.

4.6.3 Determination of the bioavailable PAHs with HPLC.

The analytical method for the PAHs determination comes from a previous study of the Analytical Chemistry Department of the A Coruña University. They were analyzed using a Waters 2695 Alliance High Performance Liquid Chromatography coupled with fluorescence detection (HPLC-FD).

High Performance Liquid Chromatography is a separation analytical technique which is based on the distribution of the analytes between two different phases, a stationary phase which is a column, and a mobile one, a solution that is continuously flowing over the column. This distribution comes from the interaction of the analytes in different proportion with these two phases. Reverse phase chromatography was used, being the stationary phase less polar than the mobile one. The detection was done with a fluorescence detector because they are more sensitive and selective than the UV one.

10 μL of the ACN extract were automatically injected in a Waters® PAH C18 (250 X 4.6 mm i.d, 5 μm) column with a 1 mL min^{-1} flow rate and a temperature of $32 \pm 5^\circ\text{C}$ maintained for all the analysis. A gradient elution using acetonitrile and water as mobile phases was performed to achieve the PAHs separation, being 50:50 ACN:H₂O the initial conditions. The elution program consists of a gradient to 100 % of ACN in 34 minutes and a return to the initial conditions in 6 minutes, both using linear curves (*Table 4*). The total analysis of a sample required 40 minutes plus 10 extra minutes for the column equilibration between runs. Before the chromatographic analysis, the column conditioning was done with a system purge using the mobile phase eluents.

Time (min)	% ACN	% H ₂ O
0	50	50
34	100	0
40	50	50

Table 4: Chromatographic elution conditions.

PAHs were monitored using a Waters 2475 fluorescence detector, whose excitation and emission wavelengths were selected in order to obtain the highest sensitivity for all the analytes and are characterized for each of them (*Table 5*).

Time (min)	Excitation wavelength (λ_{ex})	Emission wavelength (λ_{em})
0	216	330
11	260	340
12.9	252	370
14.6	250	390
16	284	466
17.8	272	390
21	276	406
26	288	410
37	300	500
42	216	330

Table 5. Fluorescence detector events.

4.6.4 Detection and quantification limits.

The limit of detection (LOD) is defined as the minimum concentration of an analyte which is capable of generating an analytical signal that cannot be confused with the blank one at 99% of confidence. It is calculated using the *Equation 1*:

$$LOD = 3SD \cdot DF \quad \text{Equation 1}$$

where SD is the standard deviation and DF, the dilution factor.

The limit of quantification (LOQ) is the minimum concentration of an analyte that generate an analytical signal which can be quantified in a trustworthy way in a confidence level of 99%. It is calculated using *Equation 2*:

$$LOQ = 10SD \cdot DF \quad \text{Equation 2}$$

where SD is the standard deviation and DF, the dilution factor.

During this study trace compounds have been analyzed, so the determined concentrations were really small, being also the limits, reaching levels of pg/m^3 (*Table 6*).

Compound	LOD (pg/m^3)	LOQ (pg/m^3)
Naphthalene	0,034	0,11
Acenaphthene	0,031	0,10
Fluorene	0,15	0,49
Phenanthrene	0,062	0,21
Anthracene	0,16	0,52
Fluoranthene	0,030	0,10
Pyrene	0,014	0,048
benzo(a)anthracene	0,010	0,027
Chrysene	0,0016	0,0050
benzo(e)pyrene	0,0056	0,019
benzo(b+j)fluoranthene	0,045	0,15
benzo(k)fluoranthene	0,0045	0,015
benzo(a)pyrene	0,0046	0,15
dibenzo(a,h)anthracene	0,0061	0,015
benzo(ghi)perylene	0,0028	0,020
indene(1,2,3-cd)pyrene	0,0053	0,0093

Table 6. LOD and LOQ of the analytical method.

4.7 Total concentration of PAHs in particulate matter.

The total concentration of PAHs in particulate matter was determined using a new green analytical method developed by the Analytical Chemistry Department of the A Coruña University with the collaboration to the Research Group on Environmental Management and Modeling of the Antioquia University (Medellín, Colombia) (Ramos-Contreras *et al.*, 2019). The method was based on a Pressurized Hot Water Extraction (PHWE) followed by a miniaturized Membrane-Assisted Solvent Extraction (MASE). The concentration determination was done using Programmed Temperature Vaporization-Gas Chromatography-Ion Trap tandem Mass spectrometry detection (PTV-GC-MS/MS).

4.7.1 Pressurized hot water extraction.

The pressurized hot water extraction, also called Subcritical Water Extraction (SWE), consists on the employ of water submitted to high temperatures, between its boiling point (100 °C, 0.1 MPa) and the critical one (374 °C, 22.1 MPa), and enough pressure to keep the water in the liquid state. Under these conditions, the physicochemical properties of water change drastically.

There are some parameters that influence the extraction efficiency, such as solubility, mass transfer and the matrix effect; however, there are four which do it in a highest way: temperature, pressure, extraction time and flow rate. Among all of them, temperature is crucial. Considering water, its dielectric constant decreases with increasing temperature, hence, solvent polarizability can be modified with temperature, being lower at higher ones. On the other hand, with the increasing of temperature, there are also an increasing in the surface tension and a viscosity degree decreasing, increasing the diffusivity. Moreover, high temperatures reduce the intermolecular interactions between analytes and matrix, being the mass transfer faster, and, as a consequence of that, improving the extraction process (Plaza, 2019). For this reason, it is recognized as a solvent-free technique that is considered a green analytical technique.

This equipment is completely automated, so the operation is very easy, it is only necessary to load the sample into the cell, and the equipment itself will control the extraction. Once the sample is loaded into the cell, it is filled with the solvent for few minutes, heated and pressurized in the oven. These conditions are maintained for a short time and new cleaned solvent is pump into the sample cell, originated a new cycle extraction. The number of cycles depends on the analyte and is a parameter that has to be optimized. Finally, part of the solvent is purged from the cell with nitrogen flow, and the extract is ready for further analysis.

An accelerated Solvent Extractor Dionex ASE 200 (*Figure 7*) was used to perform the PAHs extraction. Thirty circular portions with a diameter of 0.6 cm and 150 μL of a 200 $\mu\text{g L}^{-1}$ surrogate solution were introduced in 11 mL stainless-steel ASE cells sealed at both ends with two glass-fiber filters of diameter 19.8 mm. The surrogate solution is a deuterated PAHs cocktail that contained naphthalene d-8, acenaphthylene d-8, phenanthrene d-10, fluoranthene d-10, pyrene d-10, chrysene d-12, benzo(e)pyrene d-12 and benzo(ghi)perylene d-12.



Figure 7. Illustration of the Accelerated Solvent Extractor Dionex ASE.

These cells were previously cleaned by extraction with methanol at 150 °C and 2000 psi during 5 minutes owing to eliminate the interfering compounds that could difficult the analysis. The extraction was done using a H₂O:MeOH 75:25 (% v/v) solution as extractor solvent, static time of 5 minutes, 200 °C of temperature, pressure of 2000 psi, nitrogen purge time of 1 minute and only one extraction cycle. The PHWE extracts were brought to 40 mL with a H₂O:MeOH 3:1 solution and a 15 mL aliquot was separated.

4.7.2 Membrane-assisted solvent extraction.

The Membrane-Assisted Solvent Extraction (MASE) is based on the traditional liquid-liquid extraction and is characterized by the analyte diffusion through a membrane from a donor solution to another small amount of liquid organic phase, the acceptor solution. The membrane is composed by non-porous polypropylene (PP) in order to exclude water traces that could affect following steps.

The separated 15 mL aliquot was transferred to a 20 mL glass vial to which a high dense PP membrane was inserted using a metal funnel with a teflon ring, remaining suspended in the glass vial. The membrane was 4 cm long, 0.03 mm thickness and had a diameter of 6 mm. 500 µL of a 100 µg L⁻¹ internal standard solution of anthracene d-10 and dibenzo(a,h)anthracene d-14 in hexane were added and the vial was sealed with a metallic crimp cap with Teflon septa. The MASE device was agitated by oscillation at 750 rpm and 30 °C during 90 minutes using a commercial Combi PAL autosampler mounted on the Gas Chromatography–Mass Spectrometry system. Once it finished, the hexane extract that contained the PAHs has to be analyzed (Figure 8).

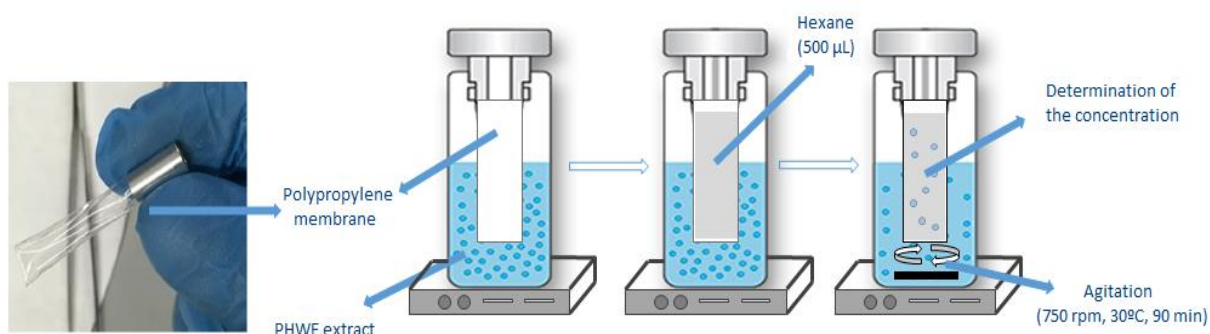


Figure 8. MASE flow diagram.

4.7.3 Determination of the total PAHs concentration using GC-MS.

The analytical method for the total PAHs determination in the hexane extract was done using a Thermo-Finngan Trace Gas Chromatograph which was equipped with a GC PAL Autosampler, a Programmed Temperature Vaporizing injector coupled to an ion trap Mass Spectrometer Polaris Q, using Xcalibur as data processor.

25 μL were injected in a DB-XLB column (60m \times 0.25mm,0.25 μm film thickness), starting the injector program at 55 $^{\circ}\text{C}$ ad increasing 3 $^{\circ}\text{C s}^{-1}$ until 300 $^{\circ}\text{C}$, temperature that was maintained for 20 minutes. The oven of the Gas Chromatograph started with a temperature of 50 $^{\circ}\text{C}$ during 3 minutes, and increased 4 $^{\circ}\text{C min}^{-1}$ to 325 $^{\circ}\text{C}$, hold in 20 minutes. The Mass Spectrometer had an electron impact of 70 eV, the transfer line temperature was set at 300 $^{\circ}\text{C}$, and the ion source one, at 270 $^{\circ}\text{C}$. Helium at 99.99% was used as collision gas at the ion trap chamber and also as a carrier gas under a constant flow rate of 1 mL min^{-1} . The parent and product ions and their retention times are shown in *Table 7*:

Compound	Retention time (min)	Parent ion (m/z)	Product ion (m/z)
Naphtalene	27,12	128	102
Acenaphthene	38,03	153	150
Fluorene	41,38	166	162
Phenanthrene	47,34	178	152
Anthracene	47,72	178	152
Fluoranthene	54,85	202	198
Pyrene	56,28	202	198
Benz(a)anthracene	63,81	228	224
Chrysene	64,06	228	224
Benzo(b+j)fluoranthene	70,23	252	248
Benzo(k)fluoranthene	70,34	252	248
Benzo(e)pyrene	71,74	252	248
Benzo(a)pyrene	72,07	252	248
Dibenz(a,h)anthracene	78,75	278	274
Indene(1,2,3-cd)pyrene	78,92	276	272
Benzo(g,h,i)perylene	80,92	276	272

Table 7. Elution order and conditions of the studied PAHs.

4.7.4 Detection and quantification limits.

The limits of detection (LOD) and limits of quantification (LOQ) of this procedure were calculated using *Equations 1 and 2*.

Compound	LOD (ng/m ³)	LOQ (ng/m ³)
Naphtalene	0,0026	0,027
Acenaphthene	0,0011	0,0011
Fluorene	0,00095	0,00096
Phenanthrene	0,0041	0,0041
Anthracene	0,0051	0,0052
Fluoranthene	0,00086	0,00087
Pyrene	0,0084	0,00085
Benzo(a)anthracene	0,00037	0,00038
Chrysene	0,00044	0,00045
Benzo(e)pyrene	0,00079	0,00080
Benzo(b+j)fluoranthene	0,0021	0,0022
Benzo(k)fluoranthene	0,0019	0,0019
Benzo(a)pyrene	0,0021	0,0021
Dibenz(a,h)anthracene	0,0065	0,00066
Benzo(g,h,i)perylene	0,0033	0,0034
Indene(1,2,3-cd)pyrene	0,0038	0,0039

Table 8. LOD and LOQ of the analytical method.

The obtained LOD and LOQ values were low enough to perform the total PAHs determination in the selected samples.

5. Results and discussion.

5.1 Bioavailable fraction.

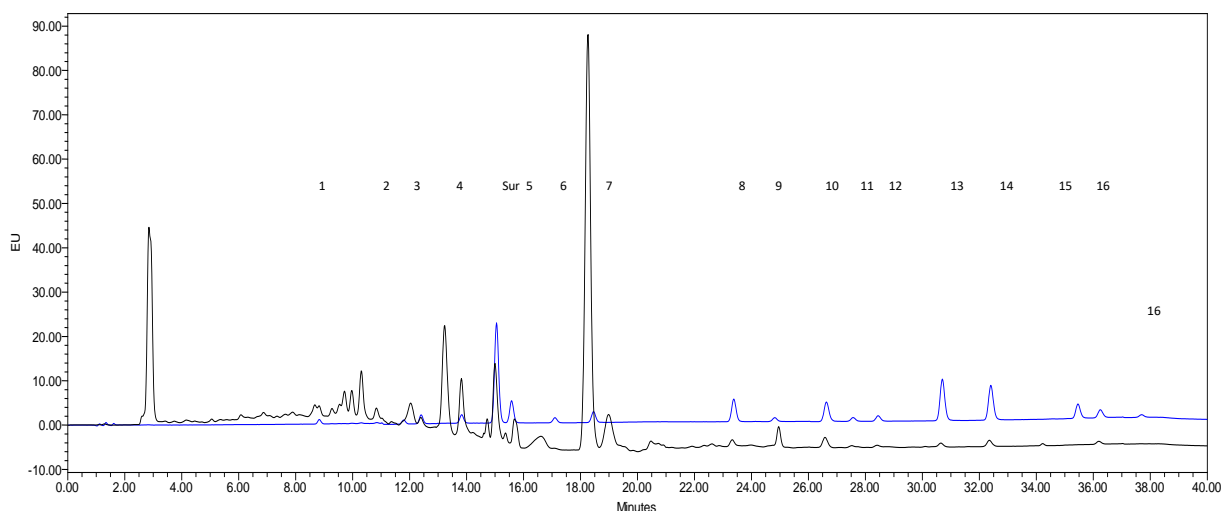
5.1.2 Quantification of the PAHs bioavailable fraction.

The PAHs identification was based on their retention times comparing to the internal standard solutions (*Table 9*) of concentrations 0.5, 1, 2, 4, 5, 7 and 10 $\mu\text{g L}^{-1}$ that were prepared using a PAH calibration mix of certified reference material.

Compound	Retention time (min)
Naphtalene	8,86
Acenaphthene	11,83
Fluorene	12,44
Phenanthrene	13,87
anthracene d-10	15,09
Anthracene	15,62
Fluoranthene	17,15
Pyrene	18,51
benzo(a)anthracene	23,44
Chrysene	24,89
benzo(e)pyrene d-12	26,72
benzo(e)pyrene	27,76
benzo(b+j)fluoranthene	28,53
benzo(k)fluoranthene	30,80
benzo(a)pyrene	32,51
dibenzo(a,h)anthracene	35,60
benzo(ghi)perylene	36,37
indene(1,2,3-cd)pyrene	37.84

Table 9. Retention times of the PAHs in the chromatographic analysis.

Due to the complexity of sample chromatograms, the integration algorithm ApexTrack of the Empower 3 Software was used in order to improve the integration area of the compounds (*Figures 9*).



Where 1 is naphthalene, 2 acenaphthene, 3 fluorene, 4 phenanthrene, 5 anthracene, 6 fluoranthene, 7 pyrene, 8 benzo(a)anthracene, 9 chrysene, 10 benzo(e)pyrene, 11 benzo(b+j)fluoranthene, 12 benzo(k)fluoranthene, 13 benzo(a)pyrene, 14 dibenz(a,h)anthracene, 15 benzo(ghi)perylene and 16 indene(1,2,3-cd)pyrene.

Figure 9. Chromatogram of a PAHs internal standard solution (blue) and a real sample dialyzate (black).

The quantification was done taking into account the Relative Response Factor (RRF), which is a measure of the analytical response of the technique to an analyte compared to its internal standard. As some internal standards were analyzed, the average of the relative response factor of each analyte was used to calculate sample concentrations, considering during all the calculations the added surrogates. The RRFs were calculated using Equation 3, with a maximum deviation standard coefficient of 4.88% (Table 10). Although two different surrogates were added, benzo(e)pyrene-d12 and anthracene-d10, and both are acceptable, the calculations were done using the anthracene-d10 values.

$$\text{RRF} = \frac{\text{Area}_{\text{internal standard}} \cdot [\text{surrogate}]_{\text{vial}}}{[\text{internal standard}]_{\text{vial}} \cdot \text{Area}_{\text{surrogate}}} \quad \text{Equation 3.}$$

where $\text{area}_{\text{internal standard}}$ is the integrated area of the standard PAH in the chromatogram, $[\text{surrogate}]_{\text{vial}}$ is the surrogate concentration in the injected vial, $[\text{internal standard}]_{\text{vial}}$ is the internal standard concentration in the injected vial and $\text{area}_{\text{surrogate}}$ is the integrated area of surrogate in the chromatogram.

Compound	RRFs	Deviation standard coefficient (%)
naphthalene	0,19	2,29
acenaphthene	0,18	2,3
fluorene	0,41	1,1
phenanthrene	0,40	1,7
anthracene	1,1	1,3
fluoranthene	0,26	2,2
pyrene	0,57	2,3
benzo(a)anthracene	1,2	1,0
chrysene	0,22	0,48
benzo(e)pyrene	0,22	2,0
benzo(b+j)fluoranthene	0,30	1,7
benzo(k)fluoranthene	2,4	1,1
benzo(a)pyrene	2,1	0,96
dibenzo(a,h)anthracene	0,81	1,2
benzo(ghi)perylene	0,48	1,1
indene(1,2,3-cd)pyrene	0,16	4,9

Table 10. Relative response factor of bioavailable PAHs.

Once the RRFs were obtained, they were used to calculate the bioavailable fraction considering the dilutions that were done during the experimental procedure using *Equation 4*:

$$[PAH]_{vial} = \frac{Area_{PAH} \cdot [surrogate]_{vial}}{Area_{surrogate} \cdot RRF} \cdot DF \quad \text{Equation 4.}$$

where $area_{PAH}$ is the integrated area of the PAH in the chromatogram, $[surrogate]_{vial}$ is the surrogate concentration in the injected vial, $area_{surrogate}$ is the integrated area of the surrogate in the chromatogram, RRF is the relative response factor and DF, the dilution factor.

The calculated bioavailable concentrations of each PAH for each month (*Table A1*) are shown in Annex 1. The variation coefficient of the different PAHs concentrations is almost all lower than 20%, excepting in some specific months for phenanthrene and pyrene. Month distribution is shown in *Figure 10*.

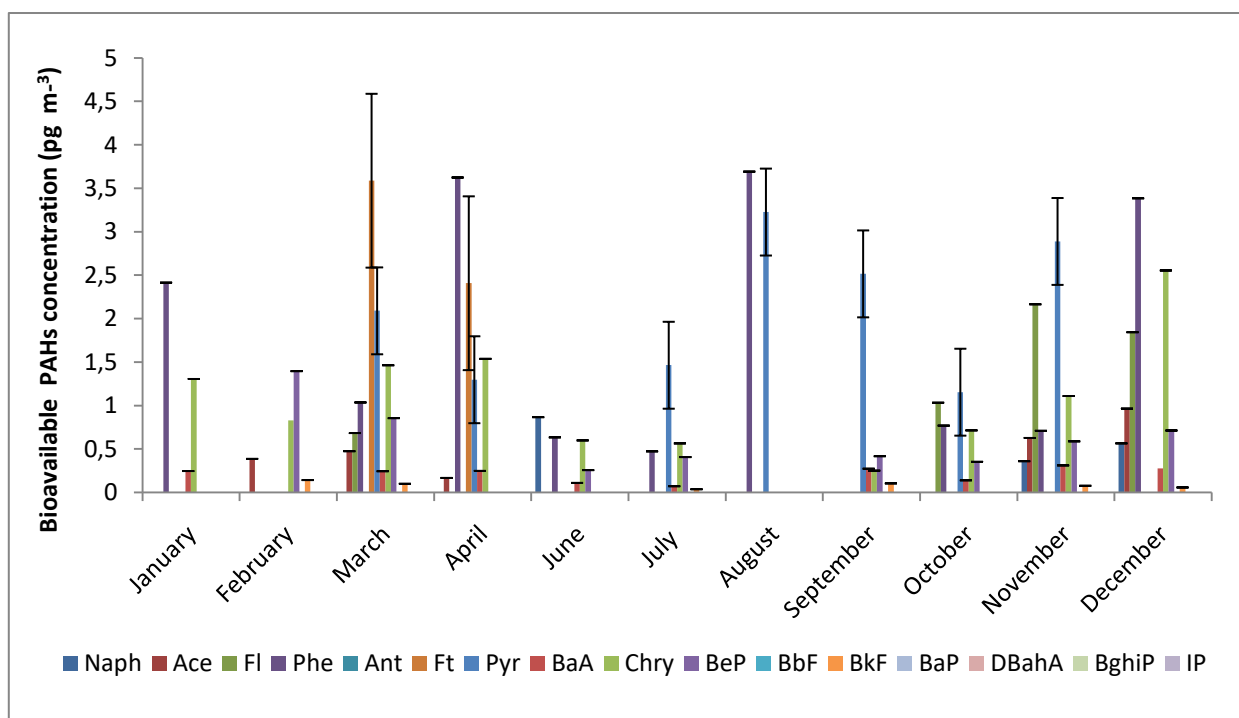


Figure 10: Monthly graph of the bioavailable PAHs concentration.

Taking into account the obtained results, first of all it is possible to distinguish the PAHs depending on if they generally have a bioavailable fraction or not. There are four that usually are in all the samples: phenanthrene, benzo(a)pyrene, chrysene and benzo(e)pyrene; and three that appear commonly but with a lower frequency: acenaphthene, pyrene and benzo(k)fluoranthene. On the other hand, anthracene, benzo(b)fluoranthene, dibenzo(a,h)anthracene, benzo(ghi)perylene and indene(1,2,3-cd)pyrene do not have a bioavailable fraction or they have such a smaller one that cannot be quantified with this method. Moreover, naphthalene, fluorene and fluoranthene appear in few samples.

Focusing on concentrations, phenanthrene has the highest bioavailable concentrations, with values of 3,7 pg m^{-3} in August and 3,6 pg m^{-3} in April. There are other PAHs that have raised concentrations, such as 3,6 pg m^{-3} of fluoranthene in March, and 3,2 pg m^{-3} of pyrene in August. Nevertheless and fortunately, there are some PAHs whose bioavailable fractions are very small, as in the case of benzo(k)fluoranthene, being the PAH with the lowest values, 0,038 and 0,059 pg m^{-3} in July and December for example, and benzo(a)anthracene, with a bioavailable concentration of 0,073 pg m^{-3} . All the other compounds are ranged between 0,11 and 2,6 pg m^{-3} . These monthly variation may be due to the variation of chemical composition of particulate matter.

The atmospheric particulate matter characteristics such as the particle size, the sources of origin and their chemical properties have to be also taking into account, because they can react into the body fluids as well as with body fluid components, occurring reactions like complexation/precipitation

with available ligands, redox reactions and dissolutions. Consequently, there are complex synergies and antagonisms effects that affect to the major and minor constituents of the atmospheric particulate matter and gastrointestinal fluid composition which alter the oral bioavailability of the pollutants (Moreda-Piñeiro *et al.*, 2019).

5.1.2 Validation of the analytical method.

The experimental procedure that has been exposed for the PAHs bioavailable determination is a new technique that has been never used. Due to that, it has to be validated in order to prove that the method is suitable for the purpose, satisfying the pre-set specifications in relation to its intended use. There are some parameters which allow us to carry out this validation, such as the limit of detection, limit of quantification, trueness and precision. The first two have just been studied in section 4.6.4, and precision, which is the mutual agreement amongst different measurements made in the same sample on the same experimental conditions, was considered with standard deviations.

This section is focused on trueness, which is defined as the degree of agreement between the experimental value and the “true value”, having different ways to evaluate it in a laboratory: the use of certified reference materials, the application of reference methods published by international organizations, interlaboratory exercises and finally, calculating analytical recoveries using standards.

The trueness of the bioavailable fraction preconcentration was evaluated with analytical recoveries. In order to do it, 20 mL of Pipes were fortified with 0.1, 1 and 10 $\mu\text{g L}^{-1}$ of a PAHs calibration mix, and they were treated following exactly the same procedure as if they were real samples. After the concentration determination, the analytical recovery (R) was calculated using *Equation 5*, obtaining the following results (*Table 11*).

$$R(\%) = \frac{[PAH]_{\text{experimental}}}{[PAH]_{\text{fortified}}} \cdot 100 \quad \text{Equation 5.}$$

where $[PAH]_{\text{experimental}}$ is the experimental obtained concentration for the PAH, and $[PAH]_{\text{fortified}}$ is the theoretical fortified PAH concentration.

The obtained results should be between 80 - 120 % to be considered correct. In 1 $\mu\text{g L}^{-1}$ and 10 $\mu\text{g L}^{-1}$ fortifications, almost all the values are below 120. However, it is possible to see that in the 0.1 $\mu\text{g L}^{-1}$ fortification, the recoveries are a little bit high because of the concentration is very low. In addition, there were problems with fluoranthene and it could be quantified. Nonetheless, the obtained results are good taking into account the concentration levels that we are dealing with.

	0,1 µg L ⁻¹ fortification	1 µg L ⁻¹ fortification	10 µg L ⁻¹ fortification
Naphthalene	109	103	101
acenaphthene	113	104	105
Fluorene	121	111	126
Phenanthrene	143	119	113
Anthracene	129	119	114
Fluoranthene	-	-	-
Pyrene	122	117	120
benzo(a)anthracene	126	118	123
Chrysene	140	129	114
benzo(e)pyrene	106	105	105
benzo(b+j)fluoranthene	125	119	124
benzo(k)fluoranthene	122	117	118
benzo(a)pyrene	128	120	123
dibenzo(a,h)anthracene	122	118	117
indene(1,2,3-cd)pyrene	126	119	122
benzo(ghi)perylene	122	115	116

Table 11. Analytical recoveries using different fortification concentrations.

5.2 Quantification of total PAHs concentration.

The identification and quantification of PAHs was done following exactly the same procedure as for the bioavailable fraction. The identification was established considering the retention time of the prepared internal standards using a PAH calibration mix, which had concentrations of 3, 10, 25, 50, 100, 200 and 300 µg L⁻¹. Moreover, in this technique, the identification was also based on their molecular weight owing to the Gas Chromatography was coupled with a Mass Spectroscopy detection.

For the quantification step, in this case several surrogates were used, and each of them was necessary and associated to the quantification of a small group of analytes, which was done using Thereupon, the naphthalene d-8 was used for naphthalene, acenaphthylene d-8 for acenaphthene and fluorene, phenanthrene d-10 for phenanthrene and anthracene, fluoranthene d-10 for fluoranthene, pyrene d-10 for pyrene, chrysene d-12 for chrysene and benzo(a)anthracene, benzo(e)pyrene d-12 for benzo(b+j)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene and benzo(a)pyrene, and finally, benzo(ghi)perylene d-12 for dibenzo(a,h)anthracene, indene(1,2,3-cd)pyrene and benzo(ghi)perylene. The RRFs were calculated using *Equation 5* and all the deviation standard coefficients are between 1.23 and 10.8%, excepting benzo(a)anthracene, with a value of the 19,5%, still being less than 20%, and therefore, correct (*Table 12*).

Compound	RRFs	Deviation standard coefficient (%)
naphthalene	0,82	5,1
acenaphthene	0,26	1,6
fluorene	0,73	11
phenanthrene	0,74	5,1
anthracene	0,56	1,6
fluoranthene	0,36	1,4
pyrene	0,42	1,2
benzo(a)anthracene	1,1	19
chrysene	1,1	5,2
benzo(b+j)fluoranthene	1,2	9,9
benzo(k)fluoranthene	0,63	11
benzo(e)pyrene	4,4	9,0
benzo(a)pyrene	0,55	6,6
dibenzo(a,h)anthracene	0,70	5,90
indene(1,2,3-cd)pyrene	0,62	8,0
benzo(ghi)perylene	0,77	5,8

Table 12. Relative response factor of total PAHs.

The determination of the total concentration of each PAH was done with its corresponding RRF and surrogate, using *Equation 3*. The standard deviation was calculated for each analyte measuring three times a known concentration (*Table A2*), located in Annex 1. The month distribution of each PAHs is shown in *Figure 11*.

Analyzing the graph, it is clearly distinguished that benzo(e)pyrene and chrysene are the ones with the higher presence in the atmosphere, whose concentrations values are ranged from 0,81 ng m⁻³ to 2,91 ng m⁻³ for the benzo(e)pyrene and 2,13 to 0,61 ng m⁻³ for the chrysene, being the higher value in February and the lower, in June. On the other hand, naphthalene, fluorene and anthracene are the least represented, showing concentrations lower than 0,0038 ng m⁻³ for naphthalene, below 0,049 ng m⁻³ for fluorene and underneath 0,084 ng m⁻³ for anthracene. Benzo(a)pyrene, the only PAH whose concentration is regulated in the atmosphere, has values between 0,33 ng m⁻³ in June and 0,96 ng m⁻³ in February, being always below the 1 ng m⁻³, which is the limit value.

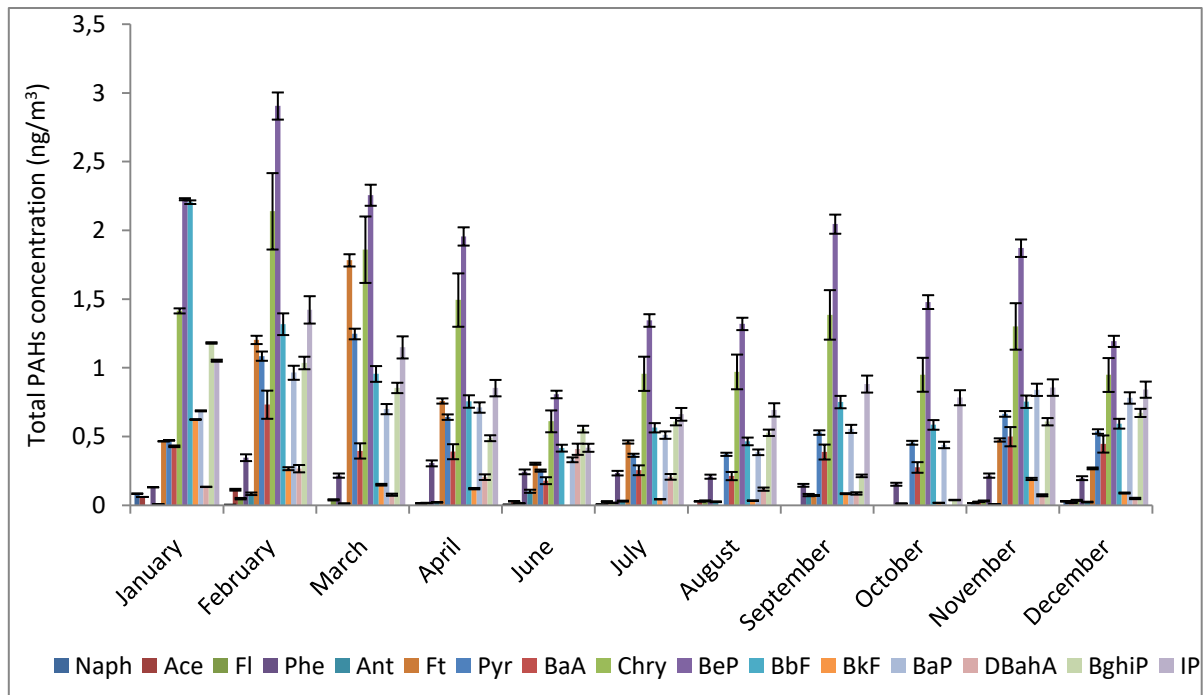


Figure 11: Monthly graph of the total PAHs concentration.

Phenanthrene and acenaphthene have similar values during all the year and all of them are lower than $0,35 \text{ ng m}^{-3}$ and $0,11 \text{ ng m}^{-3}$ respectively. The other PAHs, fluoranthene, benzo(a)anthracene, pyrene, benzo(b+j)fluoranthene, benzo(k)fluoranthene, dibenzo(a,h)anthracene, indene(1,2,3-cd)pyrene and benzo(ghi)perylene have concentration levels that are in the centre of the distribution, with values between $0,1$ and $1,8 \text{ ng m}^{-3}$.

5.3 Oral bioavailable percentage of PAHs.

Once the bioavailable and the total concentration of PAHs were determined, the bioavailable percentages of each PAH were calculating using Equation 6 (Table 13).

$$\text{Bioavailability (\%)} = \frac{[\text{bioavailable fraction}]_{\text{PAH}}}{[\text{total}]_{\text{PAH}}} \cdot 100 \quad \text{Equation 6.}$$

where $[\text{bioavailable fraction}]_{\text{PAH}}$ is the concentration of the bioavailable fraction of the PAH, and $[\text{total}]_{\text{PAH}}$ is the total concentration of the PAH in the sample.

% Bioavailability	January	February	March	April	June	July
naphthalene	- ^a	- ^a	- ^a	- ^a	18	- ^a
acenaphthene	- ^a	0,34	- ^a	1,2	- ^a	- ^a
fluorene	- ^a	- ^a	1,7	- ^a	- ^a	- ^a
phenanthrene	1,8	- ^a	0,48	1,2	0,26	0,20
anthracene	- ^a	- ^a	- ^a	- ^a	- ^a	- ^a
fluoranthene	- ^a	- ^a	0,20	0,32	- ^a	- ^a
pyrene	- ^a	- ^a	0,17	0,20	- ^a	0,40
benzo(a)anthracene	0,058	- ^a	0,062	0,064	0,062	0,029
chrysene	0,092	0,039	0,079	0,10	0,098	0,060
benzo(e)pyrene	- ^a	0,048	0,038	- ^a	0,032	0,030
benzo(b+j)fluoranthene	- ^a	- ^a	- ^a	- ^a	- ^a	- ^a
benzo(k)fluoranthene	- ^a	0,054	0,067	- ^a	- ^a	0,087
benzo(a)pyrene	- ^a	- ^a	- ^a	- ^a	- ^a	- ^a
dibenzo(a,h)anthracene	- ^a	- ^a	- ^a	- ^a	- ^a	- ^a
indene(1,2,3-cd)pyrene	- ^a	- ^a	- ^a	- ^a	- ^a	- ^a
benzo(ghi)perylene	- ^a	- ^a	- ^a	- ^a	- ^a	- ^a

% Bioavailability	August	September	October	November	December
naphthalene	- ^a	- ^a	- ^a	2,3	1,94
acenaphthene	- ^a	- ^a	- ^a	2,5	5,1
fluorene	- ^a	- ^a	- ^a	6,8	5,1
phenanthrene	1,8	- ^a	0,50	0,33	1,7
anthracene	- ^a	- ^a	- ^a	- ^a	- ^a
fluoranthene	- ^a	- ^a	- ^a	- ^a	- ^a
pyrene	0,87	0,48	0,25	0,43	
benzo(a)anthracene	- ^a	0,071	0,051	0,063	0,062
chrysene	- ^a	0,018	0,075	0,085	0,27
benzo(e)pyrene	- ^a	0,021	0,024	0,032	0,060
benzo(b+j)fluoranthene	- ^a	- ^a	- ^a	- ^a	- ^a
benzo(k)fluoranthene	- ^a	0,125	- ^a	0,041	0,066
benzo(a)pyrene	- ^a	- ^a	- ^a	- ^a	- ^a
dibenzo(a,h)anthracene	- ^a	- ^a	- ^a	- ^a	- ^a
indene(1,2,3-cd)pyrene	- ^a	- ^a	- ^a	- ^a	- ^a
benzo(ghi)perylene	- ^a	- ^a	- ^a	- ^a	- ^a

^a: Not calculated (the PAH concentration in the dialysate or in total concentration is lower than LOQ).

Table 13. Percentages of the bioavailable fraction of PAHs.

Due to the obtained results, first of all it is possible to affirm that all the oral bioavailabilities are below 7,0 %, excepting naphthalene in June, that has a very high value, 18 %. Naphthalene is the PAH

with the highest bioavailability, followed by fluorene, with bioavailabilities of 6,8 % and acenaphthene, with values that reach up to 5,0 %. The phenanthrene bioavailability has also considered values that ranged between 0,20 and 1,8 %.

Fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(e)pyrene and benzo(k)fluoranthene have all of them moderate values between 0,018 and 0,87 %. However, examining the results we can affirm that anthracene, benzo(b+j)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene and benzo(ghi)perylene have no an oral bioavailability or it is as small as cannot be determined with this method.

Regarding the showed results in *Tables 2A and 13* related to the total concentration and the bioavailability percentage of PAHs, we can conclude that there is not always a correlation between them due to the compounds with higher total concentration were benzo(e)pyrene and chrysene whereas the higher bioavailability percentages were from naphthalene and acenaphthene. That is why nowadays it is very important to consider bioavailable fractions instead of total concentrations when risk assessment is studied.

6.1 Conclusions.

- Oral bioavailability of PAHs, based on an *in-vitro* approach consisting of using a dialysis membrane during the simulated intestinal digestion, was evaluated in different PM₁₀ samples collected at urban sites.
- Total PAHs concentration in PM₁₀ samples were in the range of 0,81 ng m⁻³ to 2,91 ng m⁻³ for benzo(e)pyrene, the majority PAH, and between 0,0038 and 0,084 ng m⁻³ for the minority one, naphthalene.
- The bioavailable PAHs concentration in PM₁₀ samples are ranged from 0,47 to 3,7 ng m⁻³ for phenanthrene which is the majority PAH, and from 0,038 and 0,059 pg m⁻³ for the minority one, benzo(k)fluoranthene.
- Low bioavailability percentages (around 7,6 %) have been found for naphthalene in PM₁₀ samples, whereas fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(e)pyrene and benzo(k)fluoranthene (bioavailability percentages lower than 0,87 %) are less bioavailable.
- An important difference between concentrations and bioavailable percentages in PM₁₀ samples is shown in benzo(a)pyrene, being the one with the higher total concentration and the least bioavailable percentage.
- There is an overestimation risk if only the total concentration of a pollutant is evaluated, it is necessary to determine their bioavailable fraction for a real risk assessment.

6.2 Conclusiones.

- La biodisponibilidad oral de HAPs, basada en una aproximación *in-vitro* que consiste en el uso de una membrana de diálisis durante la simulación de la digestión intestinal, se evaluó en diferentes muestras de PM₁₀ procedentes de una zona urbana.
- Las concentraciones totales de muestras de PM₁₀ se encuentran entre 0,81 ng m⁻³ y 2,91 ng m⁻³ para el benzo(e)pireno que es el HAP mayoritario, y entre 0,0038 y 0,084 ng m⁻³ para el minoritario, el naftaleno.
- Las concentraciones biodisponibles de HAPs en muestras de PM₁₀ se encuentran entre 0,47 y 3,7 ng m⁻³ para el fenantreno que es el componente mayoritario, y entre 0,038 y 0,059 pg m⁻³ para el minoritario, el benzo(k)fluoranteno.
- Se determinaron bajos porcentajes de biodisponibilidad (sobre el 7,6 %) para el naftaleno en muestras de PM₁₀, mientras que el fluoranteno, pireno, benzo(a)antraceno, criseno, benzo(e)pireno y benzo(k)fluoranteno (con porcentajes de biodisponibilidad menores del 0,8 %) son menos biodisponibles.
- Existe una importante diferencia entre las concentraciones y los porcentajes de biodisponibilidad en muestras de PM₁₀ que aparecen reflejadas en el benzo(e)pireno, siendo el HAP con la mayor concentración y el menor porcentaje de biodisponibilidad.
- Tiene lugar una sobrestimación del riesgo si únicamente se tiene en cuenta la concentración total del contaminante, es necesario determinar su fracción biodisponible para evaluar el riesgo real.

6.3 Conclusións.

- A biodisponibilidade oral de HAPs, baseada nunha aproximación *in-vitro* que consiste no uso dunha membrana de diálise durante a simulación da dixestión intestinal, evaluouse en diferentes mostras de PM₁₀ procedente dunha zona urbana.
- As concentracións totais de mostras de PM₁₀ atópanse entre 0,81 ng m⁻³ e 2,91 ng m⁻³ para o benzo(e)pireno que é o HAP maioritario, e entre 0,0038 e 0,084 ng m⁻³ para o minoritario, o naftaleno.
- As concentracións biodisponíbeis de HAPs en mostras de PM₁₀ atópanse entre 0,47 e 3,7 ng m⁻³ para o fenantreno que é o compoñente maioritario, e entre 0,038 e 0,059 pg m⁻³ para o minoritario, o benzo(k)fluoranteno.
- Baixas porcentaxes de biodisponibilidade (sobre o 7,6 %) foron determinadas para o naftaleno en mostras de PM₁₀, mentres que o fluoranteno, pireno, benzo(a)antraceno, criseno, benzo(e)pireno e benzo(k)fluoranteno (con porcentaxes de biodisponibilidade menores do 0,8 %) son menos biodisponíbeis.
- Existe unha importante diferenza entre as concentracións e as porcentaxes de biodisponibilidade en mostras de PM₁₀ que aparecen reflexadas no benzo(e)pireno, sendo HAP coa maior concentración e a menor porcentaxe de biodisponibilidade.
- Unha sobreestimación do risco ten lugar se unicamente se ten en conta a concentración total do contaminante, é necesario determinar a súa fracción biodisponíbeis para avaliar o risco real.

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Annex 1.

Table 1A: Concentrations of the bioavailable fraction of PAHs.

	January		February		March		April	
	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)
Nap	< 0,11		< 0,11		< 0,11		< 0,11	
Ace	< 0,10		0,39	15,1	0,48	22	0,17	20
Fl	< 0,49		< 0,49		0,69	3,4	< 0,49	
Phe	2,42	1,55	< 0,21		1,0	13	3,6	30
Ant	< 0,52		< 0,52		< 0,52		< 0,52	
Ft	< 0,10		< 0,10		3,6	6,7	2,4	7,6
Pyr	< 0,048		< 0,048		2,1	7,1	1,3	3,9
BaA	0,25	14,1	< 0,017		0,25	12	0,25	15
Chry	1,3	0,85	0,83	4,4	1,5	3,7	1,5	13
BeP	< 0,019		1,4	7,8	0,86	7,9	< 0,019	
BbF	< 0,15		< 0,15		< 0,15		< 0,15	
BkF	< 0,015		0,14	9,7	0,10	2,2	< 0,015	
BaP	< 0,15		< 0,15		< 0,15		< 0,15	
DBahA	< 0,015		< 0,015		< 0,015		< 0,015	
BghiP	< 0,020		< 0,020		< 0,020		< 0,020	
IP	< 0,0093		< 0,0093		< 0,0093		< 0,0093	

	June		July		August		September	
	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)
Nap	0,87	6,1	< 0,11		< 0,11		< 0,11	
Ace	< 0,10		< 0,10		< 0,10		< 0,10	
Fl	< 0,49		< 0,49		< 0,49		< 0,49	
Phe	0,64	9,6	0,47	5,87	3,7	13	< 0,21	
Ant	< 0,52		< 0,52		< 0,52		< 0,52	
Ft	< 0,10		< 0,10		< 0,10		< 0,10	
Pyr	< 0,048		1,47	3,31	3,2	23	2,5	26
BaA	0,11	5,6	0,07	7,13	< 0,017		0,27	23
Chry	0,60	24	0,57	1,14	< 0,0050		0,25	4,5
BeP	0,26	10	0,41	12	< 0,019		0,42	7,5
BbF	< 0,15		< 0,15		< 0,15		< 0,15	
BkF	< 0,015		0,039	15	< 0,015		0,11	20
BaP	< 0,15		< 0,15		< 0,15		< 0,15	
DBahA	< 0,015		< 0,015		< 0,015		< 0,015	
BghiP	< 0,020		< 0,020		< 0,020		< 0,020	
IP	< 0,0093		< 0,0093		< 0,0093		< 0,0093	

	October		November		December	
	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)	Concentration ($\mu\text{g m}^{-3}$)	Variation coefficient (%)
Nap	< 0,11		0,36	19	0,57	9,9
Ace	< 0,10		0,63	9,7	0,97	11
Fl	1,03	19	2,2	2,9	1,85	10
Phe	0,78	32	0,71	13	3,39	30
Ant	< 0,52		< 0,52		< 0,52	
Ft	< 0,10		< 0,10		< 0,10	
Pyr	1,16	12	2,89	15	< 0,048	
BaA	0,14	2,8	0,31	9,7	0,27	6,2
Chry	0,72	23	1,1	24	2,6	12
BeP	0,35	29	0,59	0,91	0,71	10
BbF	< 0,15		< 0,15		< 0,15	
BkF	< 0,015		0,078	19	0,060	10
BaP	< 0,15		< 0,15		< 0,15	
DBahA	< 0,015		< 0,015		< 0,015	
BghiP	< 0,020		< 0,020		< 0,020	
IP	< 0,0093		< 0,0093		< 0,0093	

Table A2. Total concentrations of PAHs.

Concentration (ng m ⁻³)	January	February	March	April	June	July
naphthalene	0,084	0,0038	< 0,027	< 0,027	0,0046	0,0058
acenaphthene	0,061	0,11	< 0,0011	0,014	0,030	0,028
fluorene	< 0,00096	0,049	0,040	0,017	0,014	0,018
phenanthrene	0,13	0,35	0,22	0,31	0,24	0,23
anthracene	0,0080	0,084	0,013	0,022	0,10	0,031
fluoranthene	0,47	1,2	1,8	0,76	0,30	0,46
pyrene	0,47	1,1	1,2	0,64	0,25	0,36
benzo(a)anthracene	0,43	0,73	0,40	0,39	0,18	0,26
chrysene	1,4	2,1	1,9	1,5	0,61	0,96
benzo(b+j)fluoranthene	2,2	2,9	2,3	2,0	0,81	1,3
benzo(k)fluoranthene	2,2	1,3	0,96	0,76	0,42	0,56
benzo(e)pyrene	0,62	0,27	0,15	0,12	< 0,00080	0,044
benzo(a)pyrene	0,69	0,96	0,70	0,71	0,33	0,51
dibenzo(a,h)anthracene	0,13	0,27	0,078	0,21	0,41	0,21
indene(1,2,3-cd)pyrene	1,2	1,0	0,85	0,49	0,55	0,61
benzo(ghi)perylene	1,1	1,4	1,1	0,85	0,42	0,67

Concentration (ng m ⁻³)	August	September	October	November	December	Standard deviation
naphthalene	< 0,027	< 0,027	< 0,027	0,016	0,030	7,8
acenaphthene	0,030	< 0,0011	< 0,0011	0,025	0,020	5,8
fluorene	0,032	< 0,00096	< 0,00096	0,032	0,037	11
phenanthrene	0,21	0,15	0,15	0,22	0,20	7,1
anthracene	0,026	0,075	0,012	0,0083	0,024	11
fluoranthene		0,072	< 0,00087	0,48	0,27	2,5
pyrene	0,37	0,53	0,46	0,67	0,54	3,1
benzo(a)anthracene	0,21	0,39	0,28	0,50	0,45	14
chrysene	0,98	1,4	0,95	1,3	0,95	13
benzo(b+j)fluoranthene	1,3	2,0	1,5	1,88	1,2	3,4
benzo(k)fluoranthene	0,47	0,75	0,59	0,75	0,60	6,0
benzo(e)pyrene	0,034	0,084	0,019	0,19	0,090	4,1
benzo(a)pyrene	0,39	0,56	0,44	0,84	0,78	5,3
dibenzo(a,h)anthracene	0,18	0,086	< 0,00066	0,073	0,050	10
indene(1,2,3-cd)pyrene	0,53	0,21	0,039	0,61	0,67	4,4
benzo(ghi)perylene	0,70	0,88	0,78	0,86	0,84	7