Contribution of mass spectrometry for the detection of xenobiotics implicated in cases of drug-facilitated crimes and the quantitation of urinary metabolites of polycyclic aromatic hydrocarbons

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Summary

Current analytical methods used for screening xenobiotics and/or their metabolites in biological fluids may in some instances lack specificity. Thus, it is often difficult to identify substances used by perpetrators to commit crimes or offences against their victims (theft or rape), because these substances are administered in very small quantities, generally have short half-lives, and the analysis for these substances is often made sometime after the incident. Also, as part of exposure to environmental pollutants, it is sometimes difficult to assess the importance of exposure of employees or of the general population to compounds of regulatory concern.

In the investigation drug-facilitated crimes (DFCs), the use of Ultra-Performance Liquid Chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) provides sufficient sensitivity, particularly in multiple reaction monitoring (MRM) mode to detect substances and/or their metabolites in body fluids of victims. Also, using the same technology allows detection of the hydroxylated urinary metabolites of PAHs, compounds of significant and widespread public health concern.

The scope of this thesis was to demonstrate the applicability of mass spectrometry in two areas. The first relates to the DFCs: after describing the definitions, prevalence in Europe and English-speaking countries, description of psychoactive substances implicated, the most common difficulties encountered during investigations and recommendations to improve the detection of such substances in terms of toxicology. A case of DFC by lysergic acid diethylamide (LSD) is reported, illustrating the method of identification and quantification of this compound and its main metabolite in biological fluids by UPLC-MS/MS. During the work on the screening of substances implicated in DFCs, we prepared organic extracts both alkaline and acid pH extractions, and studied the ionizing of molecules in both negative and positive electrospray mode. It became clear that certain molecules which are more easily ionized in negative electrospray mode had too high a detection limit under basic extraction conditions; we then developed a specific acid extraction method for these molecules to improve their detection.

The second area of application was the detection of hydroxylated metabolites of PAHs. A general review of these pollutants in the environment is provided, and the development and validatation of a UPLC-MS/MS method to identify and quantify these metabolites in urine of exposed or nonexposed populations to PAHs is described.

Résumé

Les méthodes actuelles d'analyse pour rechercher des xénobiotiques et/ou leurs métabolites dans les liquides biologiques peuvent dans certaines occasions manquer de specificité. Ainsi, il est souvent difficile de mettre en évidence les substances utilisées par des agresseurs pour commettre des actes délictuels sur leurs victimes (vols ou encore viols), parce que ces substances administrées en très faibles quantités ont généralement des demi-vies courtes, ou que les recherches de ces substances se font plusieurs jours après l'agression. De même dans le cadre de l'exposition aux polluants de l'environnement, il est difficile de mettre en évidence l'importance de l'exposition de salariés ou encore de la population générale à ces composés.

Dans le cadre de la soumission chimique, l'utilisation de la chromatographie en phase liquide couplée à la spectrométrie de masse en tandem (UPLC-MS/MS) permet d'obtenir une sensibilité suffisante notamment en mode MRM pour détecter des substances ou leurs métabolites dans les liquides biologiques de victimes. L'utilisation de la même technologie permet de rechercher les métabolites hydroxylés des hydrocarbures aromatiques polycycliques, composés présentant un intêrét important en Santé Publique.

Les objectifs du travail ont été de montrer l'applicabilité de la spectrométrie de masse dans deux domaines. Le premier se rapporte à la soumission chimique, domaine dans lequel après avoir rappelé, les définitions, la prévalence des cas de soumission chimique en Europe et dans les pays anglophones, la liste des substances psychoactives incriminées, les difficultés couramment rencontrées lors des investigations et les recommandations pour améliorer la détection des substances au niveau des analyses toxicologiques, est rapporté un cas de soumission chimique par le diéthylamide de l'acide lysergique ou LSD, par mise en évidence de ce composé et de son métabolite principal par UPLC-MS/MS. Au cours du travail sur la mise en évidence des substances de la soumission chimique, nous avions préparé les extraits biologiques en mélangeant des extraits effectués à pH acide et alcalin et en ionisant les molécules en mode électrospray négatif et positif. Il est vite apparu que certaines molécules ionisées plus facilement en mode électrospray négatif avaient une limite de détection trop élevée. Ces molécules s'extrayaient pour la plupart à pH acide, nous avons alors développé une méthode spécifique pour ces molécules afin d'améliorer leur détection.

Le second domaine d'application a été la mise en évidence des métabolites hydroxylés des hydrocarbures aromatiques polycycliques. Dans un premiers temps, nous avons réalisé une revue générale de ces substances polluantes de l'environnement, puis nous avons validé par

chromatographie liquide de haute performance couplée à la spectrométrie de masse une méthode qui permet d'identifier et de doser ces métabolites dans l'urine de sujets exposés ou non à ces HAP.

List of abbreviations

DFC (s):	Drug-facilitated crime (s)
PAH (s):	Polycyclic aromatic hydrocarbon (s)
UPLC-MS/MS:	Ultra-Performance Liquid Chromatography/Tandem Mass Spectrometry
GC-MS/MS:	Gas Chromatography/Tandem Mass
UPLC-FD:	Ultra-performance liquid chromatography-fluorescence detection
HPLC-DAD:	High-Performance Liquid Chromatography with Diode-Array Detection
SVOCs:	Semi-volatile organic compounds
POPs:	Persistent organic pollutants
PCBs:	Polychlorinated biphenyls
PCDD/Fs:	Polychlorinated dibenzodioxins /furans
USEPA:	United States Environmental Protection Agency
ATSDR:	Agency for Toxic Substances and Disease Registry
IPCS/WHO:	International Program on Chemical Safety / World Helath Organization
ETS:	Environmental tobacco smoke
CYP:	Cytochrome P450
GST:	Glutathione S-transferase
Ah:	Aryl hydrocarbon
SPE:	Solid phase extraction
NAP:	Naphthalene
ACY:	Acenaphthylene
ACE:	Acenaphthene
FLU:	Fluorene
PHEN:	Phenanthrene
ANTH:	Anthracene
FLT:	Fluoranthene

PYR: Pyrene

B[a]ANTH: Benzo[a]anthracene

CHR: Chrysene

B[b]FLT: Benzo[b]fluoranthene

B[j]FLT:· Benzo[j]fluoranthene

B[k]FLT: Benzo[k]fluoranthene

B[a]PYR: Benzo[a]pyrene

B[e]PYR: Benzo[e]pyrene

B[g,h,i]P: Benzo[g,h,i]perylene

I[1,2,3-cd]PYR: Indeno[1,2,3-cd]pyrene

Db[a,h]ANTH: Dibenzo[a,h]anthracene

OH-PAHs: Hydroxylated polycyclic aromatic hydrocarbons

1-, 2-OH-NAP: 1-, 2-hydroxynaphthalene

1-OH-NAP-d_7: Deuterated 1-hydroxynaphthalene

2-OH-FLU: 2-hydroxyfluorene

1-, 2-, 3-, 4-, and 9-OH-PHEN: 1-, 2-, 3-, 4-, and 9-hydroxy phenanthrene

3-OH-PHEN-C_{13}: 3-hydroxyphenanthrene isotope

3-OH-FLT: 3-hydroxyfluoranthene

1-OH-PYR: 1-hydroxypyrene

1-OH-PYR-d_9: Deuterated 1-hydroxypyrene

1-, 2-, 3-OH-B[a]ANTH: 1-, 2-hydroxybenzo[a]anthracene

3-, 6-OH-CHR: 3-, 6-hydroxychrysene

6-OH-CHR-C_{13}: 3-hydroxychrysene isotope

3-, 7-OH-B[a]PYR: 3-, 7-hydroxybenzo[a]pyrene

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General Introduction

Modern analytical techniques used in the analysis of biological matrices such as blood and urine for xenobiotics (and/or their metabolites) are dominated by the use of Ultra-Performance Liquid Chromatography (UPLC) with tandem mass spectrometry (MS/MS). In part, this is because of the improvements in sensitivity and affordability of this technique over recent years, but it is also because of the greater specificity of LC-MS/MS, allowing unequivocal identification of compounds, when compared to techniques such as high performance liquid chromatography with fluorescence detection (HPLC-FD). We have applied LC-MS/MS in two fields: forensic toxicology and the exposure of the general population to environmental pollutants.

Over the past several years, reports of drug-facilitated crimes (DFCs) have significantly increased causing alarm among the general public. However, in many cases, the detection and quantification of the substances involved in such crimes is difficult which, in part, may be due to the lack of a forensic toxicology laboratory of well-developed sensitive and specific methods able to provide analytical proof of their presence. In this regard, we firstly described in detail, through three articles, the evolutionary process in the current understanding of allegations of such crimes or offences. In the first article, we describe definitions of DFCs, their prevalence in European and English speaking countries, common difficulties encountered in investigating such offences and recommendations to improve detection of the substances involved through toxicological analysis. In the second and third article, we explore the different aspects of the substances involved in such crimes, focusing on the relationship between the properties of these substances and their involvement in such crimes or offences. Secondly, we have developed a UPLC-MS/MS method for detection and quantification of substances involved in DFCs and we have applied it to a case of sexual assault by lysergic acid diethylamide (article 4). Finally, we have developed a method for the identification and quantification of some acidic substances which were not detected in the general unknown screening in blood and urine using UPLC-MS/MS (article 5).

In a different research field, we present an example of the application of mass spectrometry to the study of environmental pollution. Owing to their ubiquitous presence in the environment (air, food, water and soil) and their toxicological relevance (mutagenic, teratogenic and carcinogenic), there is continued concern regarding the exposure of the general population to

PAHs. This concern has driven researchers to develop methods in order to assess the exposure of the general population. In this regard, we developed a UPLC-MS/MS method to simultaneously measure the urinary metabolites of several commonly studied PAHs among non-occupationally exposed subjects from the general population (article 6).

These two research themes, and the UPLC-MS/MS methods developed within them, provide a good example of the scope that exists for the application of mass spectrometry in the assessment of a range of biomarkers for the study of human exposure to xenobiotics.

Part I

Forensic Toxicology

Introduction

In the first part of forensic toxicology section, we present a detailed description of the different aspects of DFCs in 3 published articles (article 1 through 3). The first article proposes different definitions of DFCs, their occurrence and prevalence in European and English speaking countries, a list of intoxicating substances, which have so far been incriminated or been suspected to be involved in these crimes or offences, in addition to the common difficulties encountered in investigating allegations of DFC cases and recommendations to improve detection of the intoxicating substances. The second and third articles focus on describing the intoxicating substances with regard to their pharmacokinetic, pharmacological and other properties in relation to their involvement in DFCs.

Conclusion

Over the past few years, reports of DFCs have significantly increased, which is also associated with an increase in the number of substances involved in such crimes or offences. In many cases, the detection and quantification of these substances in biological fluids of the victims are difficult. Thus, it is necessary to develop highly sensitive and specific analytical methods in order to detect them and to provide jurisdictions with the analytical proof in such allegations.

In recent years, significant attention has been given to the use of liquid chromatography (LC) or gas chromatography (GC) coupled to tandem mass spectrometry (MS/MS) for the detection of these substances, owing to their sensitivity and high specificity compared with other analytical techniques.

In the next article (article 4), we have developed and validated an Ultra-Performance Liquid Chromatography/tandem mass (UPLC-MS/MS) for the detection and quantification of lysergic acid diethylamide (LSD) in biological fluids, a substance known to be involved in DFCs, although it is rarely detected by forensic laboratories.

Article 4: Characterization of drug-facilitated-crimes by Ultra-Performance Liquid Chromatography/Tandem Mass Spectrometry (UPLC-MS/MS). A case involving Lysergic acid diethylamide (LSD).

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Abstract

An ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method has been developed and validated for the confirmation and quantitation of LSD and its metabolites in blood and urine. The procedure involves one-step liquid-liquid extraction of the analytes from 1 mL blood or urine samples. Confirmation and quantitation were done by positive electrospray ionisation with a triple quadrupole mass spectrometer operating in multiple reaction monitoring (MRM) mode. Relative retention time and ion ratios were used as identification parameters. Limits of detection (LOD) in blood and urine were 50 pg/mL for LSD and 25 pg/mL for 2-oxo-3-hydroxy-LSD (OH-LSD). Limits of quantitation (LOQ) in blood and urine were 100 pg/mL for LSD and 50 pg/mL for OH-LSD. The method was linear from 100 to 5000 pg/mL for LSD and from 50 to 5000 pg/mL for OH-LSD in blood and urine. The intra-assay reproducibilities for LSD and OH-LSD at 1ng/mL in blood were 13%, while in urine were 11% for LSD and 10% for OH-LSD. Mean recoveries were in the range 75-90% in blood and urine. The method was successfully applied to blood and urine samples in a case investigation involving a 23-year-old male who claimed to have been the victim of rape. To best of our knowledge, this is the first reported detection of LSD and its metabolite OH-LSD in biological fluids (blood and urine) in a case of drug-facilitated crimes by LSD using UPLC-MS/MS analysis.

Introduction

The criminal use of psychoactive products, first described in 1983 by the term “chemical submission”, or “submission by medication”, then in 1990 as “drug-facilitated crimes” (DFCs), corresponds in France to the following precise definition: “The administration of a psychoactive product without knowledge of the victim in order to induce incapacitation and thus facilitate criminal actions such as robbery or rape” [1].

Lysergic acid diethylamide (LSD), derived from lysergic acid which occurs naturally in the ergot of rye, is one of the most potent psychotropic substances known [2-4]. It is approximately 100-150 times as potent as psilocybin and 4500-9275 times as potent as mescaline [5, 6]. Although considered a popular psychedelic drug of the 1960s**,** the use of LSD for recreational purposes decreased dramatically in the late 1970s and 1980s. However, in the early 1990s, LSD made a comeback and again became a significant drug of abuse, especially among older teens and young adults attending dance clubs and all-night raves. An increase in seizures of the drug and its use has been seen in the United States (U.S.) and Europe, LSD is still a major hallucinogen illegally used worldwide [2, 3, 7-10]. Illicit supplies of LSD (“commonly known as acid”) appear in a wide variety of forms, usually adsorbed onto microdots or paper “blotter” with characteristic and very colorful designs including cartoon and other film characters [3, 11]. Typical street doses are in the range of 40-120 µg [12].

Mostly orally ingested, LSD is rapidly and completely absorbed from the gastrointestinal tract, and circulated throughout the body and to the brain [2, 7, 13]. When given orally (1 µg/kg) to a single male volunteer, LSD had a peak plasma concentration of 1.9 ng/mL at 3 hours post ingestion [14]. Soon after administration, LSD metabolizes extensively, with only < 1% excreted in urine unchanged [15-18]. Metabolites so far identified in animal and human urine include 2-oxo-3-hydroxy-LSD (OH-LSD), 2-oxo-LSD, lysergic acid ethylamide (LAE), lysergic acid ethyl-2-hydroxyethylamide (LEO), nor-LSD (*N*-demethyl-LSD), nor-iso-LSD, 13- and 14-hydroxy-LSD and their glucuronide conjugates, as well as trihydroxylated-LSD. The major metabolite in the urine is OH-LSD, which may be as much as 16 to 43 times higher than LSD in urine [3, 4, 16, 17, 19-21]. The plasma elimination half-life of LSD has been reported to be 3-6 hours [14, 21, 22]. Determination of urinary concentrations of LSD following a single oral dose of the drug (200 µg) in humans shows that excretion of LSD reaches a maximum approximately 4-6 hours after administration [23].

LSD possesses a complex pharmacology that includes direct activation of serotonin, dopamine, and norepinephrine receptors, activation of secondary messengers, and alteration in gene expression [11, 24, 25]. Although the mechanisms of action for the subjective or physiological effects of LSD remain uncertain [2, 7], it is likely that LSD shares mechanisms of action with tryptamine hallucinogens such as psilocybin including activation of $5HT2_A$, $5HT2_C$ and $5HT1_A$ receptors [7, 24, 26]. LSD acts at least as a partial agonist at nearly all serotonin receptors except for 5HT3 [7]. When LSD activates the $5HT2_A$ receptor, it stimulates arachidonic acid and phospholipase C. This compound-specific trigger of secondary messenger systems may play a role in producing physiological or subjective effects of LSD [27].

The effects of LSD are unpredictable [2]. The minimum recognizable dose of LSD in humans is about 25 µg orally. The usual dose of LSD provoking the classic ensemble of symptoms is estimated to be in the range of 100-200 µg orally [7]. The first effects, resulting from sympathetic stimulation, occur within 20-40 minutes post-ingestion [2, 13, 28]. Symptoms may include anorexia, dry mouth, nausea, dizziness, mydriasis, tachycardia, sweating, tremors, hyperthermia, muscle weakness, and hypertension which can last two hours [2, 11, 28- 30]. These physical symptoms usually subside by the time the psychic symptoms appear [13, 28, 30], and these emotional and sensory changes are usually much more dramatic than the physical effects in people under the influence of LSD [29]. The experience is often referred to as “trip”, starts with hallucinations and is accompanied by mood swings, rapidly cycling from ecstasy to anxiety, euphoria to confusion and despair, and “good” hallucinations to terrifying visions. Dysesthesias are common and body perception distortions allude to the depersonalizing effect of this drug. The powerful disturbance lasts from 6 to 12 hours and is generally followed by a prolonged fatigue of one to two days as the user regains their grasp of the outside world. The perceptual changes and the intense arousal induced by LSD can be used against the victim’s will as a tool to commit crimes such as robbery or rape [2, 13, 29- 31].

The detection and quantitative determination of LSD and its metabolites in body fluids is still a challenging analytical problem for forensic laboratories, due to its physical-chemical properties such as photosensitivity, thermolability, sensitivity to acidic media, ease of isomerisation, possibility of adsorptive losses [12, 32-34], and the low concentrations in biological fluids which usually drop below the sub-nanogram level caused by pharmacological and kinetic features [12,14-18,21,22]. Nevertheless, the detection of very low concentrations of LSD and its metabolites in biological fluids can be achieved either through the use of a highly selective

extraction procedure, or a highly sensitive and specific detection method, or a combination of both [35-37].

A number of analytical techniques have been reported for detecting LSD and its metabolites in biological specimens, including radioimmunoassay (RIA) [38-44], capillary electrophoresis (CE) [45-47], and high-performance liquid chromatography (HPLC) with fluorescence detection [33, 42, 48-54]. These methods suffered from interferences, lack of sensitivity and specificity, and poor detection limits [10, 37, 43]. As a general rule, these methods require large sample volumes, are technically demanding, and are poorly suited for high sample throughput [43].

Several gas chromatography- mass spectrometry (GC-MS, and GC-MS/MS) methods have been developed to identify LSD [14, 21, 33, 51, 55-66]. However, the detection of LSD using GC-MS methods has its challenges: LSD undergoes irreversible adsorption during the chromatographic process, is relatively non-volatile, and is not stable at the elevated temperatures associated with GC [65]. Furthermore, in addition to the large sample volumes required (4 mL blood or 5 mL urine), sample preparation is laborious, requiring a derivatization step [36, 65, 67]. Because of the difficulties in detecting LSD by GC-MS methods and the increase in popularity of liquid chromatography (LC), recent identification methods developed for LSD analysis have increasingly used liquid chromatography-mass spectrometry (LC-MS) [32, 59, 67-77] or liquid chromatography-tandem mass spectrometry (LC-MS-MS) [4, 10, 35, 36, 74, 78-82]. LC-MS methods proposed for the determinations of LSD in biological fluids are either complicated involving difficult and time-consuming sample preparation or lack the required sensitivity, e.g., [72, 73]. The use of LC-MS/MS clearly improves the sensitivity and specificity of the analysis **[**10, 35, 36].

This paper describes an UPLC-MS/MS method for the analysis of LSD and its metabolites in blood and urine samples. Linearity, accuracy, precision, selectivity, recovery, and reproducibility were evaluated in this method. The method was applied to blood and urine samples from a 23-year-old male who claimed to have the victim of DFSA.

Materials and methods

Chemicals and reagents

LSD and OH-LSD were obtained from Ceriliant (Round Rock, Texas, U.S.). HPLC-grade methanol and acetonitrile (ACN) were obtained from Carlo Erba (Val de Reuil, France) and JT Baker (France), respectively. Ammonium formate and formic acid were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Deionized water Versol® was obtained from Aguettant (France). All remaining organic solvents and reagents were of analytical grade and obtained from the suppliers indicated: dichloromethane (Carlo Erba, France); diethylether (Panreac France); hexane (Sharlau, France) and isoamyl alcohol (Merck, France). Methylclonazepam and β-hydroxy ethyl theophylline were used as internal standards and were obtained from Roche (France) and Sigma-Aldrich (France), respectively.

Cannabinoids detection method was adapted from a previously described and published method by Kintz et al. (1996) [83].

Standard solutions

Mixed stock solution of LSD and OH-LSD was prepared at a concentration of 1000 ng/mL in methanol. Using the stock solution, two working solutions (1 and 10 ng/mL) containing the two analytes (LSD and OH-LSD) were prepared in methanol. The working solutions were then used to prepare 5 calibration solutions (0.05; 0.1; 0.5; 1; and 5 ng/mL). Each calibration contained the two analytes spiked into drug-free human blood or urine. Mixed stock solutions of methyl-clonazepam and β-hydroxyethyl theophylline, used as internal standards (IS), were prepared in methanol at concentrations of 1.25 and 16 mg/L and stored at -20°C until analysis.

Sample preparation

To 1 mL of each sample (serum and urine), 100 µL of the IS mix and 500 µL saturated disodium tetraborate solution (pH 9-9.5) were added in a 10 mL Kimble tube and mixed for 15 sec. Samples were extracted by the addition of 3 mL organic extraction solution (dichloromethane: hexane: ether: isoamyl alcohol; 30:50:20:0.5, v/v). Samples were vortex-mixed for 2 min before centrifugation at 3000 x g for 5 min. The upper, organic layer was transferred to a clean vial. The supernatant was evaporated to dryness using a vacuum concentrator (SpeedVac Concentrator SPD1010, Thermo Electron Co, Waltham, MA, USA) at 45^{o}C. The dried extract was reconstituted in 100 µL of mobile phase and vortex-mixed for 2 min before analysis.

LC conditions

The chromatographic system comprised an Acquity UPLC (Waters Corporation, MA, USA). Analytes were separated using an Acquity UPLC HSS C_{18}, 2.1 x 150 mm, 1.8 µm column (Waters Corporation, MA, USA) maintained at 50°C. A gradient of mobile phase A (formate buffer 5 mM pH 3.0, adjusted with formic acid) and solvent B (acetonitrile containing 0.1% formic acid) was used (**Table I**). The flow rate was 400 µL/min. An injection volume of 15 µL was used throughout.

Table I. UPLC gradient conditions

Time (min)	A: Formate buffer (%)	B: Acetonitrile (%)
0	87	13
0.5	87	13
10	50	50
10.75	5	95
12.25	5	95
12.5	87	13
15	87	13

MS conditions

Detection was performed using a Waters® TQ Detector, tandem quadrupole mass spectrometer (Waters Corporation, MA, USA) equipped with a Z-Spray™ source and ES probe. Ionisation was achieved using electrospray in positive ionisation mode (ESI +). Source conditions used were as follows: source temperature was maintained at 150°C; capillary voltage: 1000 V; extractor cone: 3V; cone gas flow rate: 50 L/h; desolvatation gas flow rate and temperature: 1000 L/hr and 350°C, respectively.

In order to establish the appropriate multiple reaction monitoring (MRM) conditions, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation (CID). The collision energy adjusted to optimise the signal for the most abundant product ion (**Table II**).

Table II. UPLC-MS/MS MRM Parameters				
Compound	**Parent ion** *(m/z)*	**Daughter ion** *(m/z)*	**Collision energy** *(eV)*	**Cone voltage** *(V)*
LSD	324.3	223.1	27	40
		207.9	33	40
O-H-LSD	356.3	237.2	23	30
		222.0	35	30
β-hydroxyethyl theophylline	338.7	122.3	55	40
Methyl-clonazepam	330.0	284.3	26	45

Validation procedure

Calibration standards of 0.05, 0.1, 0.5, 1 and 5 ng/mL of LSD and OH-LSD spiked into drug-free human blood and urine were analysed in three different runs. Data collection, peak integration, and weighted (1/x) linear regression were performed using MassLynxTM version 4.1 software (Waters Corporation, MA, USA).

Results and discussion

The validation parameters for LSD and OH-LSD in blood and urine are represented in **Table III**.

Table III. Validation parameters in blood and urine

Parameter	LSD	OH-LSD
Linearity	0.1-5 ng/mL	0.05-5 ng/mL
Reproducibility (n=6)		
Blood	13%	13%
Urine	11%	10%
LOQ	0.1 ng/mL	0.05 ng/mL
LOD	0.05 ng/mL	0.025ng/mL

Linearity and range

The linear range in blood and urine was 100 to 5000 pg/mL for LSD and 50 to 5000 pg/mL for OH-LSD.

Recovery, accuracy and precision

Percentage recoveries of LSD and OH-LSD ranged from 75% to 90% in blood and urine. The intra-assay accuracy and precision were assessed at a concentration of 1 ng/mL for both analytes (LSD and OH-LSD). Quantitative analysis for blood and urine was conducted using a full calibration curve with five points. The intraday accuracy and precision for the two analytes (LSD and OH-LSD) in both blood and urine were within acceptable limits. In blood, the accuracy and precision for LSD and OH-LSD were 13%. In urine, the accuracy and precision were 11% and 10% for LSD and OH-LSD, respectively.

LOD and LOQ

The detection limit (LOD) was defined as the lowest concentration giving a response of at least three-times the average of the baseline noise. The limit of quantification (LOQ) was defined as the lowest concentration that could be measured with an intra-assay precision CV% and standard deviation less than 20%. In both, blood and urine, the LOD was 50 pg/mL for LSD and 25 pg/mL for OH-LSD, while the LOQ was 100 pg/mL for LSD and 50 pg/mL for OH-LSD.

Selectivity

The U.S. Food and Drug Administration (FDA) recommends that the response of an analyte at the LOQ of a method should be at least five times the response compared to the blank [84]. Six different blank urine and blood samples were extracted and analyzed, and no interferences were found at the LOQ for either LSD or OH-LSD.

Case report

A 23-year-old man, with a history of occasional use to cocaine and ecstasy, went with his friends to a disco. He drank half a glass before dancing and then finishing his glass. Fifteen minutes after finishing his glass, he went to toilet and suddenly had a memory loss about what happened before. When he regained consciousness, he was alone, confused, very tired, diaphoretic, and claimed to have been raped. After 4 hours, he had vivid visual disturbances. He was sent to a forensic institute for both clinical examination and toxicological specimen sampling. He claimed to have consumed "speed" and smoked cannabis that night. Blood and urine samples were collected 10 hours after the incident.

The analysis of the victim's blood and urine samples by UPLC-MS/MS showed that LSD, cocaine and its metabolites (benzoylecgonine and ecgonine methyl ester), 3,4-methylenedioxymethamphetamine (MDMA) and its metabolite 3,4-methylenedioxyamphetamine (MDA), as well as amphetamine were detected in both the blood and the urine of the victim. OH-LSD, the metabolite of LSD, was detected only in the urine of the victim (**Table IV**, **figures 1 and 2**). In addition, 11-nor-delta-carboxy-9-tetrahydrocannabinol (THC-COOH) was detected in the blood of the victim at a concentration of 3.1 ng/mL.

Table IV. Substances detected in the victim's blood and urine by UPLC-MS/MS

Substance	Blood (ng/mL)	Urine (ng/mL)
Cocaine	1.0	14.5
Benzoylecgonine	38	6769
Ecgonine methyl ester	0.5	295
MDMA	20	12544
MDA	3.5	495
Amphetamine	63	24139
LSD	0.1	0.42
OH-LSD	ND*	0.48

ND*: Not detected

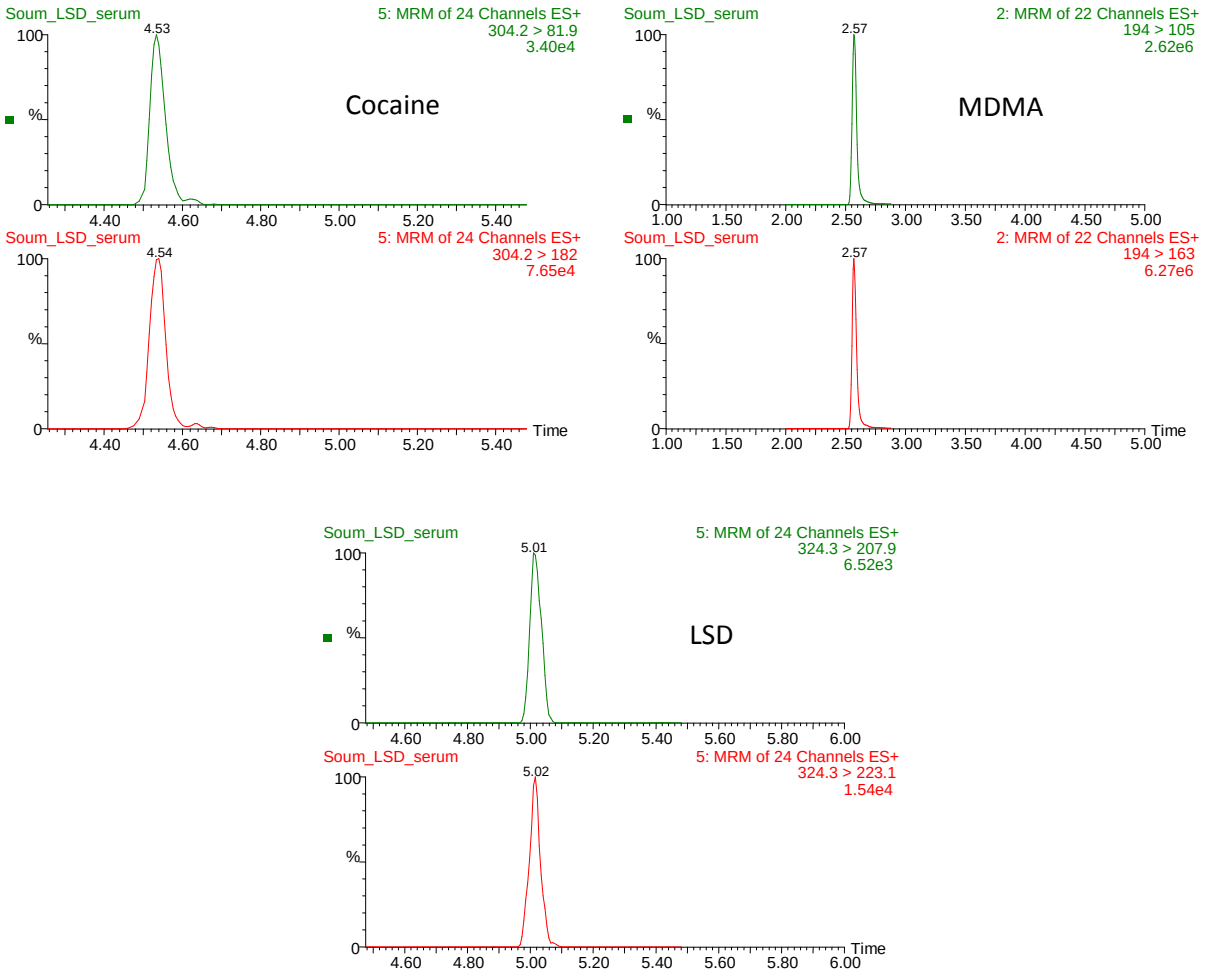

Figure 1: Chromatograms of the blood extract. On the top, the daughter ions of cocaine and MDMA, respectively. On the bottom, the daughter ions of LSD.

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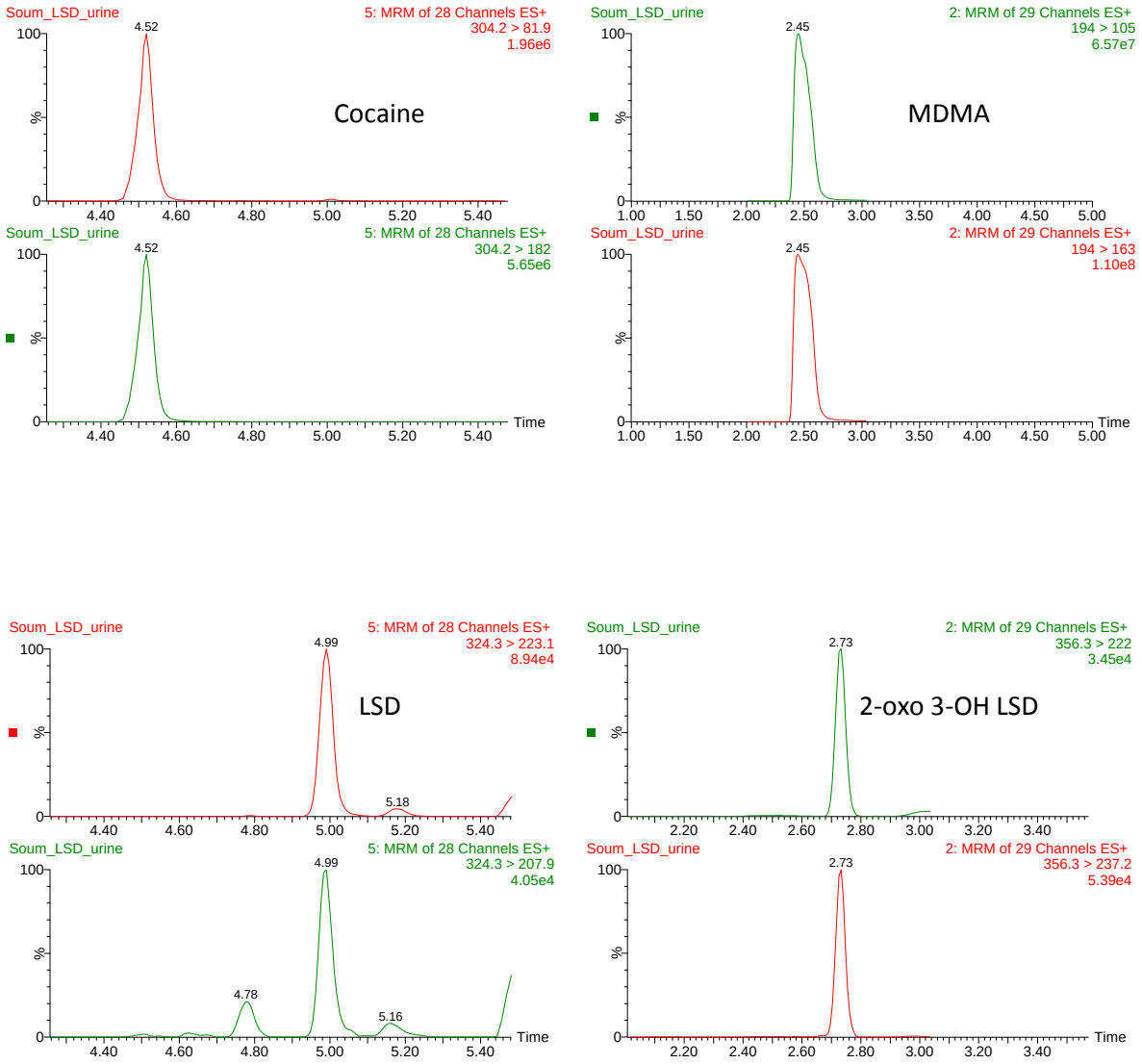

Figure 2: Chromatograms of the urine extract. On the top, the daughter ions of cocaine and MDMA, respectively. On the bottom, the daughter ions of LSD and its metabolite OH-LSD.

This case report was considered a case of DFCs by LSD. The reported scenario in this case mimics the typical scenario described by the majority of victims of DFSA by psychotropic products: they often admit to having had one or more alcoholic drinks with a friend-(s); a drink is left unattended for a period of time, after which the victim describes of losing track of events and then waking inappropriately clothed, or with sensation of having been sexually assaulted [85, 86]. As LSD is a colorless, odorless, tasteless, and water and alcohol-soluble substance [13, 28], it can be administered with ease to alcoholic beverages, without the victim's knowledge. In addition, the symptoms that the victim reported including memory loss, muscle weakness, diaphoresis, and the vivid visual disturbances, are among the characteristic effects produced by LSD. Furthermore, although other substances (cocaine and MDMA) were detected in the blood and urine of the victim that could have produced the symptoms experienced by the victim, they are unlikely to have produced symptoms of the magnitude reported, given the low concentrations of these two substances detected in his blood and urine (despite this individual's history of occasional use of these substances); this is also true of the relatively low concentrations of THCCOOH and amphetamine detected in the blood and urine of the victim. This led to the conclusion that the symptoms experienced by the victims were indeed due to LSD, but that the presence of these other substances, in addition to alcohol, might have increased the effects produced by LSD experienced by the victim.

Many liquid chromatography-mass spectrometry methods have been developed for the analysis of LSD and its metabolites in biological fluids. However, most of these studies describe methods for detection of LSD and its metabolites in urine [67-71, 73-80], due to the low concentration of LSD in blood [10, 87, 88]. Only a few studies have described analytical methods sensitive enough for determination of LSD and its metabolites in blood [4, 10, 35, 36]. However, the cases described were either cases of acute intoxication by LSD or suspected suicidal or homicidal cases in LSD abusers**,** where large quantities of LSD are suspected to have been ingested by the victims. Also, these studies did not mention the time elapsed between the collection of blood samples and the ingestion of LSD; only in one study it is indicated to be 4 hours [4]. To our knowledge, this is the first time that LSD is reported to be detected in a blood of a victim in a case of DFCs by LSD using a LC-MS/MS method. In this study, we were able to detect LSD in the blood of the victim at a low concentration (0. 1ng/mL) **(**which is the lowest concentration among the concentrations reported by other LC-MS/MS methods [4, 10, 35, 36]) 10 hours after suspected ingestion. In addition, we were able to detect OH-LSD, the metabolite of LSD, in the urine of the victim, which is consistent with other studies

reporting its presence in urine [4, 10, 35, 36, 68, 74, 78, 82], and with studies by workers who unsuccessfully attempted to identify OH-LSD in blood, although they were able to detect LSD in urine [4, 70]. One recent study [36] reported the detection of OH-LSD in the blood of a victim after LSD ingestion by a suspected LSD user. As the victim died after ingestion of LSD, it is possible that, by the time of death, some of the OH-LSD which was produced as a result of LSD metabolism before death, did not have the time to be excreted in urine and remained in the blood, and was thus detectable. But in our case, the victim did not die and, by the time the blood samples were collected (10 hours after the ingestion of LSD), most of the ingested LSD had been metabolized, and OH-LSD, was already excreted in urine: if some OH-LSD remained in the blood of the victim, it was of trace quantity that could not be detected. Nevertheless, the UPLC-MS/MS method we have developed in our laboratory for the identification and quantitation of 136 substances, incriminated or suspected of involvement in chemical submission cases **[**89], is a sensitive method for the analysis of LSD and its metabolites in the biological fluids from forensic cases.

Compared with other extraction procedures that have been proposed to determine LSD and its metabolites in biological fluids (liquid/liquid extraction at basic pH with varying solvents [4, 12, 33**,** 71], immunoaffinity purification procedures [31, 44, 54, 80], solid-phase extraction on different sorbents [64, 66, 81], or a combination of these methods [32,45**,**72,74]), we used one-step liquid/liquid extraction which seems to be the simplest and least expensive procedure, considering that a single extraction step is sufficient for UPLC-MS/MS procedure in which the high specificity of the detection balances possible partial purification of the anlaytes from the matrix.

Conclusions

LSD continues to be a significant drug of abuse, particularly in the United States and Europe, due to its wide availability and low cost. Thus, its involvement in crimes against the person including robbery or rape is to be expected. However, documented evidence regarding its involvement in these crimes is limited, which may stem partly from a lack of sensitive analytical methods available in the forensic laboratories. To the best of our knowledge, this is the first time that LSD has been reported in the biological fluids of a victim in a case of DFCs by LSD using UPLC-MS/MS analysis. This study describes an accurate, precise, rapid, selective, and sensitive UPLC-MS/MS method using positive ESI with a triple quadruple mass spectrometer to identify, confirm, and quantitate LSD and its metabolites in blood and urine. A

simple sample preparation method was used involving one-step liquid-liquid extraction with a smaller sample size than earlier described methods such as GC-MS.

Conflicts of interest: None

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Conclusion

UPLC-MS/MS is a useful technique that can provide sufficient sensitivity and specificity for the detection of a large number of substances involved in DFCs, even at low concentrations.

However, sometimes, it is difficult, for several reasons, to detect a small number of substances in the biological fluids. In the laboratory, during the work published by Humbert *et al.* [1], 42 substances, spiked in blood, were not detected. The question was: were these substances detected (or extracted) from the blood or not. Thus, we have changed the extraction procedures and have shown that the compounds were extracted under acidic conditions but not basic conditions. The method we have developed is described in article 5.

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Article 5: General unknown screening of xenobiotics: the contribution of an acidic extraction.

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Abstract

Objectives: In clinical and forensic toxicology, general unknown screening (GUS) is often used to detect and identify exogenous compounds. General screenings are often carried out, after organic extraction, by gas or liquid chromatography coupled to mass spectrometry or tandem mass spectrometry. However, according to the quality of the extraction of the xenobiotics and the mode and/or power of the ionisation of the molecule in the mass spectrometer, some molecules cannot detected.

Methods: In this study, we selected 42 molecules (drugs from various pharmacological classes and pesticides), which have not been detected previously in a general screening, either because they were not extracted or were extracted with a low yield, or because the ionization state was not optimum. We studied the conditions of a simple liquid-liquid extraction of these compounds and detected them using Ultra-Performance Liquid Chromatography/Tandem Mass Spectrometry (UPLC-MS/MS) with electrospray ionization (ESI) in scan mode.

Results: Despite these conditions, some molecules (%) were not yet detected in scan mode, however they could be detected in multiple reactions monitoring mode (MRM). Consequently, each molecule could be characterized by a combination of retention time and appropriate MRM conditions.

Conclusion: In addition to the utility of a general screening for the analysis of routine clinical and forensic samples, the developed method could be used to complete the research of molecules in biological matrix.

Introduction

General unknown screening (GUS) is often used to detect and identify exogenous compounds in various biological matrices (blood, urine or hair) in clinical and forensic toxicology, but with very different concentrations depending on the circumstances of poisoning (acute poisoning or chemical submission). The screening of xenobiotics can be carried out using untargeted separative methods and identification by GC-MS [1-3], GC-MS/MS [4-6], HPLC-DAD [7], HPLC/MS [8, 9], HPCL-MS/MS [10, 11], UPLC-MS [12], or UPLC-MS/MS [13]. In these screenings, the identity of the compounds is established by comparison of the obtained spectra (UV or mass/z) with reference spectra from commercial or in-house made libraries.

Nowadays, LC-MS (-MS) or UPLC-MS (-MS) is used routinely in the framework of analysis prescribed in clinical or forensic toxicology, because this technology allows the detection of the largest number of compounds. However, some compounds are not detected in GUS, for two main reasons. The first is the method carried out for the extraction of the unknown molecules contained in the biological matrix; the second reason is the lack of sensitivity in the detection for these molecules. For this last point, Multi Reaction Monitoring (MRM) mode in mass spectrometry improves the detection limit of these compounds, but this acquisition only allows targeted research due to a setting of many specific MRM transitions.

Another point, the sample preparation (extraction from a biological matrix) is a phase that must be evaluated and controlled, for obtaining the best limit of detection. A lot of molecules have been detected, according to the context, because the authors focused on one substance [14], or on a given therapeutic class, such as benzodiazepines [15]. It is clear that a sample preparation method which is specifically directed toward a molecule or a group of similar molecules will be the most efficient and probably more sensitive, but will require having recourse to other extractions to detect other molecules, potentially present. The repetition of extractions is laborious and requires larger sample.

A screening of xenobiotics by UPLC- MS using in-source fragmentation at increasing cone voltages has been described [13]. The xenobiotics were detected after mixture of two extractions one under acidic conditions, the other under basic conditions, by ionization in both ES^+ and ES^-. In this work, some xenobiotics (drugs or pesticides) were not detectable probably owing to the sample preparation or a lack of sensitivity of the mass spectrometer. To improve the detection of these compounds, an acidic extraction protocol was investigated for the detection of these molecules in blood and compared to a basic extraction. The detection was

also been improved using MRM mode in ES^+ or ES^-, according to the response of the compound.

Materials and methods

Standards and reagents

HPLC-grade methanol and acetonitrile were from Carlo Erba (Val de Reuil, France) and from JT Baker (France), respectively. Ammonium formate and formic acid were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Deionized water (Versol®) was obtained from Aguettant, (France). All remaining organic solvents and reagents were of analytical grade and obtained from the suppliers indicated: dichloromethane (Carlo Erba, France); diethylether (Panreac, France); hexane (Sharlau, France) and isoamyl alcohol (Merck, France).

Drug substances were supplied by various pharmaceutical companies. Individual stock solutions of the standard compounds were prepared in methanol at 1g/L; these were kept at -20°C until use.

Methylclonazepam and β-hydroxy ethyl theophylline were used as internal standards and were obtained from Roche (France) and Sigma-Aldrich, respectively. A mixed stock solution (IS mix) was prepared in methanol at 1.25 and 16 mg/L, respectively, and stored at -20°C until analysis.

Biological specimens

Blank human serum was obtained from EFS (Etablissement Français du Sang Lille, France).

Authentic samples were routine samples, these were extracted and analysed on the same day of the patient's admission to the hospital.

Liquid chromatography conditions

The chromatographic system comprised an Acquity UPLC (Waters Corporation, MA, USA). Analytes were separated using an Acquity UPLC HSS C_{18}, 2.1 x 150 mm, 1.8 µm column (Waters Corporation, MA, USA) maintained at 50°C. The mobile phase was a binary mixture of formate buffer 5 mM pH 3.0, adjusted with formic acid (A) and acetonitrile containing 0.1% formic acid (B) at a flow rate of 400 µL/min (generating a typical back-pressure of ~9000 psi) (0 min. =87% A; 6-7 min = 5 % A; 7.25-10 min = 87 % A). An injection volume of 15 µL was used throughout.

Mass spectrometry conditions

Detection was performed using a Waters® TQ Detector, tandem quadrupole mass spectrometer (Waters Corporation, MA, USA) equipped with a Z-Spray™ source and ES probe. The instrument was controlled using Waters MassLynx™ v4.1. Ionization was performed in ES^+ and in ES^-. Source conditions were as follows: source temperature was maintained at 150°C; capillary voltage: 1000 V; extractor cone: 3V; cone gas flow rate: 50 L/h; desolvation gas flow rate and temperature: 650 L/h and 350°C, respectively.

Infusions were performed at 10 µL/min with variations of the cone voltage, energies of accelerations for optimisation.

For each compound, the MRM transitions, cone voltage and collision energy are indicated in **Table I**.

Preparation of the solutions

Working solutions of the drugs or pesticides of interest were prepared by dilution of the stock solution with the mobile phase to obtain a concentration of 1000 ng/mL. Small volumes were analysed in two separate infusion analyses; one in ES+ and one in ES- to optimize and obtain two transitions MRM (**Table I**).

Data processing

All data were processed using the QuanLynx application manager (Waters Corporation, MA, USA). This software permits the integration of MRM peaks at specific retention times and the calculation of their areas.

Biological sample preparation

Blank sera were spiked with the substances approximately at high therapeutic concentrations, when these concentrations were known. When the therapeutic concentrations of the drug were unknown, or when the compound was a pesticide, concentration less than 5000 ng/mL was tested. Blank serum and spiked serum were prepared by a liquid- liquid extraction (LLE) by acidic extraction. One milliliter of biological sample, 100 µL of the IS mix and 500 µL sodium acetate buffer (1 M, pH 3.5) were added to a 10 mL Kimble tube and mixed. Samples were extracted by the addition of 3 mL organic extraction solution (dichloromethane: ether: hexane: isoamyl alcohol; 30:50:20:0.5, v/v). Samples were vortex-mixed for 2 min before centrifugation at 3000 x g for 5 min. The upper, organic layer was transferred to a clean vial.

A basic extraction was also carried out using the same procedure, by substituting the sodium acetate buffer with a saturated borate buffer (pH 9.0)

The supernatant was evaporated under nitrogen at 60^{o}C. Dried extracts were reconstituted in 100 µL of mobile phase and vortex-mixed for 2 min before analysis.

A limit of detection has been estimated, using the highest therapeutic concentration or this last concentration by dilution 1:10 or 1:100. For substances for which the therapeutic concentrations were unknown and for the pesticides, an arbitrary concentration was first tested, and then diluted 1:10 and 1:100.

Results

Table II indicates the 42 tested xenobiotics. For each compound, the therapeutic, toxic and lethal concentrations (when known), the mode of ionization and the chosen transitions for the identification of drugs are described. Twenty five substances were detected in ES^+ mode and 17 in ES^- mode.

The yield of extraction was estimated after extraction in acidic (acetate buffer) or basic (borate buffer) condition. The results show clearly a better extraction using acetate buffer (**Table II**)

A limit of detection has been estimated, permitting the detection of all the studied compounds and the measurement of low, detectable concentrations for some compounds (**Table II**).

Discussion

Several general unknown screenings have been described for the detections of xenobiotics, but often the analytical protocol targeted a specific pharmacological class of drugs [16-18] or pesticides [6, 8].

In our original, systematic GUS procedure using a double extraction (basic and acidic), some substances were not detected [13]. Extraction of the substances was then carried out in acid and basic condition. The sensitivity of the detection has been increased by using MRM mode of detection, either in positive or negative mode. In this way, all the molecules could be detected, using only the acidic, at least at therapeutic concentrations (or less) for the drugs. Therefore, currently in the laboratory, the screening described by Humbert and Colleagues [13] is used for the GUS, and an acidic extraction is also carried out for those undetectable molecules, with detection by MRM mode. The two protocols are rapid and with the use of UPLC-MS-MS, the

results are obtained in about 15 min for the routine procedure and 10 min in MRM mode for the acidic procedure.

Table I: List of the studied substances. The therapeutic, toxic and lethal concentrations (when known) are indicated. For the MRM detection, ion parent and daughter ions are also indicated.

Name	Activity	serum concentration ng/mL			Ion parent	ESI	Cone V	Transition 1		Transition 2	
		Therapeutic level	Toxic levels	Lethal levels				Daughter ion	Collision (eV)	Daughter ion	Collision (eV)
Acide Floctafenique	Analgesic				333,1	+	40	295,3	30	267,3	40
Acide salicylique	analgesic	20 000-200 000	300 000-350 000	(-400 000)500 000	179,1	-	10	137,1	8	93	18
éAdrafinil	psychotonic				167,1	+	35	152	22	167,1	16
Barbital (Arret 91)	Barbiturate	2 000-40 000	20 000-50 000	50 000	183	-	25	140,1	10	85,1	14
Bromadiolone	Rodenticide		20		525,3	-	55	250,2	36	93	38
Bumetanide	Diuretic				365,2	+	35	240,3	18	184,3	18
Bupropion	Antidepressant	25-100		7300	240,2	+	25	131,1	26	184	14
Butalbital	Barbiturate	1700-2600	10000-15000	15000-30000	223	-	30	180,3	14	84,9	18
Butobarbital (Arret)	Barbiturate	5 000-15 000	20 000	30 000	210,9	-	25	168	14	85,1	14
Carbutamide	Antidiabetic				272,1	+	25	156,2	18	74,3	12
Cetirizine	antihistaminic	appr.20-300	2 000-5 000		389,1	+	30	201	18	166	42
Chlorophacinone	Rodenticide				373	-	85	201,1	24	145	24
Diclofenac	NSAID[a]	500-3 000	50 000; 60 000		296,2	+	25	250	14	215,1	20
Diflunisal	NSAID[a]	40 000-200 000	300 000-500 000	600 000	249	-	35	205,1	20	157	34
DNOC	Fongicide	1 000-5 000	30 000-60 000	75 000	197	-	35	137	18	109,1	22
Enalipril	AHT[c]	10-100			377,3	+	35	234,4	22	303,3	18
Fenoprofen	NSAID[a]	30 000-60 000			241,1	-	20	197,1	10	93,1	22
Fluindione	Anticoagulant				239	-	60	169,1	42	145,1	40
Furosemide	Diuretic	1 000-6 000	25 000-50 000		329,3	-	35	285,1	14	205	22
Glibornuride	antidiabetic				367,1	+	25	170,2	18	152,2	18
Gliclazide	antidiabetic	4000 **			324,1	+	30	91,1	34	127,2	18

Table I continued: List of the studied substances. The therapeutic, toxic and lethal concentrations (when known) are indicated. For the MRM detection, ion parent and daughter ions are also indicated.

Name	Activity	serum concentration ng/mL			Ion parent	ESI	Cone V	Transition 1		Transition 2	
		Therapeutic level	Toxic levels	Lethal levels				Daughter ion	Collision (eV)	Daughter ion	Collision (eV)
Glipizide	antidiabetic	100-1000	> 2000		446,3	+	25	100,3	25	321,3	16
Imidapril	ACE inhibitor[b]				406,3	+	40	234	24	332,3	22
Ioxynil	Herbicide				369,9	-	40	127	34	215	32
Irbesartan	AHT[c]				429,3	+	35	207,3	30	84,2	34
Loratidine	Antihistaminic	1-20			383,1	+	45	337,1	24	267,1	34
Mecoprop	Herbicide				212,9	-	25	140,9	14	71	10
Meloxicam	NSAID[a]	400-2 000			352,1	+	35	141,2	20	115,2	20
Modafinil Acid	Stimulant	2 000-3 000			167	+	40	115	34	152	22
Naproxen	NSAID[a]	20 000-100 000	200 000-400 000		231,1	+	25	185,1	16	170	28
Nifuroxazide	ATB [d]				276,2	+	35	121,2	20	93,1	28
Pentobarbital/Amobarbital	Barbiturate	1 000-10 000	10 000-19 000	15 000-25 000	225,1	-	30	181,9	14	85	14
Perindopril	AHT[c]	80-150			369,3	+	35	172,3	20	98,2	36
Phenobarbital	Barbiturate	10 000-30 000	30 000-40 000	50 000-60 000	231,3	-	25	42,1	14	188,1	10
Piretanide	Diuretic				363,2	+	40	282,1	24	236,2	26
Piroxicam	NSAID[a]	2 000-6 000	14 000		332,1	+	30	164,3	18	95,2	22
Ramipril	AHT[c]	appr.1-10			417,2	+	35	234,3	22	117,1	39
Secobarbital	Barbiturate	1000-2200	> 3000	> 5000	237,1	-	30	194,1	12	85,3	18
Sulindac	NSAID[a]	1 000-6 000			357,2	+	45	233,3	28		
Telmisartan	AHT[c]				515,4	+	50	276,3	44	211,4	42
Thiopental	anesthetic	1 000-5 000	10 000	10 000-15 000	241,3	-	30	101	14	58	14
Tolbutamide	antidiabetic	40 000-200 000	400 000-500 000	640 000	271,3	+	25	155,2	16	74,2	16

[a] NSAID : non-steroidal anti- inflammatory drug; [b] ACE inhibitor : angiotensin-converting enzyme (ACE) inhibitor; [c] AHT : antihypertensive; [d] ATB : antibiotic

Table II : Yield of extraction after acidic or basic extraction

Name	Extraction yield		Estimated LOD
	Acetate buffer	Borate Buffer	after acidic extraction
Acide Floctafenique	61%	0%	5
Acide salicylique	68%	0%	191
Adrafinil	57%	0%	1100
Barbital (Arret 91)	54%	29%	1000
Bromadiolone	83%	33%	50
Bumetanide	62%	0%	50
Bupropion	77%	30%	10
Butalbital	54%	30%	5000
Butobarbital	63%	70%	10300
Carbutamide	54%	0%	10
Cetirizine	61%	1%	3
Chlorophacinone	47%	11%	49
Diclofenac	61%	10%	32
Diflunisal	80%	10%	9000
DNOC	80%	3%	49
Enalipril	58%	0%	0,8
Fenoprofen	100%	0%	297
Fluindione	89%	0%	5000
Furosemide	70%	0%	608
Glibornuride	73%	15%	5
Gliclazide	86%	21%	4
Glipizide	71%	3%	5
Imidapril	104%	0%	900
Ioxynil	85%	0%	49
Irbesartan	79%	0%	0,5
Loratidine	68%	50%	0,2
Mecoprop	87%	0%	5000
Meloxicam	56%	0%	21
Modafinil Acid	101%	0%	310
Naproxen	9%	0%	1088
Nifuroxazide	53%	19%	0,5
Pentobarbital/Amobarbital	83%	79%	3728
Perindopril	67%	0%	10
Phenobarbital	68%	50%	1908
Piretanide	66%	1%	5
Piroxicam	95%	3%	6
Ramipril	65%	0%	0,9
Secobarbital	33%	50%	2970
Sulindac	93%	2%	6
Telmisartan	67%	10%	50
Thiopental	44%	27%	1880
Tolbutamide	55%	9%	1008

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Part II

Polycylic aromatic hydrocarbons

Section I

Theoretical Principles

1. Definition

Polycyclic aromatic hydrocarbons (PAHs) are often considered as part of a larger group of several 'families' of organic compounds, including the polychlorinated biphenyls (PCBs) and the dioxins and furans (PCDD/Fs), designated as semi-volatile organic compounds (SVOCs) and persistent organic pollutants (POPs). PCBs, PCDD/Fs and PAHs are grouped under these two headings in order to convey their broadly similar tendencies in the environment (each family covers a range of vapour pressures, lipophilicities and persistence). However, individual compounds will behave broadly according to their own physic-chemical properties [1].

PAHs, by definition, contain only carbon (C) and hydrogen (H) and are the product of any combustion involving fossil fuels, or more generally, material containing C and H. Inefficient combustion results in PAHs being emitted to the atmosphere both as gaseous and associated with particles. PAHs are made up of two or more fused aromatic rings [1].

2. Historical context

In the late 19th and early 20th centuries, coal gas derived from the low temperature destruction of coal was a major source of fuel for heating and lighting. Coke, still used in the steel industry, was one by-product of this process, and another by-product, coal tar, provided the raw materials for the genesis of several present-day industries. The isolation of naphthalene, anthracene, benzene and toluene from coal tar in the early 19th century, and the later development of prontosil, provided the raw materials used in the stripling dyestuffs and pharmaceutical industries [2].

The carcinogenic activity of PAHs was first proposed by Sir Percival Pott (1775) in English chimney sweeps, and today they are subject to legislation arising from concerns about their teratogenic and mutagenic activity, and that of their metabolites [3, 4]. In the late 1970s, The United States Environmental Protection Agency (USEPA) listed sixteen PAHs as 'priority Pollutants' and this list was subsequently adopted by the European Union (EU). The sixteen PAHs are; naphthalene (NAP), acenaphtylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHEN), anthracene (ANTH), fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (B[a]ANTH), chrysene (CHR), benzo[b] fluoranthene (B[b]FLT), benzo[k]fluoranthene (B[k]FLT), benzo[a]pyrene (B[a]PYR), benzo[g,h,i]perylene (B[g,h,i]P, indeno[1,2,3-cd]pyrene (I[1,2,3-cd]PYR), and dibenzo[a,h]anthracene (Db[a,h]ANTH) [3] (**Figure 1**). As well as addressing contamination of soil and water by coal tar and its distillates (e.g., creosote), current EU and USEPA legislation aims to curb PAH emissions to air from

combustion processes. This latter category has become the focus of much research attention, since it promotes transport of PAHs over significant distances from their source, and has resulted in PAHs becoming ubiquitous environmental contaminants [5-8]. Sanders *etal.* [9] calculated the fluxes of PAHs from air to a dated core from an ombrotrophic peat bog in north west England, and showed that fluxes rose steadily from the start of the industrial revolution in Britain (*ca.* 1780) to a peak in the first half of the 20 century, and have been in general decline since *ca.* 1930. Other authors also have described this downward trend in air concentrations over the past 40-50 years, illustrating the impact of legislative restrictions on combustion and improvements in combustion technology on PAH concentrations in urban air [10, 11].

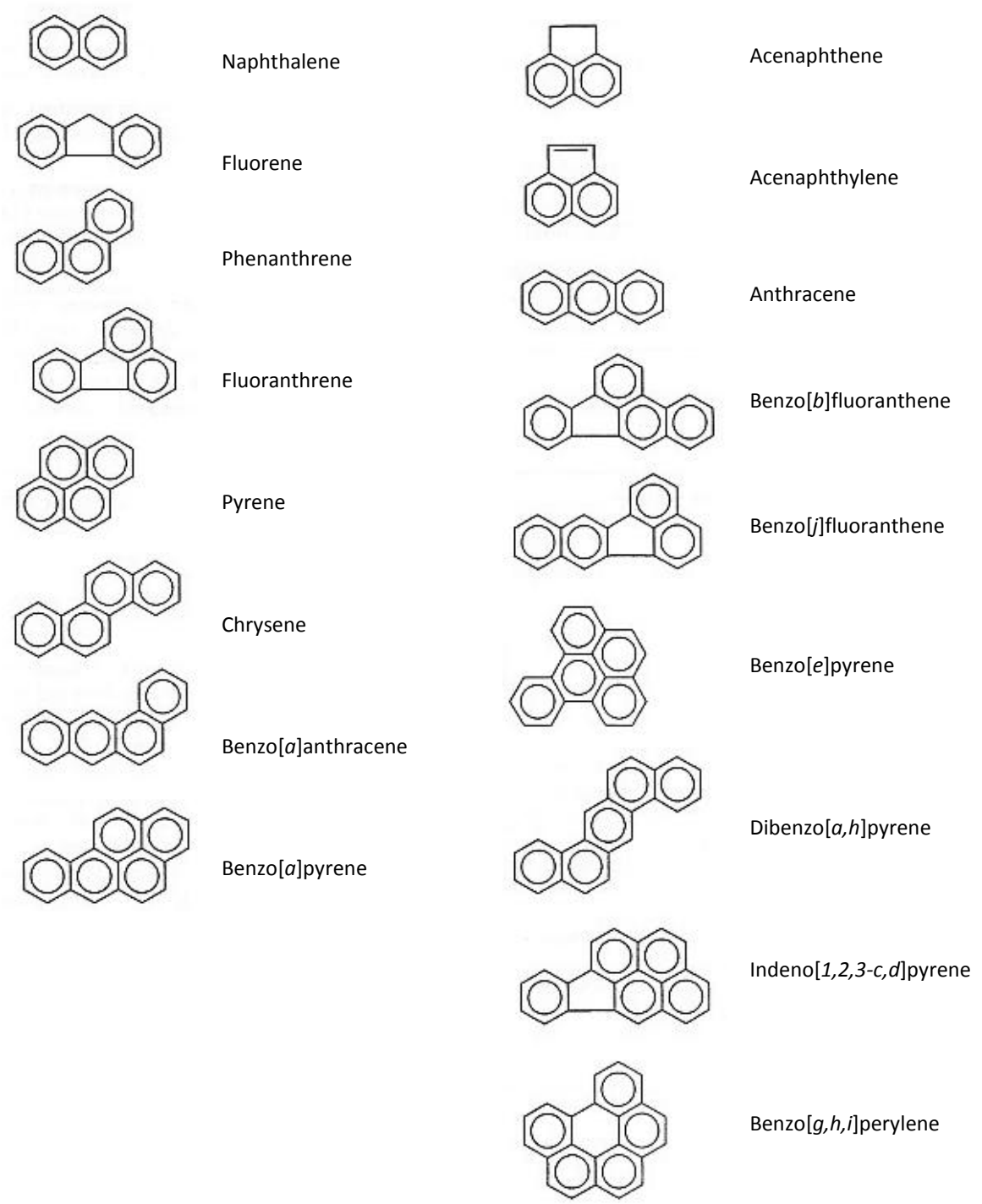

Figure 1: The 16 US EPA PAHs and Benzo[*e*]pyrene (those compounds for which urinary metabolites were measured appear on the left of the diagram).

3. Physico-chemical properties

Table I shows some physico-chemical characteristics of PAHs. As compounds increase in size, there is a concurrent decrease in volatility (P_L) and water solubility (H'), and increased attraction to environmental matrices rich in lipid or organic matter (K_{ow}/K_{oA}). These characteristics are paralleled within other families of POPs, and result in different partitioning of compounds between particle and gas phases in the atmosphere (the primary transport medium) [12].

Smaller PAHs (2 or 3 fused rings; *ca.* 130-170 a.m.u.) tend to exist primarily in the gas phase with some 3-ring and all 4-ring PAHs (*ca.* 170-230 a.m.u.) exhibiting temperature-driven partitioning between these two atmospheric phases. The heavier molecular weight PAHs (5 or more fused rings; > *ca.* 250 a.m.u.) exist primarily condensed onto particles in the atmosphere, and indeed, are often emitted absorbed into particles from combustion sources [13].

Partitioning behaviour in the atmosphere determines, to a large extent, the distance that POPs of different sizes will be transported from the source, as well as the dominant mode of deposition for a given compound. In general, 2-ring PAHs, classified in the high mobility category of POPs, undergo world-wide atmospheric dispersion with limited deposition. 3-ring PAHs, included in the relatively high mobility category of POPs, preferentially deposit and accumulate in polar latitudes. 4-ring PAHs, included in the relatively low mobility category of POPs, are deposited and accumulate mainly in mid latitudes. PAHs with > 4 rings, included in low mobility category of POPs, subjected to rapid deposition and retention close to the source [14].

The susceptibility of individual PAHs to degradation in the environment by biotic or a biotic means is also related to their physico-chemical properties, since these not only determine the reaction rates of the compound, but also affect the availability of PAHs to biotic and abiotic degradation processes [15].

Table I: Physico-chemical properties of the 16 USEPA PAHs and Benzo[e]pyrene at 298K (adapted from Mackay *et al*. 1992[12]).

PAH	No. rings[a]	Mol Wt[b]	PL[c]	Log K_{OW}[d]	Log K_{OA}[e]	H'[f]
Naphtalene	2	128	36.8	3.37	5.13	0.017
Acenaphthylene	2*	152	4.1	4.0	6.47	0.0034
Acenaphthene	2*	153	1.5	3.92	6.23	0.0049
Fluorene	2*	166	0.72	4.18	6.68	0.0032
Phenantrene	3	178	0.11	4.57	7.45	0.0013
Anthracene	3	178	0.078	4.54	7.34	0.0016
Fluoranthene	3*	202	0.0087	5.22	8.60	4.2×10^{-4}
Pyrene	4	202	0.012	5.18	8.61	3.7×10^{-4}
Benzo[a]anthracene	4	228	6.1×10^{-4}	5.91	9.54	2.4×10^{-4}
Chrysene	4	228	1.1×10^{-4}	5.86	10.44	2.6×10^{-5}
Benzo[b]fluoranthene	4*	252	-	5.80	-	-
Benzo[k]fluoranthene	4*	252	4.1×10^{-6}	6.0	11.19	6.5×10^{-6}
Benzo[a]pyrene	5	252	2.1×10^{-5}	6.04	10.77	1.9×10^{-5}
Benzo[e]pyrene	5	252	2.4×10^{-5}	-	-	8.1×10^{-6}
Benzo[g,h,i]perylene	5*	276	2.3×10^{-5}	6.50	11.02	3.0×10^{-5}
Indeno[1,2,3-cd]pyrene	5*	278	-	-	-	-
Dibenzo[a,h]anthracene	5	276	9.2×10^{-8}	6.75	-	-

[a]-No of aromatic rings (* indicating the presence of a non-aromatic structure or substitution)
[b]-Molecular weight (a.m.u)
[c]-Sub-cooled liquid vapour pressure (Pa)
[d]-Natural logarithm of the octanol-water partition coefficient
[e]-Natural logarithm of the octanol-air partition coefficient
[f]-Dimensionless Henry's law constant
- Indicating data unavailable

4. Sources of human exposure

There is a continuous concern regarding the exposure of the general population to PAHs. This concern arises from the fact that PAHs are ubiquitous environmental pollutants that possess mutagenic, teratogenic and carcinogenic effects [3, 16, 17]. This concern has driven the researchers to develop methods in order to assess the exposure of the general population to PAHs; one method that is highly used being the identification and quantification of urinary metabolites of PAHs.

It should be noted that the general population is generally exposed to mixtures of PAHs rather than to a single PAH. The International Agency for Research on Cancer (IARC) has classified a number of individual PAHs compounds, based on animal experiments, as probable human carcinogens (Category 2A) [16, 17] **(Tables II and III),** and a number of common mixtures of substances that include PAH compounds as carcinogenic to humans (Category 1), based on occupational studies in workers in different industrial settings [17-21] **(Table IV).**

For the general population, the major routes of exposure to PAHs are from inhalation of ambient and indoor air, ingestion of food, water and soil, and dermal absorption of products containing PAHs [3, 4, 22].

Table II: The degree of evidence for carcinogenicity of "alternant" PAHs in experimental animals, and overall evaluations of carcinogenicity to humans (evaluated by IARC and IPCS/WHO) [16, 17].

PAH compound	No. rings	IARC		WHO
		Animals	Humans	
Phenanthrene	3	I	3	(+/-)
Anthracene	3	I	3	-
Pyrene	4	I	3	(+/-)
Benzo[a]anthracene	4	S	2A	+
Chrysene	4	L	3	+
Benzo[a]pyrene	5	S	2A	+
Benzo[e]pyrene	5	I	3	+/-
Dibenz[a,h]anthracene	5	S	2A	+
Anthanthrene	6	L	3	+

Abbreviations: I, Inadequate evidence;L, limited evidence;S, sufficient evidence;2A, probably carcinogenic to humans;2B, possibly carcinogenic to humans;3, not classifiable;-, negative;+, positive; +/-:questionable; ( ), limited number of studies.

Table III: The degree of evidence for carcinogenicity of "non-alternant" PAHs in experimental animals, and overall evaluations of carcinogenicity to humans (evaluated by IARC and IPCS/ WHO) [16, 17].

PAH compound	No. rings	IARC		WHO
		Animals	Humans	
Fluoranthene	4	I	3	(+)
Benzo[b]fluoranthene	5	S	2B	+
Benzo[k]fluoranthene	5	S	2B	+
Benzo[g,h,i]fluoranthene	5	I	3	(-)
Indeno[1,2,3-cd]pyrene	6	S	2B	+

Abbreviations: I: Inadequate evidence; L: limited evidence; S: sufficient evidence; 2A: probably carcinogenic to humans; 2B: possibly carcinogenic to humans; 3: not classifiable; -: negative; +: positive; +/-: questionable; (): limited number of studies.

Table IV: IARC evaluations of certain complex mixtures and occupational exposures involving exposure to PAH compounds [17-21].

Mixture/exposure	IARC classification			
	Group 1: carcinogenic to humans	**Group 2A: probably carcinogenic to humans**	**Group 2B: possibly carcinogenic to humans**	**Group 3: not classifiable**
Bitumen				x
Bitumen extracts			x	
Carbon black				x
Carbon black extracts			x	
Coal dust				x
Coal tar pitches	x			
Coal tars	x			
Creosotes		x		
Crude oil				x
Diesel fuels				
Light				x
Marine			x	
Fuel oils				
Heavy			x	
Light				x
Gasoline			x	
Jet fuel				x
Mineral oils				
Untreated	x			
Mildly treated	x			
Highly treated				x
Petroleum solvents				x
Shale oils	x			
Soots	x			
Diesel exhausts		x		
Gasoline exhausts			x	
Tobacco smoke	x			
Aluminium production	x			
Coal gasification	x			
Coke production	x			
Petroleum refining		x		

4.1. PAHs in ambient and indoor air

The European Directive No. 2004/107/CE, concerning environmental exposure to PAHs, states that B[a]PYR should be used as a marker of carcinogenic risk to humans and imposes a target value of 1 ng/m^3 for B[a]PYR (annual average for PM_{10} fraction of the atmosphere) [23]. In general, the data on PAH concentrations in ambient air are sparse and not always directly comparable. Because sampling is costly and technically challenging, no community-wide and consistent set of data is available for the EU at present. In the 1990s, typical annual mean levels for B[a]PYR in rural background areas varied between 0.1 and 1 ng/m^3, and for urban areas was between 0.5 and 3 ng/m^3 (traffic sites at the upper boundary of this range) or sometimes up to 30 ng/m^3 within the immediate vicinity of certain industrial installations (i.e., a cokery). Few measurement data exist for rural communities burning coal and wood domestically; however, these measurements suggest levels similar to those found in cities [22].

The main sources that contribute to the emission of PAHs to ambient and indoor air originate from either anthropogenic (industrial, transport and domestic heating/cooking emissions) or natural (forest fires and volcanoes) sources. The contribution of anthropogenic sources is generally higher than that of natural sources [3, 4]. The risk associated with human exposure to PAHs is thus highest in urban areas, compared with suburban or rural areas, considering the density of population, greater vehicular traffic and industrial activity [22]. For example, in the most urbanized and industrialized (coal-mining) Upper Silesia region of Poland, the mean annual PAH concentrations were reported to be in the range 150-900 ng/m^3, and that in the industrialized town of Kosice in Slovakia was found to be 689 ng/m^3. Unpolluted (rural) regions of these two countries were characterized by much lower levels of PAHs in air: 20 ng/m^3 in Biala Podska in Poland, and 36 ng/m^3 in Starina in Slovakia. Highly urbanized cities in the U.S. show levels of carcinogenic PAHs of 15-50 ng/m^3, whereas in rural areas the median carcinogenic PAH content in air ranged from 2.6-4.4 ng/m^3 [24]. In Beijing, China (2003), the total concentrations of 17 PAHs ($\sum$17 PAHs) in urban area were 1.1-6.6 times greater than those measured in a suburban area, mainly due to heavy traffic and domestic coal combustion [25]. In Kuala Lumpur**,** Malaysia (2000), the $\sum$17 PAHs in an urban area were 20 times greater than those in a rural area, mainly due to heavy vehicular traffic [26].

The most important industrial sources of PAHs include primary aluminium production (in particular plants using Söderberg process), coke production (e.g., as part of iron and steel

production), creosote and wood preservation, waste incineration, cement manufacture, petrochemical and related industries, bitumen and asphalt industries, rubber tire manufacturing, and commercial heat/power production [3, 22]. In general, aluminium smelters and industrial processes for the pyrolysis of coal, such as coking operations and steel mills, result in higher levels of PAH than most other point industrial sources [3, 4]. For example, the average concentration of individual PAHs in samples collected 100 meters directly downwind of a coke plant in Chicago, USA, were: NAP (22400 ng/m^3), ACY (474 ng/m^3), ACE (23 ng/m^3), FLU (502 ng/m^3), PHEN (500 ng/m^3), ANTH (158 ng/m^3), FLT (88.3 ng/m^3), PYR (56.3 ng/m^3), B[a]ANTH (7.59 ng/m^3), B[b]FLT (4.77 ng/m^3), B[k]FLT (8.1 ng/m^3) B[a]PYR (5.3 ng/m^3), B[e]PYR (11.6 ng/m^3), B[g,h,i]P (0.69 ng/m^3), and I[1,2,3-cd]PYR (1.1 ng/m^3) [4].

Sources of transport emissions include motor vehicles (automobiles, lorries, motor scooters and motor cycles), trains, aircrafts, shipping, and off-road vehicles (e.g., garden/agricultural engines and military vehicles) [22]. These modes of transport are reliant on internal combustion engines that have varying degree of PAH emission characteristics dependent on engine temperature (particularly cold-start), load, age, fuel quality, speed, driving mode and presence of PAHs in the fuel [22, 27]. Urban areas with congested traffic conditions and with vehicles often only travelling short journeys promote the emission of PAHs [22]. Diesel-derived aerosols are enriched in lower molecular weight PAHs, whereas higher molecular weight PAHs are associated with gasoline engine-derived aerosols [28, 29]. Diesel fuelled vehicles have higher particulate emissions than gasoline fuelled vehicles [22]. The use of turbo-charging and intercooling for heavy-duty diesel engines reduces diesel particulate emissions, and catalytic converters are very efficient at reducing particle-bound organic emissions [22]. Many studies have reported that motor vehicles' exhaust contribute appreciable amounts of inhalable fine particulate matter to the atmosphere and that motor vehicles can be considered as a major source of atmospheric PAH emissions in some urban areas [28-37]. The use of motor scooters and motor cycles may present a significant PAH emissions sources in some countries, where they are used in large numbers [22].

Trains, aircrafts, and large ships/ferries can also contribute to the emission of ambient PAHs. The main source of PAHs emissions in rail transportation is the use of diesel and diesel/electric locomotives. Coal-fired steam locomotives no longer represent a large proportion of the rolling stock operation in Europe, but in developing countries it may contribute to PAHs emission but

no measurement data are available [22, 28]. It has been estimated that around 139 tons of ∑16 USEPA PAHs are emitted by the creosoted ties of the Swiss railway network annually, with predominance of emissions of the volatile 2-and 3-ring PAHs [38]. According to the European Commission standard, average emission factors for an aircraft gas turbine engine are 1.24 mg per landing-take off (LTO) cycle for B[a]P. As air travel increases, the proportion of total PAH emissions, which are attributable to air transport, could increase, though it is unlikely that it will become a major contributor to total PAH emissions [22]. In a recent study, the total emission factors for B[a]PYR for an helicopter turbo shaft engine has been found to be 2.19 mg/ LTO, and the authors suggested that appropriate measures should be taken to reduce PAH emissions from such engines in the future [39]. Larger ships/ferries may contribute to PAHs emission to air. However, there are a limited number of studies focusing on PAH emissions from shipping and further investigations would be needed [22]. For example, within Lloyds Marine Exhaust Emissions Programme (1995), the emissions of B[a]PYR and the genotoxic dibenzo[a,i]pyrene, in the exhaust of different ships using marine distillates and heavy bunkers, were in the range of 0.2-0.65 μg/m^3, and < 0.02 to 0.3.20 μg/m^3, respectively [22]. The concentration of ∑24 PAHs measured in the engine exhaust of on-board high-speed, diesel-fuelled passenger ferries were in the range of 27-867 μg/m^3 and 45-916 μg/m^3, during whole voyages and during manoeuvring and harbouring, respectively, while total concentrations of B[a]PYR were in the range of < 0.03 < 0.1 μg/m^3 [40].

The domestic emissions of PAH which can influence ambient air quality (indoor and outdoor) are, in the main, heating, cooking and environmental cigarette smoke (ETS) [3, 4, 22]. Domestic heating and cooking varies among countries and ranging from the use of modern (cleaner) fuels (natural gas, liquefied petroleum gas "LPG", paraffin, heating oils, electricity, kerosene) to solid fuels, such as coal, brown coal, peat and biomass fuels (wood, animal-dung-cake, crop wastes) [3, 4, 22, 27]. The results of a recent comprehensive assessment of household fuel use from 181 countries (2006), indicate that in sub-Saharan Africa, south-east Asia, and the western Pacific region, the use of solid fuels prevails over cleaner fuel options, reaching 77%, 74%, and 74%, respectively. The eastern Mediterranean countries use of solid fuels represents 36% of all fuels used, while in Latin America, the Caribbean and in Central/Eastern European countries, solid fuels represent only 16% of all fuels used [41].

The emissions rates of PAHs from domestic fuels are influenced by the fuel type, combustion conditions (e.g., temperature, moisture, and availability of oxygen) and ventilation [27]. In general, PAH emissions due to the domestic combustion of solid fuels (biomass or coal) in open fireplaces or stoves make a significant contribution to indoor total PAH emissions and, in certain geographic zones and seasons, also to outdoor total PAH emission, compared to cleaner fuels [22, 42]. Given the fact that a large of fraction of total suspended particulate emissions (50-80%) from solid fuels burning is mainly associated with a respirable fraction of ≤ 2 μm size, with a large amount of PAHs (> 75%) belonging to this fraction, and the large number of people potentially affected, the continued use of solid fuels, represents a major health concern, especially in developing countries [43]. A model calculation for Germany showed that 5000 oil-heated houses contributed to the pollution of ambient air by B[a]PYR to the same extent as one coal-heated house. It was assumed that one German household consumes annually about 5000 litre of heating oil, producing a maximum of 5 mg of B[a]PYR (about 1μg/litre combusted oil). On the basis of a consumption of a similar amount of hard coal, the same household would have an output of 25 g B[a]PYR (about 500 μg/kg combusted hard coal) annually [4]. In homes in suburbs of Maputo, Mozambique, the total concentrations of PM_{10} in indoor air were 1200±131 μg/m^3, 942± 250 μg/m^3, 540±80 μg/m^3, and 200±110 μg/m^3, and 380±94 μg/m^3 for wood, coal, charcoal, LPG and electricity, respectively [44]. In rural Tamil Nadu, India, average concentrations of PM_{10} were found to be 76 μg/m^3 and 101 μg/m^3 in kitchens using kerosene and gas, respectively, contrasted with concentrations of 1500 to 2000 μg/m^3 in kitchens where biomass fuels (wood or animal dung) were used [45]. The concentration of $\sum$7 PAHs (B[a]ANTH, CHR, B[b]FLT, B[k]FLT, B[a]PYR, I[1,2,3-cd]PYR, Db[a,h]ANTH) were found in the range 4.34-26.81 μg/m^3, 5.72-70.67 μg/m^3 and 1.97-10.87 μg/m^3, in indoor air in homes in rural area in India, using wood, cow-dung cake or LPG, respectively [46]. The emission factors of $\sum$10 PAHs (FLT, PYR, B[a]ANTH, CHR, B[b]FLT, B[k]FLT, B[a]PYR, B[g,h,i]P, I[1,2,3-cd]PYR, Db[ah]ANTH) from combustion of wood, briquette**,** and dung cake ranged from 2.0-3.2, 2.8-3.0, and 3.1-5.5 mg/kg, per mass fuel burnt, respectively [47].

In a rural area in China, where crop residues (65%) and wood (20%) are the main energy sources for cooking and heating, the concentration of $\sum$16 USEPA PAHs in the ambient air (outdoor air) during cooking periods ranged from 72.1 to 554.4 ng/m^3, with the mean concentrations of 299.3 and 372.3 ng/m^3 in $PM_{2.5}$ and PM_{10}, respectively. Higher proportions of

5- and 6-ring PAHs (46-70%) as well as 4-ring (25-47%) species were detected during the cooking periods. The concentrations of B[a]PYR were in the range 1.5-32.7 ng/m^3, with average concentrations of 17.29 and 23.9 ng/m^3 in $PM_{2.5}$ and PM_{10}, respectively [42].

Besides the active smoking of cigarettes, exposure may occur by passive smoking through inhalation of ETS, which is generated by the combustion of tobacco presents in tobacco products, including cigarettes, cigars and pipes, as well as from waterpipe smoking. ETS is comprised primarily of diluted sidestream smoke and exhaled mainstream smoke [3, 4]. For a filter cigarette, the mean concentration of $\sum$14 PAHs (NAP, ACY, ACE, FLU, PHEN, ANTH, FLT, PYR, B[a]ANTH, CHR, B[b]FLT, B[k]FLT, B[a]PYR, and B[e]PYR]) in the mainstream smoke was estimated to be in the range of 806-934 ng /cigarette [48], while the levels of individual PAHs in sidestream smoke ranged from 42-2400, with PHEN, FLT, PYR, ANTH, CHR, B[a]PYR, B[b]FLT and B[b]FLT as the major components. Levels of 10.9 and 103 ng/cigarette B[a]P were found in mainstream smoke and sidestream smoke , respectively [49]. An active smoker generally retains 90% or more of mainstream particles, and because of its proximity to the source, usually inhales more of ETS, whereas a non-smoker exposed to ETS appears to retain a smaller percentage of ETS particles [50]. In studies in healthy male smokers, aged 20-40 years, the B[a]PYR intake from the smoking of 24 cigarettes/day was calculated to be 150-750 ng/day, assuming deposition rate for particulate matter of 75%, while B[a]PYR intake in non-smokers exposed to ETS was 1-11 ng/day, assuming breathing volume of 0.5 m^3/h and a deposition rate for particulate matter of 11% , and an exposure time of 8 hours in an unventilated, 45-m^3, furnished room [51]. Indoor air concentrations of B[a]PYR in smoking and non-smoking homes were reported to be 1.0 ng/m^3 and 0.4 ng/m^3, respectively. In commercial buildings, mean B[a]PYR concentrations of 1.07 ng/m^3 and 0.39 ng/m^3 in smoking and non-smoking environments, respectively, have been found. Therefore, the additional ETS-related B[a]PYR exposure is about 0.6 ng/m^3. Assuming a daily ETS exposure duration of about 3 hours, a respiration rate of 1m^3/h, would result in a maximum intake of 1.8 ng B[a]PYR /day due to passive smoking, which accounts for about 1% of the overall daily B[a]PYR intake in non-smokers (120-2800 ng/day) [52]. These results are in agreement with the findings that, for smokers, the main contribution to total daily PYR intake came from food consumption (53%) and mainstream smoking (47%), while for non-smokers, food consumption is the main source for total daily PYR intake (99%) and the contribution of passive smoking was very small (less than 1%) [53].

Natural sources of PAHs include the accidental burning of forests, woodland, moorland, etc. due to lightning strikes, etc. Meteorological conditions such as wind, temperature, humidity and fuel type (moisture content, green vs. seasoned wood, etc.) may play an important role in the degree of PAH production [22]. The influence of wildfire emissions on surrounding areas can be considerable. In fact, rural wildfires commonly are the main source of PAH emissions to the local environment [22, 27]. For example, High PAH emissions from burned savannas (17 and 600 ton/year for the particulate and the gaseous PAHs, respectively exceeded PAH inputs from anthropogenic sources in parts of Africa [54]. Pollutants from distant rural wildfires can problematic even in large cities where episodes of regional haze caused by wildfires have repeatedly raised substantial concern. Boreal forest fire emissions have travelled from their source in Canada and cause extensive haze in Europe. Hemi-spheric scale transport has also been reported to carry smoke from forest fires in Russia to North America and Europe [55]. No data are available in Europe regarding the PAHs emissions from forest fires and volcanic eruptions in the atmosphere [22].

4.2. PAHs in food

Foodstuffs can be contaminated by PAHs present in polluted air, soil or water. Vegetables, fruits and cereals obtained from polluted environments (e.g., near a highly industrialized area, or near areas with high traffic) showed higher PAH concentrations than those from unpolluted areas (e.g., rural areas) [3, 4, 56, 57].

PAHs are also formed in food as a consequence of processing (drying and smoking) and cooking methods (grilling, broiling, frying, roasting, or baking) [56, 57]. Generally, smoked and cooked fish and meat contain the highest PAH levels [56]. The maximum residue limit of 5 µg/kg of B[a]PYR is fixed by the European Commission for smoked seafood products, when smoke comes from wood pyrolysis [58]. The European Directive 88/388/EEC has limited the maximum residual levels of B[a]PYR to 0.03 µg/kg for foodstuffs as the result of the use of smoke flavourings [59]. In France, B[a]PYR concentrations in smoked fish, poultry and pork products were found in the range of < 0.2-1.9, 0.3-1.9, and < 0.2-7.2 µg/kg, respectively; 36% of the samples analyzed had B[a]PYR concentration > 1 µg/kg [3]. In a recent study from France, smoked salmon obtained by liquid smoke flavouring contained 0.1 µg/kg (which is 3 times higher than the legal limit for smoke flavouring set by the European Commission) [60]. In Italy, the concentration of total PAHs in smoked seafood ranged from 46.5 µg/kg in swordfish fish to 124 µg/kg in herring, while that of B[a]PYR, undetected in several fish, reached 0.7 µg/kg in Scottish salmon. B[a]ANTH was found in all samples and at particularly high levels in salmon (32.2 µg/kg) [61]. In a detailed analysis of smoked food in USA, total PAH concentrations in smoked meat ranged from 2.6-29.8 µg/kg, while in smoked fish, the range was 9.3-86.6 µg/kg. Concentrations of 5 carcinogenic PAHs (B[a]ANTH, B[a]PYR, B[b]FLT, Db[a,h]ANTH, I[1,2,3-cd]PYR) had maxima of 16.0 µg/kg in salmon. In liquid smoke seasonings, the total PAH concentrations and concentrations of the 5 carcinogenic PAHs ranged up to 43.7 µg/kg and 10.2 µg/kg, respectively [62]. In Brazil, B[a]PYR was detected in smoked meat samples at concentrations 0.1-5.9 µg/kg [3].

Cooking foods at high temperatures (grilling, broiling, frying, roasting, or baking) has been shown to result in the production of PAHs in the food. The amount of PAHs produced during cooking rises with increased fat content, longer exposure to flames, proximity to the heat source [57]. Normal roasting or frying food does not produce copious quantities of PAHs; some of the highest levels of PAHs reported in foods have been detected in food cooked over an open flame [56]. For example, in barbecued meat, total PAHs were found to present at levels up to 164 µg/kg, with B[a]PYR being present at levels of 30 µg/kg [56]. Also, it has shown that charcoal

grilling of duck samples with skin contained higher amount of total PAHs compared to charcoal grilling of duck samples without skin [63]. The highest concentrations of PAHs were detected in charcoal grilled (9.36-132 µg/kg), followed by flame-gas grilled (direct heat) (4.36-20.4 µg/kg) and oven grilled (indirect heat) (3.51-9.66 µg/kg) meat, chicken and fish. PAH concentrations of flame-gas grilled samples were found to be low when the flame-gas source was vertical [64]. No PAHs were detected in bread samples toasted using electric oven and toaster, while charcoal and flame grilling toasting presented very high levels of PAHs [65]. Concentrations of B[a]PYR in the range of 2.4-3.12 µg/kg were found in commonly consumed Nigerian smoked/grilled fish and meat using trational systems which use a wood fire, while when smoked/grilled in the laboratory using a charcoal fire, the concentrations of B[a]PYR were in the range 0.7-2.8 µg/kg [66].

The contribution from individual food groups to the total dietary intake of PAHs has been studied by analysis of PAHs in the diet. Findings from different countries (England, Italy, Netherlands, Spain, Sweden, USA) show that, the main contributors of PAHs to the total dietary intake appear to be 'cereal group' and 'oils and fats group'. The 'oils and fats group' has high individual PAH levels, whereas the cereal group, although never containing high individual PAH concentrations, is main contributor by weight to total intake in the diet. Cooked and smoked fish and meat products, although containing the highest PAH levels, appear to be low to modest contributors, as they are minor components of the usual diet [56, 57, 67, 68]. However, it should be noted that various countries and cultures have very different diets and methods of cooking, which may result in exposure to very different amounts of PAHs [56, 57].

4.3. PAHs in water

The presence of PAHs in drinking-water may be due to the surface or groundwater used as raw water sources or the coating of the drinking-water distribution pipes [4, 69].

Apart from highly polluted rivers, the concentrations of individual PAHs in surface and coastal waters are generally 50 ng/L. Concentrations above this levels (sometimes into the 10 µg/L range) indicate contamination by PAHs mainly through atmospheric deposition, industrial effluents, urban runoff, municipal effluents, shipyards, urban runoff and oil spillage or leakage [3, 4, 69]. PAH levels in uncontaminated groundwater are usually in the range of 0-5 ng/L; however, high levels of PAHs were found in contaminated groundwater, particularly in groundwater near industrial plants (e.g., wood preserving plant) [70].

European Community directive 80/778/EEC states a maximum level for PAHs in drinking water of 0.2 µg/L with FLT, B[b]FLT, B[k]FLT, B[a]PYR, B[g,h,i]P and I[1,2,3-cd]PYR as reference compounds [71]. The WHO recommends concentration of B[a]PYR lower than 0.7 µg/L in drinking water for additional carcinogenic risk of 10^{-5} over life [72]. Generally, PAH levels in drinking-water are usually below 1ng/L [57], and drinking-water samples analyzed from different European countries shows that the PAHs are present below the standard for PAHs of 0.2 µg/L [69]. Although not recommended by WHO, coal tar-coated pipes in public water supply systems are still used in some developing countries, which can result in high levels of PAHs in drinking-water, either after the passage of drinking-water through those pipes or after repair work. For example, 2.7 µg/L PAHs was detected in one sample of such water [69].

4.4. PAHs in Soil

Soil is perhaps the most important long-term repository for PAHs in the environment. Accumulation of PAHs in soils may lead to further potential contamination of vegetables and food chains, and then cause direct or indirect exposure to humans. Moreover, leaching, evaporation and migration are possible PAHs sources of atmospheric or ground water contamination [73]. In addition, ingestion of contaminated soil is considered to be an exposure pathway in humans, especially in young children (ages 1.5-3.5 years), who are capable of unintentionally swallowing minute amounts of foreign substances and it is estimated that up to 1200 mg soil/day can be ingested in this manner. Therefore, children playing in and on contaminated ground may represent a subpopulation at increased exposure risk [74]. For example, the concentrations of $\sum$12 PAHs in the surface soil sampled of a playground in a kindergarten situated in an area with a high traffic density was found to be 132 ng/g, which is twice that of a playground in a kindergarten situated in a residential zone with only local traffic (65 ng/g) [74].

Sources of PAHs in soil include sludge disposal from public sewage treatment plants, vehicular exhausts and emissions from wearing of tires and asphalt along highways and roads, industrial plants, power plants and blast furnaces, hydrocarbon spillage, forest and prairie fires, plant debris, and use of organic waste as soil compost and fertilizers. However, most of the PAHs in soil are believed to result from atmospheric deposition after local and long-range transport. The presence of PAHs in the soil of regions remote from industrial activity supports this theory [3, 4, 73].

A classification of soil contamination by PAHs, as "non-contaminated" **(**< 200 µg/kg dw), "weakly contaminated" (200-600 µg/kg dw), "contaminated" (600-1000 PAH µg/kg dw), and "heavily contaminated" (> 1000 µg/kg dw), has been proposed. The threshold values of this classification (200, 600, and 1000 µg/kg) were expressed as the absolute sum of the content of 16 USEPA PAH compounds in the soil samples, regardless of PAH composition and soil characteristics, and were derived from the results of determinations of PAH concentrations in European soils, as well as from estimation of risks of human exposure and the average intake rates [75].

PAH concentrations are strongly linked to the land use of the site. For example, analysis of soil samples collected at seven sites along the seine River basin, France, from 4 different types of sites (i.e., industrial, urban, suburban, and remote rural sites) showed that, the industrial sites Notre-Dame de Gravenchon and Oissel had the highest total PAH concentrations (5650 and 3390 µg/kg, respectively), followed by the urban site Rouen (2780 µg/kg), suburban sites Harfleur and Bois-Gullaume (1670 and 2690 µg/kg, respectively), and remote rural sites Honfleur and forest of Brotonne (450 and 940 µg/kg, respectively) [76]. Similar findings were reported in studies of soil samples in France, Switzerland, China, India and Malaysia [26, 77-80].

5. Assessment of human risks in relation to sources of exposure to PAHs

Table V represents the relative contribution of different sources of PAHs to the total daily intake of PAHs in humans. The contribution by food ingestion to the total intake of PAH was compared with the intake from drinking water and soil, as well as inhalation of air. From the average American diet**,** the median daily dietary intake was estimated to be 3 µg/day (total PAHs) for non-smokers. Exposure via inhalation of ambient air was estimated to be 0.16 µg/day, assuming inhalation rate of 20 m^3/day. Smoking one pack of nonfiltered cigarettes per day increases this estimate by an additional 2-5 µg/day; smokers consuming 3 packs per day increase their exposure by an estimated 6-15 µg/day. The estimated median for drinking water exposure was estimated to be 0.006 µg/day, assuming consumption rate of 2 L per day. The estimated median soil intake was 0.06 µg/day, assuming accidental ingestion of 50 mg soil per day, which may be more typical for children than most adults [3]. In the Netherlands, for smokers it is estimated that on average 53% of total PYR came from food consumption and 47% from mainstream smoking. For non-smokers, PYR intake through food consumption accounted for 99% of total daily PYR intake. The contribution of passive smoking and inhalation of indoor and outdoor air to total PYR intake was very small (less than 1%) [53]. In

Italy, the calculated total dietary PAH intake was 3 µg/day per person (89%), which was significantly higher than the calculated respiratory intake of PAHs from polluted urban air (370 ng/day) (11%) [81]. In Canada, food intake contributed 93-97% to the total daily PYR absorbed dose for children living in big cities that are not heavily polluted by PAHs from industrial sources, which was much more important than that from inhalation (3-7%) and intake from soil (0.032 %) [74]. In Czech Republic, food intake contributed 85-99% of the total PYR absorbed dose, inhalation contributed 0.3-15% and intake from soil contributed 0.010-0.022%, for children living in polluted and unpolluted areas [82]. All these results confirm that food is the major source of exposure of the general population to PAHs in areas which are not highly polluted [3, 4, 56, 57, 73].

Table V: Contribution of different sources of exposure to daily intake of PAHs

Source of exposure	Daily intake						
	USA [3]		**The Netherlands** [53]		**Italy** [81]	**Canada** [74]	**Czech Republic** [82]
	Smokers	Non-smokers	Smokers	Non-smokers			
Food	3 µg/day (44.6%)	3 µg/day (93%)	53%	99%	3 µg/day (89%)	0.167 - 0.186 µg/day (93-97%)	0.169 - 1.36 µg/day (85-99%)
Air	0.16 µg/day (2.4%)	0.16µg/day (4.9%)	< 1%	< 1%	0.37 µg/ day (11%)	0.0089 - 0.0054 µg/day (3-7%)	0.0042 - 0.0294 µg/day (0.3-15%)
Water	0.006µg/day (<0.01%)	0.006 µg/day (0.2%)	-	-	-	-	-
Soil (accidental ingestion)	0.06 µg/day (1%)	0.06 µg/day (1.9%)	-	-	-	0.000061 - 0.000104 µg/day (0.032–0.059%)	0.000026 - 0.000311 µg/day (0.011–0.022%)
Cigarette smoke	3.5 µg/day (average 2–5 µg/day) (52%)	-	47%	<1% (passive smoking)	-	-	-

6. Occurrence and fate in the environment

PAHs have half-lives in the environment that are generally shorter than other POPs (PCDD/Fs and PCBs for example), and a range of organisms, can metabolise PAHs such that they tend not to undergo biomagnifications in food webs. The 2- and 3-ring PAHs have half-lives of days in air, of weeks in water and of about a year in soil and sediments. Some 3 and 4- ring PAHs have half-lives about double these values, while the greater affinity of the larger compounds for organic matter in the environment limits their exposure to degradative processes, resulting in half-lives for PAHs with 5 or more rings of weeks in air, months in water, and years in soils and sediments [83]. It has been calculated that, depending on the compound, 92-97% (50 000 tonnes) of the UK environmental burden of PAHs resides in soil, with fresh water sediments accounting for further 3-8% (2800 tonnes), air containing 0.02-0.3% (38 tonnes), and vegetation constituting 0.01-0.4% (52 tonnes). Thus, soils and sediments are the ultimate environmental 'sink' for PAHs, while their fate in environmental tends to be compound specific [84].

Once PAHs have released into the atmosphere, they are removed by chemical reactions in the atmosphere (i.e., photolysis and oxidation reactions) as well as by atmospheric deposition (i.e., dry and wet deposition) [3, 4, 27, 84].

Reactions of PAHs in air are influenced by the atmospheric phase in which the PAHs exist, as well as temperature, humidity and sunlight , the surface area and nature of the available aerosol, and the subcooled vapour pressure of the compound [3, 4, 27, 83]. PAHs are subject to reactions with oxides of nitrogen (NO_x) and sulphur (SO_x), photochemical reactions with oxygen (O_x), and reactions with photolysis products such as ozone (O_3), peroxyacyl nitrates (PANs-R-$COO_2^{\bullet}NO_2$), hydroxyl ($OH^{\bullet}$) and peroxyl ($HO_2^{\bullet}$) radicals [27, 83]. The most important mechanism for removal of gaseous PAHs is reaction with $OH^{\bullet}$ radicals, with rate constants for reactions with $NO_x^{\bullet}$ and O_3 an order of magnitude lower than that for $OH^{\bullet}$. Direct photolysis of gaseous PAHs is likely to occur but is poorly characterized [83]. Particle-bound PAHs are subject to photolysis, which is the most important mechanism for their decay in the atmosphere, under sunlight irradiation [85]. The photolysis reaction rate constants of particle-bound PAHs are strongly influenced by the nature of the substrate to which they sorbed [83, 85]. Carbonaceous particles appear to protect PAHs from photolysis reactions, while the presence of certain organic species such as methoxphenols present in particles from wood combustion have been shown to enhance rates of photolysis [3, 83]. The sorption of PAHs to

particles also protects them from oxidising reactions mentioned above for gaseous PAHs [83]. Also, the photolysis reactions decrease with increasing molecular weight of PAHs [85].

Generally, winter air concentrations of PAHs are higher than in summer because of increases in the number and magnitude of combustion sources, and a reduction in the reaction rates of photolysis and oxidation [3, 4, 84]. Atmospheric deposition of PAHs is a significant source of loading PAHs onto surface waters, soils, and of special interest, regarding human exposure, is the deposition on food crops [3,4, 57, 86]. The smaller PAHs are subject to deposition dissolved in precipitation, as wells as dry gaseous deposition. With increasing molecular weight, wet and dry deposition of particles becomes more important as a deposition pathway [83].

In water, PAHs evaporate, disperse in the water column, become incorporated into bottom sediments, concentrate in aquatic biota, or experience degradation [3, 4, 87]. The most important degradative processes for PAHs in aquatic systems are photooxidation, chemical oxidation, and biodegradations by aquatic organisms [3]. Most PAHs in aquatic environments are associated with particulate materials; only about 33% are present in dissolved form [87]. Dissolved PAHs will probably degrade rapidly through photooxidation, and degrade most rapidly at higher concentrations, at elevated temperatures, at elevated oxygen levels, and at higher incidences of solar radiation [3, 87]. The ultimate fate of those PAHs that accumulate in sediments is believed to be biodegradation by benthic organisms [87].

PAHs in soil may be assimilated by plants, degraded, or accumulated to relatively high levels in the soil [3, 4, 87]. Microbial degradation is the major process for degradation of PAHs in soil environment, while photolysis, oxidation and hydrolysis generally are not important processes in the degradation of PAHs in soil [3]. While the small, more water-soluble PAHs will be relatively more available for biotic or abiotic degradation**,** with increasing molecular weight of the PAHs (≥ 3 rings), there is increased attraction for soil organic matter, as well as increased resistance to biodegradation**.** This results in different half-lives in soil for compounds of different sizes and physico-chemical properties. For example, NAP has been calculated to have a half-life in soil of *ca.* 2 years, while B[g,h,i]P had a half-life of *ca.* 10 years [83].

Atmospheric deposition on leaves is the main route of PAHs accumulation in plants [3, 83, 87]. The larger, more lipophilic PAHs tend to accumulate in vegetation from air and are subsequently retained by plants more effectively than the smaller compounds, which are more susceptible to (re)volatilisation [83].

7. Toxicokinetics of PAHs

7.1. Absorption

PAHs are more or less lipophilic compounds that may be readily absorbed from the lungs following inhalation, or from within the gastrointestinal tract (GIT) following ingestion and the skin following dermal exposure [3, 4, 88].

7.1.1. Absorption by inhalation

For PAHs to be inhaled as gases, they must be sufficiently volatile to be in the gas phase, or the source must emit PAHs in gas form. The smaller gaseous PAHs are deposited in the lung mostly as soluble vapours and therefore are rapidly absorbed [88]. For particle-bound PAHs to be inhaled, they must be associated with very small particles, i.e., < 12 µm to pass the larynx, or < 5 µm to pass the alveoli. PAHs in the atmosphere have shown to be usually associated with particles of the inhalable range [3, 57, 88]. The rate and extent of the absorption of particle-bound PAHs following inhalation is highly dependent on the type and particle size of particles onto which they are adsorbed [3, 4, 57, 88]. For example, in rodents, B[a]PYR adsorbed to 0.5-1µm carbon particles was eliminated from the lung approximately 4 times faster than B[a]PYR adsorbed to 15-30 µm carbon particles [3].

Animal inhalation studies on inhalation exposure to PAHs showed rapid absorption of B[a]PYR through the lungs in rats, guinea pigs and hamsters**,** and after inhalation exposure of rats to diesel exhaust [3, 4]. The epithelia lining of the nasal cavities of hamsters, dogs, and monkeys has shown the ability to metabolize about 50% of B[a]PYR instilled as an aqueous suspension [3, 4]. In humans, it was reported that B[a]PYR measured in the lungs following inhalation of soot particles was much lower than expected. This may be due to the ability of the pulmonary epithelial cells to metabolise B[a]PYR thereby facilitating its absorption and clearance from the lungs. Also, it may also due to the fact, owing to its lipophilicity, this larger PAH prefers to remain on the particle and is therefore relatively unavailable for uptake and subsequent metabolism, though is dependent on the nature of the particle (i.e., its carbon/lipid content) [3, 4].

Pulmonary absorption often occurs in parallel with mucociliary clearance, by which PAHs that are adsorbed onto inhaled particles are cleared out of the pulmonary tree and subsequently swallowed, which thereby changes the exposure route from inhalation to exposure [3, 4, 88].

7.1.2. Oral absorption

Animal studies have shown evidence for oral absorption of different PAHs. In general, low absorption occurred through the GIT following administration of low doses of PAHs (e.g., 30% for B[a]PYR [3]), whereas slightly higher absorption occurred following administration of high doses of PAHs given by gavage or in the diet [3, 57]. Absorption from the GIT occurs rapidly. Rapid absorption of B[a]PYR with highest levels seen in the thoracic lymph nodes after 3-4 hours, was demonstrated following intragastric administration in rats. Oral administration of FLT, PYR, and B[a]ANTH to rats caused peak concentrations of these compounds in the blood after 1-2 hours [3, 57, 88].

Few data are available regarding the absorption of PAHs in humans following ingestion, but in general it is thought to be low [3, 4]. However, one study reported that most of a low oral dose of B[a]PYR **(** 9 µg contained in broiled meat) was systemically absorbed as no B[a]PYR was detected in faeces, although the number of volunteers in the study was low (8 volunteers) [3].

The absorption of the individual PAHs of the GIT is highly dependent on their aqueous solubility, their lipophilicity, the presence of bile, and the lipid content of the various PAH-containing foods ingested [3, 4, 88].

7.1.3. Dermal Absorption

PAHs in gas or liquid forms are likely to be taken up by the skin relatively quickly, though the composition of the vehicle will be also important. Particle-bound PAHs are likely to diffuse extremely slowly from the particles to the skin before passing through it to the blood [3, 4, 88]. Percutaneous absorption of PAHs appears to be quite rapid in both animals and humans [3, 4, 88]. Extensive skin absorption of PAHs (PHEN, ANTH, PYR, and B[a]PYR) has been demonstrated in mice as almost all of the applied dose appeared in the faeces following application to the skin. Similarly, rapid absorption was demonstrated in rats, monkeys and guinea pigs [3, 4]. Also, rapid dermal absorption of PAHs in humans was reported. For example, workers exposed to the wood preservative creosote oil in wood impregnating plant, workers exposed to coal tar fume in coke oven plant, dermatology nurses who apply ointments containing coal tar to patients, and patients undergoing skin treatment with coal tar in petroleum jelly [89-92].

7.2. Distribution

In vivo animal studies indicate that PAHs appear to be widely distributed following both and inhalation and ingestion, having been detected post exposure in several organs [3, 4]. For example, following oral exposure in rats, B[a]PYR was measured in the kidney, caecum, small intestine, trachea, stomach and testes, whereas following inhalation, levels were measured in the liver, oesophagus, stomach and small intestine, and later in the large intestine and caecum [3]. The evidence of the distribution of PAHs to organs or tissues following dermal exposure is limited [3, 4]. Organs rich in adipose tissue can serve as storage depots to which PAHs may be gradually absorbed and from which they are then released [3]. PAHs can readily cross the placenta following oral, inhalation or dermal administration. Studies with B[a]PYR reported that when pregnant rats were exposed to B[a]PYR via inhalation or given oral B[a]PYR on day 21 of pregnancy, an increase in B[a]PYR and its metabolites were measured in both maternal and fetal blood tissues [3, 4].

No data are available regarding the distribution of PAHs in humans [3, 4]. Limited data are available of tissue levels of PAHs in autopsy or biopsy samples from humans. B[a]PYR was found in liver, lung, spleen, kidney, heart and skeletal muscle [93]. Autopsies performed on cancer-free patients reported that several PAHs (i.e., ANTH, PYR, B[k]FLT, B[a]PYR, B[e]PYR and B[g,h,i]P) were detected in fat samples; in post mortem liver samples, these PAHs were also detected, except B[e]PYR [94].

7.3. Metabolism

The metabolism of PAHs follows the general scheme of xenobiotic metabolism, in that PAHs are first oxidized by microsomal cytochrome P450 (CYP) - dependent mono-oxygenase to form phase-I metabolites, primary metabolites such as epoxides, phenols, and dihydrodiols, and then secondary metabolites, such as diol epoxides, tetrahydrotetrols, and phenol epoxides. The phase-I metabolites are then conjugated with glutathione, sulfate, or glucuronic acid to form phase-II metabolites, which are much more polar and water-soluble than the parent PAHs [3, 4].

The metabolism of PAHs has been studied extensively in tissues and cells following ingestion of food containing PAHs, or inhalation or ingestion of environmental PAHs. Consequently studies have been carries out in the bronchus, colon, keratinocytes, monocytes, macrophages and lymphocytes [3].

The metabolic process involves several possible pathways with varying degrees of enzyme activity. The activities and affinities of the enzymes in a given tissue determine which metabolic route will prevail. The enzyme system primarily responsible for PAH metabolism, mainly in the liver, is the CYP - dependent mono-oxygenase, which converts the non-polar PAHs into polar hydroxyl and expoxy derivatives [3, 4, 88]. Studies with a few prototype compounds such as B[a]PYR and its metabolites and 7,12-dimethylbenz[a]anthracene indicate that several CYP are involved in PAH metabolism and one or more member of each CYP family is capable of metabolizing one or more PAHs [4]. CYP1A1 can metabolize a wide range of PAHs, but other CYPs, including CYP1A2 and members of the CYPIB, CYP2B, CYP2C and CYP3A families of enzyme, have been demonstrated to catalyze the initial oxidation of B[a]PYR and other PAHs to varying extents [4, 88]. All of these CYP enzyme families are inducible, and their level of expression can be enhanced by foreign as well as endogenous stimuli either through the aryl hydrocarbon (Ah) receptor or other receptors [4]. The mammalian CYP genes that encode CYP1A1, CYP1A2, and CYP1B1 are regulated in part by the Ah receptor, which can be activated by numerous PAHs [4, 88].

The metabolism of alternant PAHs (e.g., B[a]ANTH, B[a]PYR, Db[a,h]ANTH) differs from nonalternant PAHs (such as B[b]FLT, B[k]FLT, and I[1,2,3-cd]PYR). B[a]PYR metabolism has been extensively studied and will be used as a model for alternant PAHs metabolism, while the metabolism of B[b]FLT will be used as a model for nonalternant PAH metabolism [3, 4, 88].

The metabolism of an alternant PAH B[a]PYR is summarized in **Figure 2**. B[a]PYR is metabolized initially by the microsomal CYP-dependent mono-oxygenase system to several arene oxides (epoxides) [3, 4]. Once formed, these epoxides may spontaneously rearrange to phenols, be hydrated to the corresponding dihydrodiols in a reaction catalyzed by microsomal epoxide hydrolase, or be conjugated with glutathione (GSH), either spontaneously or in a reaction catalyzed by cytosolic glutathione-S-transferases (GST) [3, 4]. Phenols may also be formed by the CYP system by direct oxygen insertion, although unequivocal proof for this mechanism is lacking [3]. 6-Hydroxy-B[a]PYR is further oxidized either spontaneously or metabolically to the 1,6-, 3,6-, or 6,12-quinones, and this phenol is also a presumed intermediate in the oxidation of B[a]PYR to the three quinones catalyzed by prostaglandin endoperoxide synthetase [3]. Two additional phenols may undergo further oxidative metabolism: 3-Hydroxy-B[a]PYR is metabolized to the 3,6-quinone and 9-hydroxy-B[a]PYR is further oxidized to the K-region 4,5-oxide, which is hydrated to the corresponding 9-hydroxy-

4,5-dihydrodiol (4,5,9-triol) [3, 4]. These phenols, quinones, and dihydrodiols can all be conjugated to glucuronides and sulfate esters, and the quinines may also form glutathione conjugates [3, 4].

In addition to being conjugated, the dihydrodiols can undergo further oxidative metabolism. The CYP system metabolizes B[a]PYR-4,5-dihydrodiol to a number of uncharacterized metabolites, while the 9,10-dihydrodiol is metabolized predominantly to its l- and/or 3-phenol derivative with only minor quantities of a 9,10-diol-7,8-epoxide being formed. In contrast to the 9,10-dihydrodiol metabolism, the principal route of oxidative metabolism of B [a]PYR- 7,8-dihydrodiol is to a 7,8-diol-9,10-epoxide, and phenol-diol (triol) formation is a relatively minor pathway. The diol epoxides can themselves be further metabolized to triol expoxides and pentols and become conjugated with glutathione either spontaneously or by a GST-catalyzed reaction. They may also hydrolyze spontaneously to tetrols, although epoxide hydrolase does not appear to catalyze this hydration. Further oxidative metabolism of B[a]PYR-7,8-diol can also be catalyzed by prostaglandin H synthase, by myeloperoxidase system, or by lipoxygenases. These reactions may be of particular importance in situations in which there are relatively low levels of CYP (i.e., in uninduced cells and tissues) or when chronic irritation and/or inflammation occur, as during cigarette smoking. The products detected have included diol epoxides and tetrols [3, 4, 88].

A proposed metabolic scheme for the metabolism of the nonaltenant PAHs, e.g., B[b]FLT, is presented in **Figure 3**. Nonalternant PAHs, in contrast to several alternant PAHs, do not appear to exert their genotoxic effect primarily through the metabolic formation of simple dihydrodiol epoxides. In the case of B[b]FLT, there is evidence to suggest that metabolism to the dihydrodiol precursor to its bay-region dihydrodiol occurs. Rather than this metabolite being converted to its dihydrodiol epoxide; however, it appears to be extensively converted to its 5-hydroxy derivative. It is the further metabolism of this phenolic dihydrodiol to 5, 9, 10-trihydroxy-11,12-epoxy-9,10,11,12-tetrahydrobenzo[b]FLT that has been linked to the genotoxic activity of B[b]FLT in mouse skin**.** In the case of B[j]FLT, two potentially genotoxic metabolites have been identified. These are the trans-4,5- and 9,10-dihydrodiols of B[j]FLT. It is the conversion of trans-4,5-dihydro-4,5-dihydroxybenzo[j]FLT to anti-4,5-dihydroxy-5,6a-epoxy-4,5,6,6a-tetrahydrobenzo[j]FLT that is principally associated with DNA adduct formation in mouse skin**.** B[k]FLT in rat microsomes was shown to result in the formation of 8,9-dihydrodiol. This dihydrodiol can form a dihydrodiol epoxide that is not within a bay region. This may represent an activation pathway of B[k]FLT that may be associated, in part,

with its genotoxic activity. In the case of nonalternant PAHs, then, reactive metabolites that deviate from classical bay region dihydrodiol epoxides have been linked to tumorigenic activity [3].

Taken together, these reactions illustrate that B[a]PYR in particular, and PAHs in general, can undergo a multitude of simultaneous or sequential metabolic transformations and they also illustrate the difficulty in determining which metabolites are responsible for the various biological effects resulting from treatment with the parent PAHs [4, 88].

An additional complexity of PAHs metabolism stems from the fact that PAH compounds are stereoselectively metabolized to optically active products. Taking B[a]PYR-7,8-diol-9,10-epoxide as an example, 4 isomers can be generated as there are two enantiomers of each diastereoisomer. In rat liver microsomes, the (+) 7,8-epoxide of B[a]PYR is formed in excess relative to the (-) isomer, such that more than 90% of the B[a]PYR-7,8-oxide formed consists of the (+) enantiomer. The (+) 7, 8-epoxide is then stereospecifically metabolized by epoxide hydrolase to the (-) 7, 8-dihydrodiol. This metabolically predominant dihydrodiol is metabolized in turn, primarily to a single diol epoxide isomer, the (+) anti-B[a]PYR- 7,8-diol-9,10-epoxide (anti-PADB). This metabolically predominant isomer is also the isomer with the highest tumour-inducing activity and that which was found predominantly to be covalently bound to DNA following exposure to B[a]PYR in various mammalian cells and organs [4].

A number of factors may affect the metabolism of PAHs. The route by which PAHs and other xenobiotics enter the body may determine their fate and organ specificity. For example, an inhaled compound may bypass the first-pass effect of the liver and reach peripheral tissues in concentrations higher than one would see after oral exposures**.** The effects of various factors that can modify the hepatic clearance of PAHs, specifically B[a]ANTH and CHR, were studied. The hepatic clearance and rate constants of these PAHs were significantly reduced in the livers of fasted rats relative to those of fed rats. In contrast, *in vivo* pre-treatment of the rats with these PAHs significantly increased clearance of both PAHs when compared to control rats. It was also noted that hepatic clearance of B[a]ANTH was significantly higher in male rats then female rats of the same age, perhaps suggesting a sexual difference with aryl hydrocarbon hydroxylase activity**.** Similar findings regarding sexual differences in the metabolism of B[a]ANTH and CHR by rat livers were also reported [3, 4].

Furthermore, it was suggested that age may play a role in PAHs metabolism. The hepatic clearance of PAHs in older rats (2 years) was significantly less than the hepatic clearance in

younger rats (8 weeks). However, activation of B[a]PYR to mutagenic derivatives by hepatic microsomes from male rats from 3 weeks to 18 months of age showed no age-dependent changes [3].

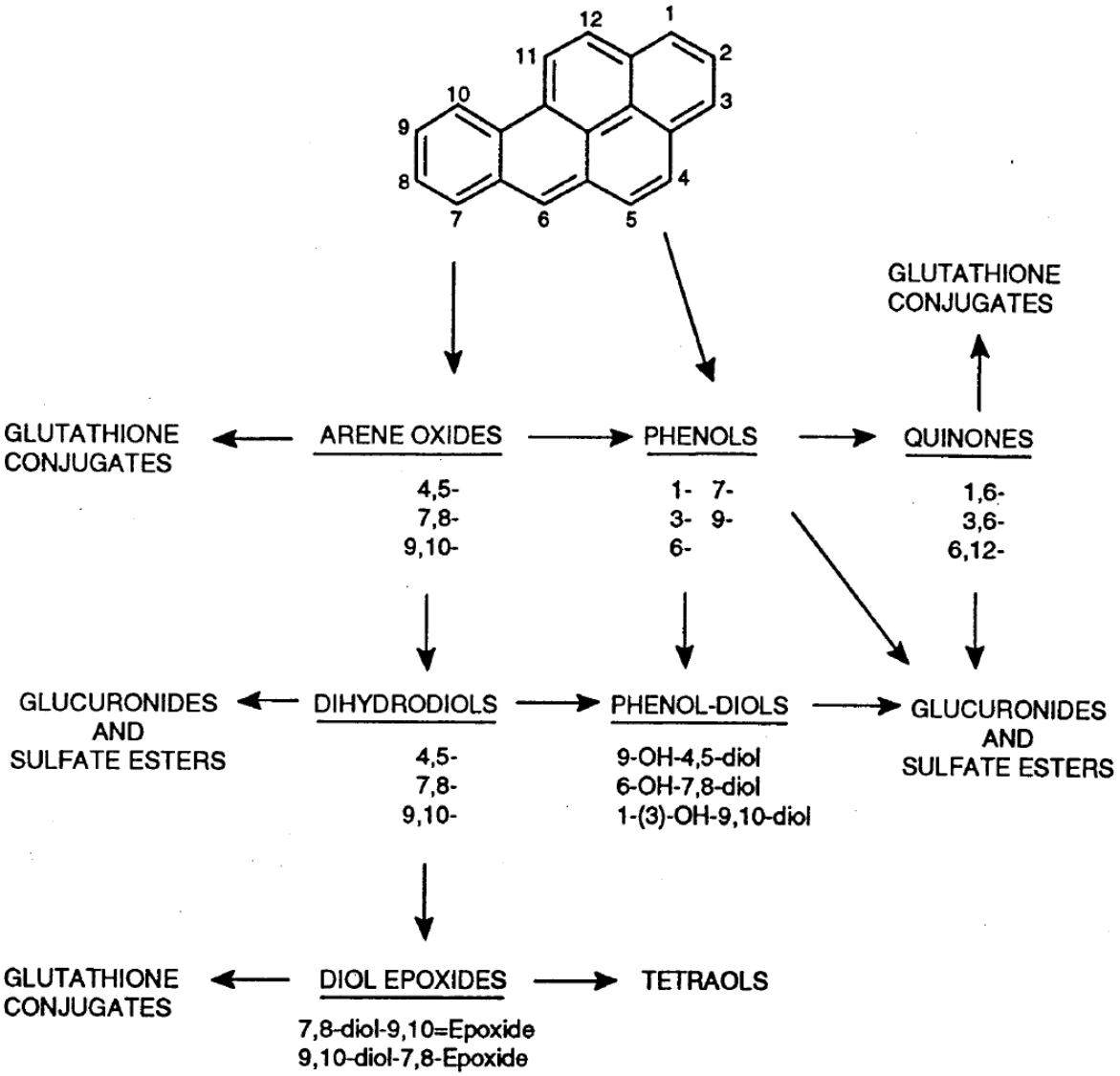

Figure 2: Metabolic scheme proposed for Benzo[a]pyrene (alternant PAH) (adapted from ATSDR [3]).

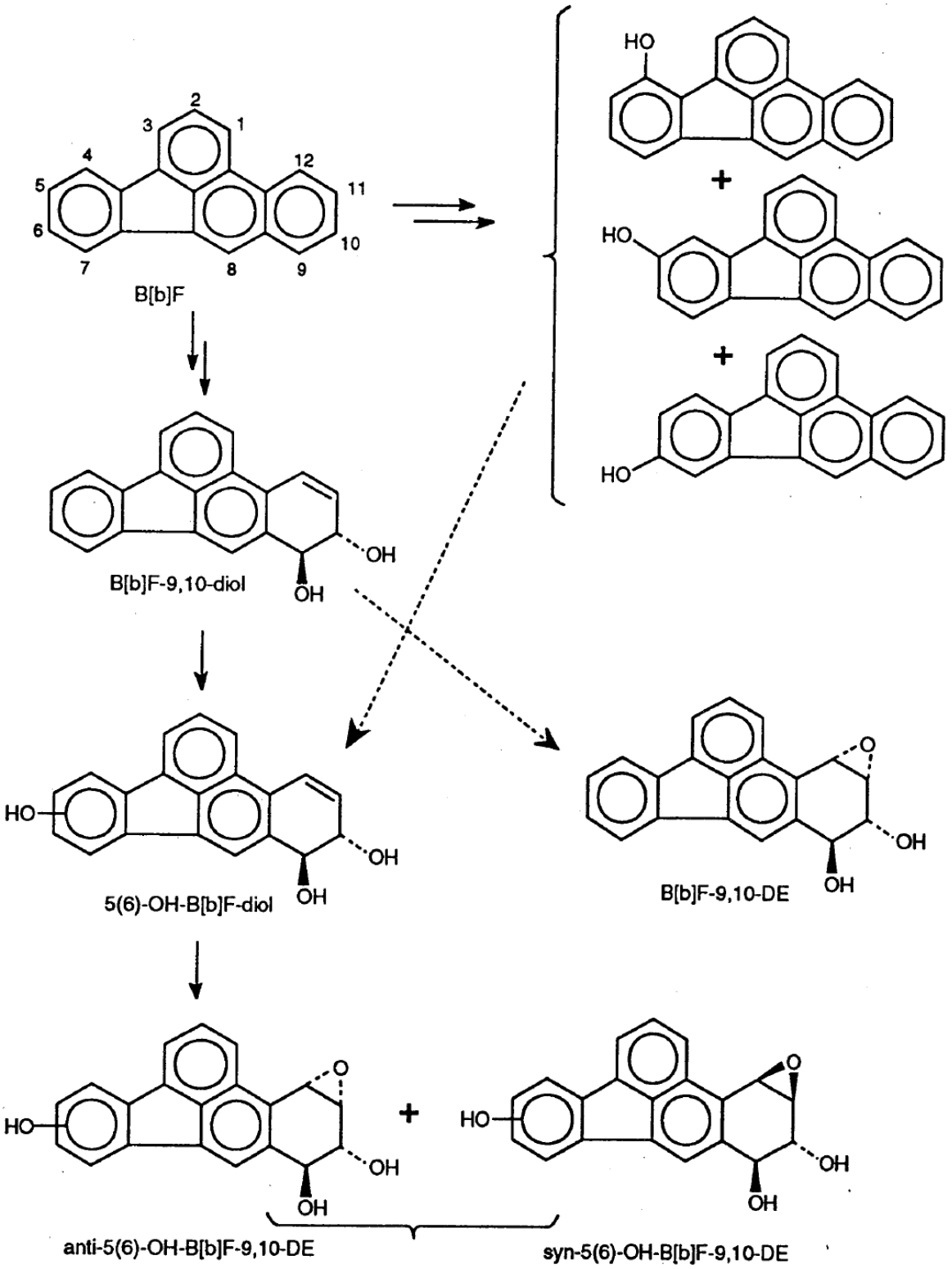

Figure 3: Metabolic scheme proposed for Benzo[b]fluoranthene (non-alternant PAH) (adapted from ATSDR [3]).

7.4. Excretion and elimination:

Quantitative data on the excretion of PAHs in humans are lacking**.** Results from animals exposed to PAHs indicate that PAHs and their metabolites are excreted as conjugates of glutathione (GSH), glucuronic acid or sulphate in the faeces and in the urine following inhalation, oral or dermal exposure [3, 4, 88]. The hydroxylated urinary metabolites of PAHs are used widely as biomarkers for routine biological monitoring of human exposure to PAHs in both occupationally-exposed and nonoccupationally exposed subjects [3, 4] (see section 9).

8. Health effects

PAHs can produce a range of health-related effects in animals and humans. These effects range from systemic effects, immunological effects, reproductive and developmental effects, to genotoxic and carcinogenic effects. The most often studied PAH in animal models is B[a]PYR because it is one of the most potent carcinogens among the PAHs [3, 4, 86, 88].

8.1. Systemic effects

Adverse haematological effects including a plastic anaemia, pancytopenia, severe reduction in peripheral blood leukocytes, and severe bone marrow depression with almost complete destruction of pluripotent haematopoietic stem cells have been observed in animals following oral exposure to high doses of B[a]PYR. FLU and FLT administered by gavage to mice caused haematological effects including decreased packed cell volume and decreased haemoglobin content [3].

In vitro and *in vivo* experiments have demonstrated adverse effects on the respiratory tissues following exposure to PAHs. Severe, long-lasting hyperplasia, cell proliferation in airway and alveolar regions of the lungs, small foci of hyperplastic alveolar cells, oedema and/granulocyte infiltration, fibrosis and hyalinization have been reported. The effects were more severe with increasing dose of PAHs and were most sever and long-lasting with B[b]FLT and B[a]PYR, compared to ANTH, PYR and B[e]PYR, [3, 57, 95].

A decrease in ventilary function, which was well correlated with prolonged and high exposure to particulate matter and B[a]PYR was reported in employees working in various areas of a rubber factory. However, no attempt was made to separate the effects of exposure to B[a]PYR and particulate matter, or to identify possible simultaneous exposure to other toxic chemicals [3].

Mild adverse hepatic effects (e.g., moderate induction of cystolic aldehyde dehydrogenase and mild increase in liver weight) have been observed in animals following acute oral, intraperitoneal, or subcutaneous administration of various PAHs (FLU, PHEN ANTH, B[a]ANTH, CHR, B[a]PYR, D[bah]ANTH), although much higher doses were required to induce these effects for the noncarcinogenic among these PAHs [3]. Long-term oral administration of PAHs in mice has been reported to result in more serious adverse effects. For example, increase in liver weight correlated with hepatocellular hypertrophy with exposure to ACY, and increased liver weight and a dose-related centirilobular pigmentation accompanied by an increase in liver enzymes were observed with exposure to FLT [3]. No adverse hepatic effects have been reported in humans following exposure to PAHs [3, 4, 57, 88].

Adverse cardiovascular effects have been reported in animals. *In vitro* studies conducted using bovine, rabbit, and human smooth muscle cells from arteries demonstrated that B[a]P caused a decreased secretion of newly synthesized collagen from bovine arterial smooth muscle cells, and an increase in cellular toxicity were noted in both animal and human cell cultures [3, 95]. Also, B[a]PYR, B[e]PYR, and Db[a,h]ANTH promoted the development of pre-existing atherosclerotic plaques in chickens, and B[a]PYR enhanced the formation of arterial lesions in pigeons [3, 57, 95].

There is evidence that occupational exposure to combustion products, including PAHs, might also be associated with an increased risk of cardiovascular disease [96].

Adverse dermatological effects have been noted in animals in conjunction with acute and intermediate-duration dermal exposure to PAHs, which included destruction of sebaceous glands, skin ulcerations, hyperplasia, hyperkeratosis, alterations in epidermal cell growth, and erythema [3, 95]. Regressive verrucae (warts) were reported in humans following up to 120 dermal applications of 1% B[a]PYR over a 4 month period. B[a]PYR application also apparently exacerbated skin lesions in patients with pre-existing skin conditions such as pemphigus vulgaris and xeroderma pigmentosum. Workers exposed to substances that contain PAHs (e.g., coal tar) experienced chronic dermatitis and hyperkeratosis [3].

Acute oral doses of FLT and B[a]PYR in rats produced a variety of neurobehavioural deficits, including decreased motor activity, neuromuscular, physiological and autonomic abnormalities and decreased responsiveness to sensory stimuli [97, 98].

8.2. Immunological effects

There are reports in the literature concerning the immunosuppressive effect of PAHs from *in vitro* and *in vivo* laboratory studies. However, most of the reports published in the literature have used either subcutaneous or intraperitoneal injection of B[a]PYR, while few studies used oral administration, as the route of exposure. Also, regardless of the route of exposure, the resulting effects have been considered mostly at the systemic level; very few studies have looked for alterations of the local gut immune system even though the carcinogenic PAHs, as a group, have immunosuppressive effects (though the data are limited), and suggestions that the degree of immunosupression correlates with carcinogenic potency [3, 4, 57, 95]. For example, in cultured cells, B[a]PYR, but not B[e]PYR, has been shown to suppress humoral and cell-mediated immunity and to inhibit interferon induction by viruses by 60-70% [3, 57]. Very little information is available on the immunological effects of other PAHs: mice treated with high doses of Db[a,h]ANTH exhibited a reduced serum antibody level in response to antigenic challenge by comparison to controls, while B-cell lymphopoiesis in mouse bone marrow has been shown to be inhibited by incubation with FLT *in vitro.* [3, 57].

Conversely, PAHs have also been shown to induce immunostimulation in animals. Exposure to low doses of PAH may augment T-cell signalling pathways, resulting in immune enhancement or an adjuvant effect [57]. ANTH, FLT, PYR, and B[a]PYR had an adjuvant activities on the production of anti IgE antibodies to allergens, and PHEN and ANTH were capable of enhancing IgE and IgG1 production [3, 57].

The immunological effects of PAHs in humans have been assessed in some occupational studies. A marked depression of mean serum IgG and IgA and a significant increase in IgE and tumour necrosis factor-α levels were observed in the coke oven workers, compared to cold-rolling mill workers with exposure levels 3-5 magnitudes lower than the coke oven workers [99, 100]. Significant, but slight, alterations such as decreased mitogenic reaction to T-cells to phytohemagglutinin, reduced expression of the interleukin-2 receptor, impairment of B-cell activity and a decreased oxidative burst in monocytes after challenge, were reported in coke oven workers, compared to healthy unexposed workers [57]. The $CD4^+$ percentage and the $CD4^+/CD8^+$ ratio, serum IgM levels and the percentage of monocytes were significantly higher in road paving workers, compared to unexposed controls [101].

8.3. Reproductive and developmental effects

Data from animal studies has shown that B[a]PYR is embryotoxic in some strains of animals but not others, largely dependent on their Ah receptor status and the inducibility of cytochrome P450 enzymes [3, 57]. Sub-acute and sub-chronic exposures to inhaled B[a]PYR (75 µg/m^3) reduced plasma testosterone concentrations, reduced testicular weight, reduced sperm number and motility, and reduced epididymal function of the exposed male rats [102, 103]. Following oral administration of B[a]PYR to pregnant mice, reduced survival of the pups, reduced body weight, total sterility in 97% of the F1 offspring, and a dramatic decrease in the size of gonads, were observed [57]. *In utero* exposure to B[a]PYR has also produced several serious effects in the progeny of mice such as interstitial cell tumours, immunosupression and tumor induction [95]. NAP, ACE, FLU, ANTH, and FLT had no adverse reproductive or developmental effects when administered orally to rats and mice [3, 57].

Recent epidemiological studies have found associations between prenatal exposure to airborne PAHs (1.8-36.47 ng/m3) and adverse reproductive or child health outcomes in humans, including low birth weight and length, preterm birth, reduced head circumference at birth, and lower scores on childhood (3-5 years) tests of neurodevelopment [104-108]. Elevated first trimester dietary B[a]PYR was associated with a significant reduction in birth weight, compared to low first trimester dietary B [a]PYR [109]. However, in one study, although 7 PAHs (B[a]ANTH, CHR, B[b]FLT, B[a]PYR, B[g,h,i]P, I[1,2,3-cd]PYR, Db[a,h]ANTH) were detected in the placentas of 200 women living in two industrialized cities, there were no associations found between any of the placental PAH concentrations and birth weight of the infant [110].

8.4. Genotoxic effects

PAHs have been thoroughly studied in genetic toxicology test systems. PAHs induce genetic damage in prokaryotes, eukaryotes, mammalian cells *in vitro* and *in vivo* produced a wide range of genotoxic effects, including gene mutations in somatic cells, chromosome damage in germinal and somatic cells, DNA adduct formation, unscheduled DNA synthesis, sister chromatid exchanges, and neoplastic cell transformation [3, 4, 57].

The genotoxic profiles of PAHs, considered by International Programme on Chemical Safety (ICPS) in 1988, have been updated and reevaluated by the Scientific Food Committe of the European Commission in 2002 on the basis of literature available at the Environmental Mutagen Center. For classification, the same criteria used under the Directive 91/325/EEC

(labelling Guide) were applied [57]. Overall evaluations on genotoxicity of the 16 USEPA (plus B[e]PYR) are listed in **Table VI.**

Genotoxicity studies have been carried out on environmental samples containing complex PAH mixtures, such as air particulate, combustion emissions and water sediment extracts. However, in only a few cases has the genotoxity of crude PAH fractions been correlated to the content of genotoxic PAH. For example, in coal gasification residues, both increasing and decreasing levels of genotoxicity with increasing B[a]PYR (or B[a]PYR-equivalents) content have been observed, highlighting the difficulties in the toxicological evaluation of complex mixtures, where the final genotoxic effect may be modulated by toxic or inhibitory components present in the mixture [57].

The genotoxicity studies of PAHs in humans in occupationally exposed workers and environmentally exposed populations have investigated mainly the occurrence PAH-DNA adducts and to a lesser extent gene mutation. In some occupational studies, increased levels of PAH-DNA adducts were observed and a significant correlation between the estimated PAH exposure and adduct levels were obtained, while in other occupational exposure studies, although PAH-DNA adduct levels were slightly increased as compared to controls, there was no correlation between exposure and adduct formation even though the exposure levels were high [86, 95, 111-114]. Also, the interindividual variation in the levels of adducts observed has been large, over 10-fold. The variation is usually larger in the exposed than in the control populations, suggesting that exposures as well as constitutional factors contribute to such a variation [95].

Clinical and occupational studies on smoking-related DNA adduct formation have shown conflicting results. Some studies have found a significant effect of smoking habits on DNA adduct levels measured from blood cells, while other studies have not detected a remarkable effect, if at all, of smoking on DNA adduct levels measured in blood cells [113, 114]. Studies have shown that PAH-DNA adduct levels increased markedly in the white blood cells of 2 out of 4 subjects [115], and in 4 out of 10 subjects ingested charbroiled meat [116].

Increased rates of hypoxanthine-quinine phosphoribosyl transferase (*HPRT)* mutations were found in lymphocytes of workers in an iron foundry and in heavily exposed, non-smoking bus maintenance workers exposed to diesel exhaust, compared with controls [117, 118].

Table VI: Overall evaluation of the 16 USEPA individual PAH and Benzo[e]pyrene (adapated from Food and Scientific Committee, EU [57]).

Compound	Conclusion of evaluation
Naphtalene	**Probably not genotoxic** (mainly negative results *in vitro*; limited negative data *in vivo*)
Acenaphtylene	**Database inadequate for evaluation** (mixed results from bacterial studies)
Acenaphthene	**Database inadequate for evaluation** (mixed results from few *in vitro* studies)
Fluorene	**Database inadequate for evaluation** (mixed results from few *in vitro* studies; no *in vivo* data available)
Phenanthrene	**Equivocal** (mixed results *in vitro*; negative or borderline positive *in vivo* cytogenetics)
Anthracene	**Not genotoxic** (negative results in the majority of *in vitro* test systems and in all *in vivo* assays)
Fluoranthene	**Equivocal** (mixed results *in vitro*; evidence of DNA binding *in vivo* after intraperitoneal administration, negative in mutagenicity/ geotoxicity tests by oral route)
Pyrene	**Not genotoxic** (mainly negative results *in vitro*; extensive negative database *in vivo*)
Benzo[a]anthracene	**Genotoxic** (positive results i*n vitro* and *in vivo* for multiple end-points; positive also at germ cell level)
Chrysene	**Genotoxic** (positive results *in vitro* and *in vivo* for multiple end-points; positive also at germ cell level)
Benzo[b]fluoranthene	**Genotoxic** (positive results in assays *in vitro* and *in vivo* for different end-points)
Benzo[k]fluoranthene	**Genotoxic** (positive results in assays *in vitro* and for DNA binding *in vivo*)
Benzo[a]pyrene	**Genotoxic** (positive results *in vitro* and *in vivo* for multiple end-points; positive also at germ cell level)
Benzo[e]pyrene	**Equivocal** (mixed results *in vitro*, inconsistent results *in vivo*)
Benzo[g,h,i]perylene	**Genotoxic** (positive results in assays *in vitro* and for DNA binding *in vivo*)
Indeno[1,2,3-cd]pyrene	**Genotoxic** (positive results in assays *in vitro* and for DNA binding *in vivo*)
Dibenzo[a,h]anthracene	**Genotoxic** (positive results in assays *in vitro* and *in vivo* for multiple end-points)

8.5. Carcinogenic effect

There are several lines of evidence from *in vitro*, *in vivo* and human studies showing a mechanistic link between the formation of PAH-DNA adducts, mutations, and cancer outcome following long-term exposure to PAHs [57, 111]. Also, the evidence of genotoxicity and carcinogenicity of several PAH compounds show considerable similarities, highlighting the pivotal role of genetic damage in the mechanism of carcinogenicity of PAHs [3, 4]. However, studies with laboratory rodents have shown that the correlation between DNA adduct formation, mutagenesis and carcinogenesis is not straightforward. For example, comparable levels of PAH-DNA adducts and gene mutations have been detected in tumour target and nontarget tissues of rodents treated with B[a]PYR [57, 111], suggesting that genotoxic end-points are not solely responsible for tissue-specific cancers, but rather that the potent biological activity of PAHs seems to rely on different characteristic properties [57, 86]. Extensive mechanistic studies have shown that many PAH compounds - including some that occur in ambient air - are complete carcinogens, i.e. they possess both genotoxic and epigenetic effects. The epigenetic effects of PAH compounds involve binding to the Ah receptor in the cytoplasm, translocation of the PAH-Ah complex into the nucleus, binding to a nuclear transcription factor and activation of genes that regulate the expression of factors that control cellular growth and differentiation [22, 57, 86]. PAH compounds may also affect the production of cancer by triggering an inflammatory response and generating intracellular oxidative stress through free-radical production [22, 86].

Most animal studies to assess the carcinogenic potential of individual PAHs were carried out following dermal, subcutaneous, or inhalation exposure. Complete carcinogenic studies in the laboratory animals have demonstrated the ability of PAHs to induce skin tumours in mice **[**3, 4]. Only a limited number of studies dealt with oral administration [3, 4, 57]. Overall, results from oral exposure to PAHs indicate a statistically significant increase in the proportion of animals with forestomach, alimentary tract and lung tumours, as well as leukaemia, compared with controls [3, 4].

B[a]PYR is the only PAH that has been tested for carcinogenicity following inhalation. A significant increase in lung tumours was reported in mice exposed 0.05 or 0.09 $\mu g/m^3$ B[a]PYR. Similarly, respiratory tract tumours were induced in a dose-dependent manner in the

nasal cavity, trachea, oesophagus and forestomach in hamsters exposed to 9.5 μg/m^3 or 46.5 μg/m^3 B[a]PYR for 109 weeks [3, 57].

In most carcinogenicity studies, the site of the tumour development was related to the route of administration, i.e. oral administration induced gastric tumours, dermal application induced skin tumours, inhalation and intratracheal instillation resulted in lung tumours, and subcutaneous injection resulted in sarcomas. However, the observations that mammary tumours are induced following intravenous injection in Sparague-Dawley rats, the susceptibility to tumour development on the skin after dermal application is not similar in mice and rats, and oral cavity tumours are not observed when B[a]P is administered in the diet, suggest that the point of first contact may not always be the site of PAH-induced tumours [3, 4].

Based on data from animal experiments (as little or no human data exist for individual PAHs), the IARC, in 1983 and 1987 has evaluated the carcinogenicity of several individual PAHs. The evidence that the compound is carcinogenic to experimental animals is classified by the IARC as either inadequate (I), limited (L), or sufficient (S) [16, 17]. In 1998, the IPCS/WHO [4], reevaluated the carcinogenicity of several individual PAHs. These classifications, together with the overall evaluations concerning carcinogenicity to humans, are show in **Tables II and III**.

The carcinogenicity of complex mixtures containing PAHs has been assessed. In a feeding study that compared the tumourigenicity of coal tar with that of B[a]PYR in mice, the major site of tumour formation was the forestomach when B[a]PYR administered alone. When B[a]PYR was part of coal tar mixtures, the formation of forestomach tumours seemed to be in accordance with the B[a]PYR content of the mixtures. However, in addition to the forestomach tumours, the coal tar mixtures also produced increased incidence of alveolar and bronchiolar adenomas and carcinomas, liver tumours, tumours of the small intestine, and haemangiosacromas. The overall carcinogenic potencies of the complex coal tar mixtures were 2-5 times higher than that of B[a]PYR [57].

The 4-7 ring PAHs fraction of condensate from car exhaust (gasoline and diesel), domestic coal stove emissions, and tobacco smoke contained almost all the carcinogenic potential of PAHs. This was in a series of studies using skin painting, subcutaneous injection and intrapulmonary implantation of different fractions. It was concluded from the skin painting tests of different

condensates that B[a]PYR represented 5-15% of the carcinogenic potency of exhaust condensates from car exhaust and coal-fired domestic stoves. When tested by lung implantation in the rat, B[a]PYR contributed a somewhat lower percentage of the total carcinogenicity potential (< 1-2.5%)**.** The most pronounced lung carcinogenic effect of sidestream smoke in lung was caused by the fraction represented only 3.5% by weight of the total sidestream condensate. The database from rat inhalation bioassay suggest that the rat lung tumour response to diesel soot, might predominantly be a nonspecific effect unrelated to the mutagenic substances (e.g., PAHs, dinitropyrenes) present in diesel particulates, and the cancer presumably result from lung overloading of diesel soot particles, and the ensuing inflammatory response [57, 95]. This is apparently supported by the observation that a dose-related lung carcinogenic effect was observed in rats exposed by inhalation to coal tar/pitch condensation aerosol (no particles) at a concentration of either 20 or 46 $\mu g/m^3$. The lifetime lung tumour risk for rats exposed to $1\mu g/m^3$ B[a)]PYR as a constituent of the complex PAH mixture was calculated to be 2% (2×10^{-2} per $\mu g/m^3$). In comparison, the WHO estimated a unit lung cancer risk for human of nearly 9% (8.7×10^{-2} per $\mu g/m^3$) B[a]PYR in ambient air based on epidemiological data on workers exposed to coke-oven emissions from coking plants. Thus, it was suggested that, in the evaluation of the lung carcinogenicity of PAH adsorbed on inhaled fine particles, the likely enhancing properties of the inflammatory effects of particles on lung tissue should be considered [57, 95].

Occupational studies have demonstrated that workers exposed to mixtures of PAHs in certain industrial settings are at a definite risk of lung, skin, bladder, stomach cancers as well as leukemia [3, 4, 119, 120]. However, some studies in certain occupational settings (e.g., exposure to coke, aluminium, coal tars, etc.) have shown exposure-response relationships, which allowed IARC to classify these industrial settings as carcinogens to humans (Group 1 carcinogens), while inconsistencies between studies, lack of control of confounding factors, potential bias, and uncertainty regarding a dose-response relationship precluded any definitive conclusions for these occupations in other studies (e.g., exposure to bitumen fumes, engine exhausts, carbon black, etc) [17-21] (**Table IV**).

Some studies were carried out in order to assess the risk of carcinogenicity of PAHs in the general population. In a U.S. clinic based-control study, increased risks of colorectal adenomas were found with high intake of B[a]PYR from well-done and grilled meat and from all food

sources. The authors concluded that, although their study is relatively small and the findings require replication in large, epidemiological studies, their findings provide evidence that dietary B[a]PYR plays a role in colorectal adenomas etiology [121]. The findings from a large-scale case-control study of gastric cancer in Spain have suggested that the exposure to wine stored in tar impregnated leather bottles may participate in the etiology of gastric cancer, especially with consumption of more than 2 litres of wine/week, although the study population was too small to reveal a statistically significant increase [57].

A high rate of lung cancer has been described for women in Xuan Wei county in China as a result of cooking with smoky coal without proper ventilation, and other investigations from China also suggest that fumes from cooking, particularly from rape seed oil, may contribute to an increased risk of lung cancer**.** A case-referent study of risk factors for lung cancer among women in Los Angeles, California, US, showed a positive a association with coal burning in the home during childhood [120].

9. Assessment of exposure to PAHs

Owing to the widespread presence of PAHs in the environment and their toxicological relevance, the assessment of exposure to PAHs is important, both in the work place and among the general population [122]. In this context, biological monitoring of exposure to PAHs, that is the identification and quantification of PAH compounds and/or of their metabolites in biological fluids, is a useful tool to better detect associations between conditions of exposure to PAHs and human health risks [123, 124]. The advantage of the use of a biomarker to assess exposure to PAHs is not only that it provides an integrated measurement of several routes of exposure to PAHs (i.e., by inhalation, dermal, or digestive routes) but also that it covers unexpected or accidental exposures and reflects interindividual differences in uptake or genetic susceptibility [125, 126]. However, the concurrent disadvantages of this approach are that it does not distinguish between different sources of exposur**e** and that it may be confounded by interindividual variation [127].

In addition to hydroxyl urinary PAH metabolites, measurement of unmetabolised (parent) PAHs, tetrols, polar fragments, PAH-DNA adducts, PAH-protein adducts, and cytogenic alterations are used as biomarkers to assess the exposure to PAHs [3, 4, 112, 124, 127].

In order to assess occupational and environmental exposures to PAHs, measuring unmetabolized (parent) PAHs in urine or blood of occupationally and non-occupationally exposed subjects has been attempted [123, 128-131]. Measurement of unmetabolized PAHs, instead of metabolites, is supported by several reasons: they are less susceptible to intra-individual variability; their elimination is directly associated to previous exposure; they are characterized by a high degree of specificity**.** On the other hand, some drawbacks, are to be taken account, such as the tiny amount of these compounds presents in the blood or excreted in urine, the possibility of contamination of the biological specimen, and the loss of the analytes which, in the case of PAHs, may be absorbed on the walls of glass and plastic vials used to collect and store specimens [123].

Some authors have suggested that, the B[a]PYR tetrols would be used as biomarkers for human biomonitoring investigations. These tetrols are the direct metabolic products of the ultimate carcinogen 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-BaPYR [132]. The metabolite r-7, t-8, 9, c-10-tetrahydroxy-7, 8, 9, 10-tetrahydrobenzo[a]pyrene (trans-anti-Bap-tetraol) was detected in urine of coke- oven workers, psoriasis patient treated with coal tar-containing ointment and smokers [133, 134], and it is found that urinary trans-anti-B[a]PYR-tetraol concentrations were significantly and positively correlated with ambient PAHs and urinary 1-hydroxypyrene concentration [134]. No information is given in these studies on the detection of B[a]PYR tetrols in the urine of non-smokers, and no further investigations into the excretion of B[a]PYR tetrols in the general population exist at the present time [132].

Sometimes, the polar fragments (i.e., thioether or D-glucaric acid) to which hydroxylated metabolites may be conjugated, were used as biomarkers to PAHs exposure [135]. However, these conjugate fragments provide evidence of exposure to any electrophilic compound that the individual has been exposed to and is not specific for electrophilic metabolites of PAHs [112].

Measurement of PAH-DNA adducts have been used as an indicator of exposure to PAHs and also of the dose of the ultimate reactive metabolite in peripheral lymphocytes and other tissues (e.g., alveolar macrophages) [3, 4, 111, 112]. Overall, the results obtained thus far on the relationship between the exposure of humans to complex mixtures containing PAHs and the extent of PAH- DNA adduct formation indicate a correlation, although weak in most cases. There are still problems with regard to the sensitivity, specificity, and the stability of different

adducts, as well as substantial interindividual variation in PAH-DNA adduct levels (greater than that prescribed for 1-hydroxypyrene) observed in populations studied, rendering such methods less suitable for routine biological monitoring. However, because DNA adducts are associated with mutation and cancer, measurement of an increase in DNA adducts relative to the corresponding control levels, is, in principle, an indication of increased risk [57, 86, 111, 112].

The assessment of PAH-blood protein adducts have also been considered as possible markers of exposure to PAHs [136]. For example, higher levels of covalent PAH serum albumin adducts were found in foundry workers than non-exposed controls [137, 138]. However, only a few studies have been carried out and the data is limited; in addition, as with PAH-DNA adduct determination, PAH-protein adducts determinations are still problematic with regard to their sensitivity and specificity [57, 86].

Cytogenetic alterations in cultured peripheral blood lymphocytes, such as chromosomal aberrations, sister chromatid exchanges, and micronuclei, have for many years been applied as biomarkers of genotoxic exposure and early effects of genotoxic carcinogens [139]. The relevance of increased frequency of cytogenetic alterations, as biomarkers of PAHs exposure and cancer risk, has been corroborated by evidence from epidemiological studies showing the high frequency of chromosome aberrations in peripheral lymphocytes as predictive of increased risk of cancer, while micronuclei and sister chromatid exchanges in peripheral blood cells are, at present, unsatisfactory biomarkers for increased cancer risk from exposure to PAH. However, additional data for PAH exposures are necessary to confirm this [112, 124, 140].

Any genetic polymorphism that affects PAHs metabolism or cellular response to DNA damage may alter individual susceptibility to genotoxic carcinogen PAHs. Thus, interindividual differences in the enzyme systems participating in the metabolism of PAHs are expected to play an important role in the tumour susceptibility of an individual [86, 124]. As an example, it is observed that non-smoking GSTM1-null bus drivers exposed to urban air pollution show a high frequency of chromosomal aberrations as compared to GSTM1-positive drivers, in the Copenhagen metropolitan area [141]. Other studies of DNA adducts have suggested that the GSTM1 and GSTT1-null DNA genotype predisposes carriers to increased genotoxic effects from PAH polluted air [124].

Studies on the effect of CYP polymorphism on chromosomal damage are limited, and their interpretation is complicated by unclear association between the CYP genotype and the phenotype and rarity of variant alleles [142].

Since PAHs requires metabolic activation to express their carcinogenicity, the determination of their metabolites excreted in the urine (phenol or hydroxyl-PAH metabolites) is useful to assess PAH activation and potentially provides a more accurate profile of an exposed individual's response to PAH [143], as they provide more appropriate estimates of total exposure as compared to exposure assessments based on environmental data [124]. In addition, the greater advantages connected with the use of predominant, primary metabolites in the biological matrices are their stability and the absence of contamination by other substances [144]. Hence, the hydroxyl-PAHs metabolites, compounds that are formed in phase I metabolism and are excreted, mainly as conjugates, in urine, can be used as reliable biomarkers of exposure to PAHs [112, 124, 127]. The choice of a urinary metabolite as a reliable biomarker for PAHs exposure is a crucial point because PAHs are always present as a complex mixture whose composition varies depending on source and temperature of emission. Moreover, each compound, once inside the human body, is metabolized to more than one metabolite constituting different positional isomers [122]. Thus, the choice of the urinary metabolite to be used as a biomarker should consider the constituents of the most common PAH profile to which people are exposed. In cases of complex mixtures, more than one biomarker should be considered to ensure adequate evaluation [124]. Smoking is often monitored by analysing for cotinine (a hydroxyl metabolite of nicotine), which permits data to be normalized for smoking behaviour in biological monitoring of PAH exposure using urinary PAH metabolites [112, 127].

In 1985, urinary 1-hydroxypyrene (1-OH-PYR) was proposed as a reliable biomarker of PAH exposures [145]. Since then, 1-OH-PYR has been widely used as a reliable indicator of exposure to PAHs in numerous occupational and environmental investigations across the world [112, 132, 127], on the basis that PYR is one of the most abundant PAH in the majority of complex PAH mixtures usually encountered, in both professional and general population exposure scenarios, and that is rapidly distributed, metabolized and eliminated from the body [146]; 1-OH-PYR, the principal metabolite of PYR, which is formed in mammals [127], represents in urine a constant fraction (2%) of total PYR intake [146] and the half-life of

urinary 1-OH-PYR excretion has been reported to range from 4 to 35 hours [147], declining to a base line within 48 hours [148].

A number of studies have shown significant correlations between urinary 1-OH-PYR excretion and levels of PYR and B[a]PYR in ambient air in workers of coke, steel and aluminium plants [127].

Similarly, significant correlations were found between urinary 1-OH-PYR excretion and Exposure to PAHs for non-occupationally exposed populations. Significant correlations were found between urinary 1-OH-PYR levels with concentration of PYR and B[a]PYR in the ambient air in several cities residents where coal burning occurs [149]. A clear difference in urinary 1-OH-PYR excretion levels were observed between non-smoking inhabitants from a city with PM_{10} levels (> 120 $\mu g/m^3$) compared to those from less polluted city (< 70 $\mu g/m^3$) [150]; in smokers compared to non-smokers [151, 152]; in non-smokers exposed to ETS compared to non-smokers without ETS exposure [153]; in children living in urban residences compared to those living in rural residences [154]. In addition, a controlled human exposure study showed that a 100- to 250-fold increase in a dietary B[a]PYR dose paralleled a 4 to 12-fold increase in urinary 1-OH-PYR excretion [148]. Based on these studies, and results of other studies, it was suggested that the identification and determination of 1-OH-PYR in the urine could serve as a sensitive and a suitable biomarker of PAHs exposure, in estimates of individual exposure to PAHs in the exposed and the general population [132, 155-158]. However, other studies have failed to demonstrate significant correlations between urinary 1-OH-PYR excretion and exposure to PAHs in subjects from urban areas and those from suburban areas [159, 160], and between non-smoking traffic police officers and non-smoking controls [153]. Also, no associations were reported between1-OH-PYR excretion and personal exposure to $PM_{2.5}$ or black smoke in 50 non-smoking students living and studying in central parts of Copenhagen [161], and between 1-OH-PYR excretion and actual measurements of personal exposure to particles in non-occupationally exposed subjects [160]. In addition, recent studies in non-smoking, non-occupationally exposed populations have shown no significant correlation between PYR (gas and particulate phase) with urinary 1-OH-PYR levels either in non-smoking subjects exposed to ETS [126], or in an atmospheric exposure scenario relevant to the general population [125]. According to these observations, it is suggested that 1-OH-PYR is an

equivocal biomarker of exposure to atmospheric PAHs in the non-occupationally exposed population [125, 126, 162].

Given the significantly stronger carcinogenic effect of high molecular weight PAHs, particularly B[a]PYR, compared with low molecular PAHs, investigators have turned their attention to the determination of metabolites of carcinogenic PAHs, such as B[a]PYR and B[a]ANTH. However, it should be noted that, one major problem in determining urinary levels of PAH metabolites that for the group of PAHs with ≥ 5 rings, which includes the carcinogenic PAHs, is that they are present in urine at only very low levels, partly because they are present at extremely low concentrations in particulate fraction of air, and the fact that a large proportion of the metabolites formed is preferentially excreted in faeces via the bile [127, 132, 163]. Some studies have been carried out to detect 3-hydroxy-B[a]PYR (3-OH-B[a]PYR) in the urine of occupationally [164-169] and non-occupationally [125, 143, 166, 167, 170, 171] exposed subjects in order to assess its role as a biomarker of PAH exposure, either alone or simultaneously with 1-OH-PYR. In general, the results of these studies showed that the traditional methods using high performance-liquid chromatography-fluorescence detection (HPLC-FD) used in these studies were able to measure 3-OH-B[a]PYR in urine of occupationally exposed subjects, but their application for the general population was unsuccessful because of the relatively high detection limits and even in studies attempted to improve the sensitivity of the traditional methods to be applicable for measurement of 3-OH-B[a]PYR from the general population, only traces of 3-OH-B[a]PYR were detected compared to the relatively facile detection of higher concentrations of simultaneously measured 1-OH-PYR in these studies. In addition, in some recent studies attempted measuring 3-OH-B[a]PYR in the general population, even with highly sensitive analytical methods [125], although it was possible to find a significant relation between urinary 3-OH-B[a]PYR levels and the concentrations of B[a]PYR in the ambient air, a complete and reliable quantitative statistical analysis of the results were impossible owing to the high percentage of samples with concentrations of urinary 3-OH-B[a]PYR below the detection limits [125, 171, 172]. Thus, it is concluded that while far from being a completely satisfactory biomarker of B[a]PYR exposure in the general population, the presence or absence of detectable urinary 3-OH-B[a]PYR may serve as a qualitative indicator of exposure [125]. In addition to 3-OH-B[a]PYR, and as a biomarker of carcinogenic PAHs, a few studies attempted to measure 3-hydroxy-B[a]ANTH (3-

OH-B[a]ANTH) in urine of occupationally and non-occupationally exposed workers [164, 168].

The results (in 2000) concerning the carcinogenic potency of NAP in animal investigations have meant that this substance has increasingly also been a focus of attention and assumed to be a substance that contributes considerably to human cancer risk, both in workers at certain industrial workplaces and in the general population [132, 173, 174]. As NAP is the most volatile member of PAHs and predominantly airborne, thus it is nearly always present in the atmosphere of these workplaces as well as in the wider environment [174]. NAP is metabolized in human body to more than 30 different metabolites, with the phenolic metabolites and their conjugates considered to be the major metabolites from human metabolism. The majority of researchers have used urinary 1- naphthol (1-OH-NAP) and/or 2-naphthol (2-OH-NAP) as biomarkers for assessment of human exposure to PAHs [174], although one study also dealt with the determination of 1-naphthylglucuronide and 2-naphthylsulfate in human urine samples. Generally speaking, 2-naphthol seems to be more specific biomarker for PAHs exposure because 1-naphthol may also result from possible exposure to the pesticide Carbaryl; nevertheless, simultaneous determination of both urinary metabolites seems to provide a sufficient measure [174].

The results from biological monitoring by means of naphtol concentrations in the urine of subjects occupationally exposed to PAHs at their workplaces, show that in those studies dealing with the simultaneous determination of both metabolites, 1-naphthol prevails over 2-naphthol with respect to the mean values [175, 176-178]. Furthermore, extremely high urinary naphthol concentrations, for example, in the naphthalene oil distillation industry [176] in the creosote-impregnation sector [179] and the distillation department of coking plants [175], were found. On the other hand, shipyard and aircraft maintenance workers, as well as iron-foundry workers, showed urinary naphthol concentrations in the same range as observed in the general population [180-183]. There are some studies showing fairly good linear correlation coefficients between urinary 1-naphthol and/or 2-naphthol and airborne naphthalene concentrations at different workplaces: in the coal-tar and naphthalene distillation sector [184, 185], and in coking plants [175], and aircraft maintenance sector [177].

Some studies have been published that deal with the determination of 1-naphthol and/or 2-naphthol in the urine of persons from the general population [180, 182, 185-193]. In these studies, tobacco smoking was the most relevant source of NAP exposure. Overall, not all of all these studies showed a difference between smokers and non-smokers, although considerably increased naphthol concentrations have been be found in the urine of smokers compared with non-smokers (*ca.* 2.5- to 14--times higher**)** [182, 186, 188, 193]. In one study, a significant relationship particularly between urinary 2-naphthol levels and the number of cigarettes smoked per day was found [189].

The usefulness of 2-hydroxy-FLU (2-OH-FLU) as a biomarker for PAHs exposure was demonstrated in a study measuring 2-hydroxyfluorene in urine samples of smokers and non-smokers [194].

Metabolites of PHEN in urine have also been used as biomarkers for PAHs exposure [127, 132]. PHEN can be metabolized to five different phenols (1-, 2-, 3-, 4- and 9-OH-PHEN) and three dihydrodiols (1, 2-, 3,4- and 9,10-dihydroxydihydrophenanthrene) which are predominantly excreted in the form of their sulfates and glucuronides [127]. Since PHEN is oxidised at three different regions and, in contrast to pyrene, also converted into dihydrodiols, the urinary metabolite profile can provide information on the balance of the enzymes involved in these processes [127]. In addition, PHEN possesses a bay region, a feature closely associated with carcinogenicity and its metabolism is similar in many ways to B[a]PYR, but with the advantage that the products are far more abundant in human urine, and PHEN metabolites have therefore been suggested to be a useful biomarkers for PAHs exposure [195].

Some studies have used urinary metabolites of PHEN (phenols and dihydrodiols) for monitoring human exposure to PAHs among different population groups and to assess the role of metabolic activation of PAHs to the increased risk of lung cancer among smokers as well as for comparison of polymorphisms in genes involved in PAHs metabolism. In general, these studies show that the whole population is subject to exposure to PHEN, which finds expression in urinary OH-PHEN excretions that are easily detected by urine analysis [195-205].

Owing to the complexity of PAH mixtures in the atmosphere, the difference in PAH profiles among different environmental and occupational (industrial) locations, and since multiple

exposures to different PAHs always occurs, simultaneous measurement of hydroxylated metabolites coming from different parent compounds has been proposed in recent years in order to obtain a direct estimate of the total PAHs exposure [122, 123]. Some studies reported the simultaneous determination of NAP, PHEN, and PYR (because of the metabolites of these PAHs present at higher concentrations in urinary excretions and can give insight into metabolism and carcinogenicity of PAHs) in exposed workers and general population [178, 183, ,193, 206]. Other studies have reported the simultaneous quantification of other PAHs metabolites, such as NAP, FLU, PHEN, FLT, PYR, B[a]ANTH, CHR and 3-OH-B[a]PYR in occupationally exposed subjects and in the general population, using LC-MS/MS or gas-chromatography-tandem mass spectrometry (GC-MS/MS) [122, 163, 207-211].

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Section II

Experimental

Determination of hydroxylated urinary metabolites of Polycyclic aromatic hydrocarbons in the general population using UPLC-MS/MS.

In order to assess the health risks associated with the environmental exposure to PAHs, we have developed and validated a UPLC-MS/MS method to identify and quantify 16 hydroxylated urinary metabolites in urine samples collected from subjects of the general population.

The following method is one that was developed from that used by Onyemauwa and colleagues [163].

Chemicals and supplies

2-OH-NAP, 1-OH-NAP-d_7 (> 99.5 % isotope purity), 1-OH-NAP, and 2-OH-FLU, were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). 2- OH-PHEN , 3- OH-PHEN, 4-OH-PHEN, 3-OH-FLT, 1-OH-B[a]ANTH, 2-OH-B[a]ANTH, 3- OH-CHR, 6-OH-CHR, and 7-OH-B[a]PYR were purchased from Midwest Research Institute (Kansas City, MO, USA), while 3-OH-PHEN-C_{13} (> 99.5 % isotope purity), 1- OH-PHEN , 9-OH-PHEN, 1-OH-PYR-d_9 (> 99.5 isotope purity), 1-OH-PYR, and 6-OH-CHR-C_{13}(> 99.5 % isotope purity), were purchased from Cambridge Isotope Laboratories (Sainte Foy La Grande, France), and 3-OH-B[a]PYR was purchased from EC-JRC-IRMM (Geel, Belgium). β-glucuronidase/aryl sulfatase type H-1 enzyme from *Helix pomatia* was purchased from Roche Applied Science (Meylan, France). Methanol, acetic acid and sodium acetate were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) and were of the highest available purity. EnvirELut PAH solid phase extraction (SPE) cartridges were from Agilent (Les Ulis, France). Atmosphere vials, inserts and the column (ThermoGold C_{18}, 1.9µm, 100 x 2.1 mm) for liquid chromatography were from Thermo Electron (Les Ulis, France).

Subjects and urine collection

Urine samples from method development were collected from anonymous healthy non-smoking donors. All samples were frozen and stored at -80ºC until analysis. No personal PAH exposure data were available for the subjects on the days of urine collection.

Analysis of urine for OH-PAHs

Three millilitres of urine (if sample highly colored/turbid, 1.5 mL of urine used) were dispensed into a glass 30 mL tube and buffered with 5 mL of 0.1 M acetate buffer, pH 5 (6.5 mL buffer if only 1.5 mL of urine used). After adding 20 µL of β-glucuronidase/aryl sulfatase, the mixture was incubated in *bain marie* at 37ºC, with lateral shaking overnight (at least 15 hours). Twenty microliters of an internal standard mixture in methanol was added to the incubation tube to give final concentrations in urine of 13.3 ng/ml 1-OH-NAP-d_7, 0.133 ng/ml 3-OH-PHEN-C_{13}, 1.33

ng/ml 1-OH-PYR-d_9, 0.133 ng/ml 6-OH-CHR-C_{13}. Samples were purified on SPE cartridges as follows. The digested urine sample was loaded onto a SPE cartridge that had been washed with 5 mL of methanol and equilibrated with 5 mL acetate buffer using a Pasteur pipette and drawn through under vacuum (Varian vacuum manifold, *ca.* 1.5 mL/min.). The cartridge was successively washed with 3 mL of 1:1 methanol: water (v/v), used to rinse the incubation tube before adding to the cartridge, then with 1 mL of methanol. OH-PAHs were eluted using 5 mL of methanol, and collected in a 10 mL glass tube. Under a gentle stream of nitrogen with tubes resting in a block heated to 40ºC, the methanol was evaporated to almost dryness (*ca.*50 µL). Samples were then quantitatively transferred to 300 µL insert with at least three 50 µL rinses of methanol, giving a final volume of ca. 200 µL.

Liquid chromatography-tandem mass spectrometry

Analyses were performed on a TSQ Vantage instrument (Thermo Electron, Les Ulis, France). A Thermogold C_{18} analytical column (100 x 2.1 mm, 1.9 µm particle size) at 30ºC was used for analyte separation. The mobile phase was a binary mixture of water (A) and methanol (B) at a flow rate of 400 µL/min. (0 min. =65% A; 15 min. = 10% A; 15-20 min. = 65% A). The mass spectrometer employed a "HESI" heated electrospray ionization probe that was operated in the negative ion mode with a spray voltage of – 3000 V and a vaporizer temperature of 370ºC. The capillary temperature was set at 270ºC**.** To optimize mass spectrometric parameters, standard solutions of 1 or 10 µg/mL of individual OH-PAHs in 1:1 methanol: water (v/v) at 400 µL/min was used. The ion source was operated in the negative ion mode and a predominant $[M-H]^-$ was observed for each OH-PAHs in Q1. Product ion spectra, obtained by collision induced dissociation of the parent ion, indicated a major fragment ion of $[M-H-28]^-$ in Q3 for all OH-PAHs except 2-OH-FLU. This neutral loss of 28 Da has been observed by other investigators and can be attributed to loss of CO from the parent ion [1]. The collision energies and tube lens voltages were then further optimized for individual parent-to-product ion transitions using the Excalibur software. For quantitation of each OH-PAH, the loss of 28 Da $[M-H-28]^-$ from $[M-H]^-$ was monitored for all but for 2-OH-FLU. Acquisition was in "single reaction monitoring" (SRM) mode monitoring the transitions given in (Table I). Quantification was by internal standard (deuterated or C_{13} labelled), according to the sub groups shown in Table I; some compounds could not be separated chromatographically (e.g., 2- and 3-OH-PHEN), meaning results were given as the sum of the co-eluting compounds.

Standard calibration curves

Stock solutions of 1or 2 mg/mL OH-PAHs and isotopically labelled OH-PAHs were prepared in methanol, and were subsequently diluted with methanol to prepare working standards between 0.12 and 12 µg/mL. Six point standard calibration curves were prepared by spiking 3 mL of the volunteer's urine with concentrations between *ca.* 0.002-0.02 and *ca.* 0.1-1µg/L (depending on the compound), and following the incubation/clean-up described. Calibrations were performed for every 3-4 incubation series (18 samples/series). The mean calibration slope for each compound obtained during the whole period of analysis was used for the quantification (see **Table I** for internal standards used for each compound sub-group), and the R^2 for the calibration curves was always greater than 0.99, indicating good linearity of the method.

Recoveries, limits of quantitation, limits of detection and precision

Limits of detection (LODs) and quantification (LODs) were determined from 10 blanks (3 mL of the volunteer's urine), or, in the absence of a compound in the urine, from the concentration giving a signal: noise ratios of at least 3 or 10, respectively.

Responses of calibration standards were checked before each analytical run, the analysis proceeding only if the responses were within 15% of that measured in the previous analysis of the same extract.

Quality Assurance and Control

Recovery of compound after incubation and clean-up

Volunteer's urine was spiked with ca. 0.85 µg/L urine (1- and 2-OH-naphthalene) or ca. 0.085 µg/L urine (all other compounds) and analysed in triplicate to determine recovery of the compounds after both incubation and SPE, and after SPE only (**Table II**).

Percentage recoveries were between 56-98% (3- and 7-OH-benzo[a]pyrene and 1-OH-pyrene, respectively) after both incubation and SPE, with coefficients of variation (CVs) all <15%.

Percentage recoveries after SPE only were between 72-100% (3-OH-PHEN-C_{13} and 2- and 3-OH-PHEN, respectively) with CVs all <15%.

Coefficients of variations for the analysis of the samples

Fourteen randomly chosen samples were analysed in duplicate by different analysts on different days (**Table III**).

The range of concentrations in these samples was representative of the values measured in the 300 samples from the general population. Coefficients of variation for these duplicate analyses were between 0.1-27% (minimum and maximum for 1 or 2-OH-NAP and the sum of 1-, 9- and 4-OH-PHEN, respectively), with mean CVs for the 14 duplicates between 5-12% (2-OH-NAP or 1-OH-PYR and 2-OH-FLU or the sum of 2- and 3-OH-PHEN, respectively).

Certified Reference Material For 1-OH-pyrene

A reference urine with certified values for 1-OH-PYR (ClinCheck® from Recipe, Precision Instruments, France) was analysed with every calibration set (n=6) with excellent recoveries (+/- s.d.) of 93% (+/- 13) and a CV of 14%.

Representative chromatograms obtained from urine spiked with the OH-PAHs and carried through the analysis are presented in **figure 1.**

Results

Results are currently undergoing statistical analysis and will be the subject of a publication in the near future.

Table I: Compounds grouped with their internal standards; MS^1 – MS^3 transitions and collision energies; retention times; LODs and LOQs.

	SRM transition [a]		Collision Energy	Retention Time (min.)	LOD [b]	LOQ [b]
	m/z "parent" ion	m/z "daughter" ion				
2-OH-naphthalene	143	115	26	5.90	0.0025	0.0083
1-OH-naphthalene-d_7*	150	122	26	6.00	-	-
1-OH-naphthalene	143	115	26	6.30	0.0010	0.0032
2-OH-fluorene	181	180	26	8.75	0.0019	0.0063
3-OH-phenanthrene-C_{13}*	199	171	29	9.35	-	-
2- and 3-OH-phenanthrene	193	165	29	9.50	0.0003	0.0010
1-, 9- and 4-OH-phenanthrene	193	165	29	9.80	0.0002	0.0006
3-OH-fluoranthene	217	189	32	11.00	0.0005	0.0015
1-OH-pyrene-d_9*	226	198	32	11.20	-	-
1-OH-pyrene	217	189	32	11.40	0.0003	0.0010
3- and 6-OH-chrysene and 2-OH-benzo[a]anthracene	243	215	32	12.30	0.0005	0.0017
6-OH-chrysene-C_{13}*	249	221	32	12.40	-	-
1-OH-benzo[a]anthracene	243	215	32	12.50	0.0002	0.0008
3- and 7-OH-benzo[a]pyrene	267	239	35	14.00	0.0003	0.0010

* – internal standard used to quantify all the compounds in the sub-group indicated.

Table II: Recovery after Incubation and Clean-up by SPE – % recovery after Incubation and SPE and after SPE only.

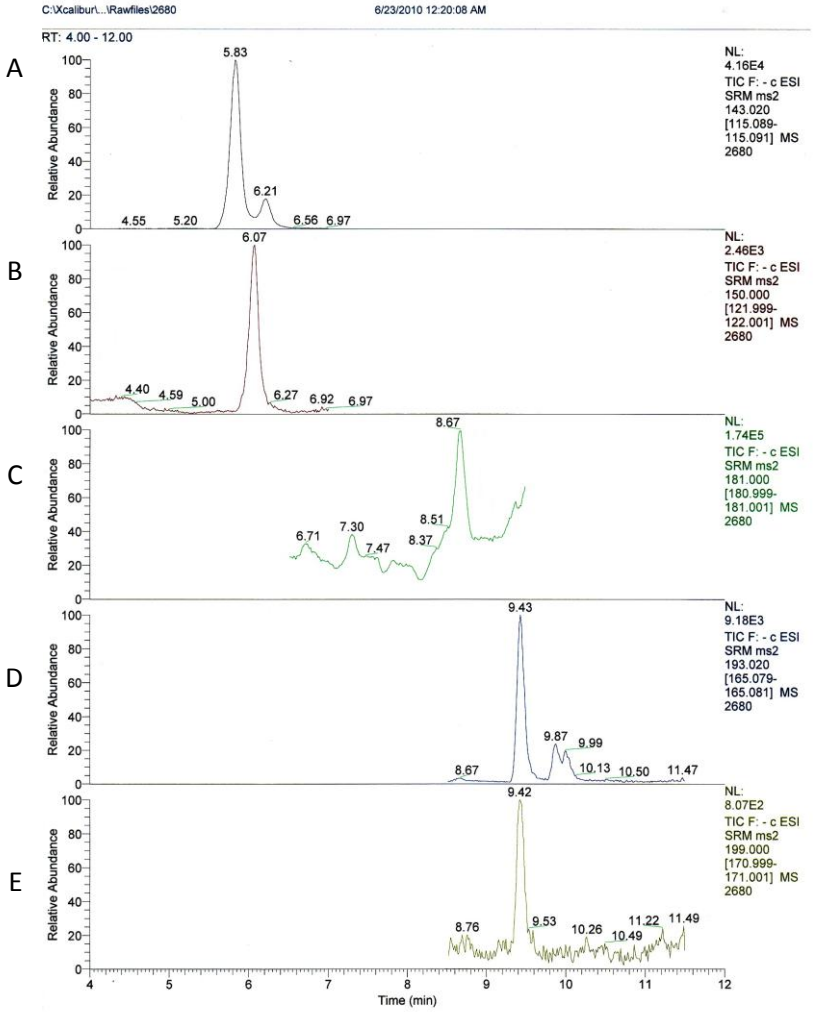
	% recovery after Incubation and SPE [a]			% recovery after SPE only [b]		
	Mean	s.d. [c]	*CV* [d]	Mean	s.d.	*CV*
2-OH-naphthalene	76.8	8.6	*11*	87.1	8.5	*10*
1-OH-naphthalene-d_7*	87.3	12.1	*14*	82.3	9.3	*11*
1-OH-naphthalene	74.5	6.0	*8*	79.0	5.2	*7*
2-OH-fluorene	96.5	7.8	*8*	96.2	4.8	*5*
3-OH-phenanthrene-C_{13}*	69.0	6.9	*10*	72.3	9.6	*13*
2- and 3-OH-phenanthrene	94.8	5.7	*6*	99.8	6.2	*6*
1-, 9- and 4-OH-phenanthrene	85.8	4.0	*5*	98.3	6.7	*7*
3-OH-fluoranthene	68.0	3.8	*6*	94.1	6.2	*7*
1-OH-pyrene-d_9*	73.5	9.4	*13*	79.9	10.8	*13*
1-OH-pyrene	98.2	8.2	*8*	98.5	8.0	*8*
3- and 6-OH-chrysene and 2-OH-benzo[a]anthracene	69.2	7.0	*10*	81.2	7.2	*9*
6-OH-chrysene-C_{13}*	96.5	5.2	*5*	97.1	6.5	*7*
1-OH-benzo[a]anthracene	63.1	5.8	*9*	80.4	7.2	*9*
3- and 7-OH-benzo[a]pyrene	55.6	1.7	*3*	76.2	6.8	*9*

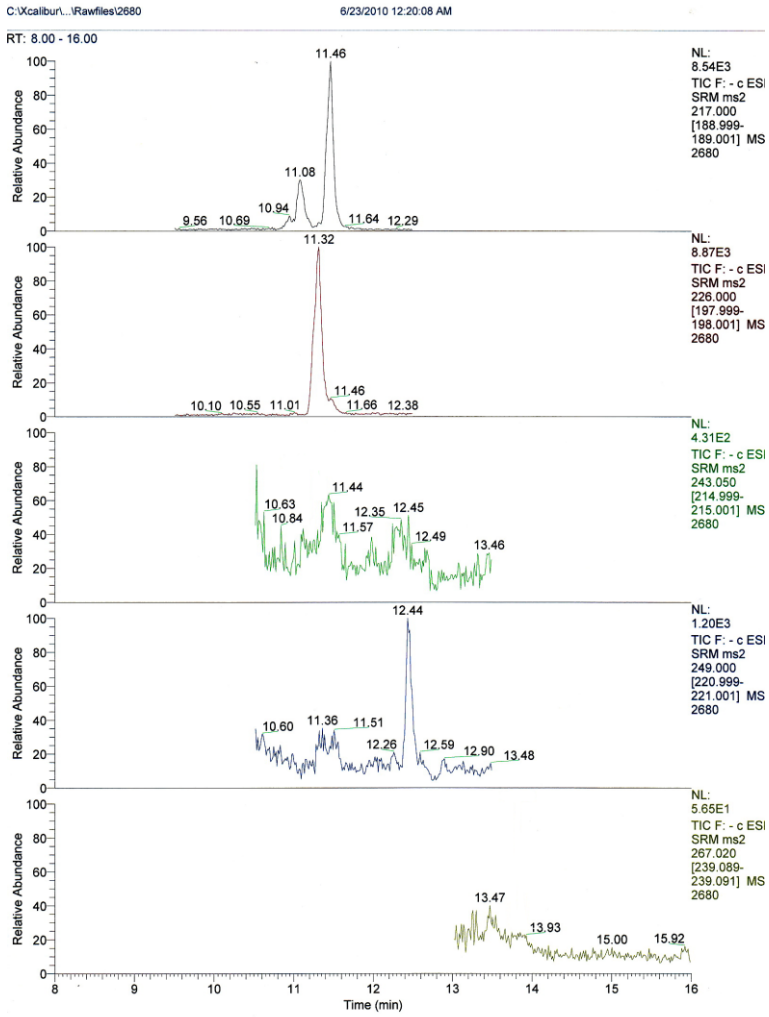
[a] – spike of *ca.*0.85 – 0.085 µg / mL volunteer's urine, depending on the compound, made before Incubation ; [b] – spike made after Incubation and before SPE ;[c] – standard deviation ; [d] – coefficient of variation (%) ; * – internal standard used to quantify all the compounds in the sub-group indicated.

Table III: Range of concentrations and coefficients of variation for the duplicate analysis of 14 samples.*

	Range of concentrations measured (µg / L urine) [a]		Coefficient of variation of duplicate analyses (%) [b]		
	Max.	Min.	Max.	Min.	Mean
2-OH-naphthalene	35.6	2.2	18	0.1	5
1-OH-naphthalene	36.2	0.70	23	0.4	9
2-OH-fluorene	2.40	<LOD	25	0.5	12
2- and 3-OH-phenanthrene	1.60	0.13	22	3.5	12
1-, 9- and 4-OH-phenanthrene	1.80	0.20	27	0.6	8
3-OH-fluoranthene	2.62	0.07	18	1.8	9
1-OH-pyrene	1.65	0.16	12	0.4	5
3- and 6-OH-chrysene and 2-OH-benzo[a]anthracene	nd [c]	nd	–	–	–
1-OH-benzo[a]anthracene	nd	nd	–	–	–
3- and 7-OH-benzo[a]pyrene	nd	nd	–	–	–

* - Samples analysed in duplicate: 136, 137, 167, 168, 170, 177, 182, 1103, 1117, 1123, 1130, 1136, 1142, 1152 ;
[a] – maxima and minima of concentrations measured in the 14 samples analysed in duplicate;
[b] – maxima, minima and mean of coefficients of variation (CVs) of duplicate analyses ;
[c] – Not detected (no peak).





A – 2-OH-NAP (5.83 mins) and 1-OH-NAP (6.21 mins) ;
B – 1-OH-NAP-d_7 ;
C – 2-OH-FLU ;
D – 2- and 3-OH-PHEN (9.43 mins) and 1-, 9- and 4-OH-PHEN (9.87 and 9.99 mins) ;
E – 3-OH-PHEN-C_{13}.

F – 3-OH-FLT (11.08 mins) and 1-OH-PYR (11.46 mins) ;
G – 1-OH-PYR-d_9 ;
H – 3- and 6-OH-CHR and 2-OH-B[a]ANTH ;
I – 6-OH-CHR-C_{13} ;
J – 3- and 7-OH- B[a]PYR.

Figure 1: UPLC-MS/MS SRM transitions used in the analysis of a single sample for urinary OH-PAHs

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General discussion and Perspectives

UPLC (LC)-MS/MS is an indispensable tool for the detection of substances that are present at low concentrations in biological fluids in DFC cases as these substances are usually given to the victim in low doses, have very short half-lives, and there is often a significant delay in victim's reporting of the incident (rape or theft).

In France, in late 2002, SFTA published a special issue on the topic of DFCs addressing the necessity for separation techniques coupled to mass spectrometry, and if possible tandem mass spectrometry, which is the preferred approach [1].

We have shown the utility of this technique in a DFC case involving LSD, which is in accordance with other recent published studies that have also demonstrated the indispensable character of UPLC-MS/MS to identify, confirm, and quantitate a large number of substances and/or their metabolites in biological fluids (blood, urine, hair, etc.) (e.g., [2, 3]).

Nowadays, UPLC (LC)-MS/MS technique has become less expensive and can also be used in the toxicology laboratory in different applications (e.g., therapeutic drug monitoring) and should be available in forensic toxicology laboratory engaged in the detection of substances involved in DFCs. Nevertheless, in addition to the sensitivity and specificity of tandem mass spectrometry, the availability of skillful and experienced analysts and experts and the use of efficient extraction methods are also important in this regard.

In addition to its usefulness in the field of forensic toxicology, we have also demonstrated the usefulness of UPLC-MS/MS in the field of environmental toxicology and public health. We have developed and validated a UPLC-MS/MS method for the detection and quantification of hydroxylated urinary metabolites of PAHs that are used as biomarkers of exposure of the general population to PAHs, which is important owing to the toxic effects of PAHs (teratogens, mutagens, and carcinogens).

UPLC (LC)/MS/MS technique can also be used for the detection of a wide range of substances in other matrices, such as hair (e.g., [4, 5]), particularly when the substances involved in DFCs cannot be detected or are absent in the blood or urine, or to show the accumulation of environmental substances in the hair.

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