DEVELOPMENT OF A 1-HYDROXYPYRENE QUANTIFICATION METHOD FOR USE AS A BIOMARKER IN ASSESSMENT OF POLYCYCLIC AROMATIC HYDROCARBON EXPOSURE

An Undergraduate Research Scholars Thesis

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ABSTRACT

Development of a 1-Hydroxypyrene Quantification Method for Use as a Biomarker in Assessment of Polycyclic Aromatic Hydrocarbon Exposure

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A common phenolic metabolite of pyrene, 1-hydroxypyrene (1-OHP), is often used as a biomarker to assess polycyclic aromatic hydrocarbon (PAH) exposure. The developed high-performance liquid chromatography with mass spectrometry (LC-MS/MS) method showed sufficient sensitivity to quantify 1-OHP in non-smoker urine samples, obtained in an ongoing project studying the effects of traffic related air pollution exposure in a population of pregnant women in South Texas. Data obtained through the developed method will be used to assess the effectiveness of 1-OHP as a biomarker for PAH exposure and will be compared to urinary concentrations of nitrated pyrene metabolites and airborne pyrene concentrations, collected through the use of personal monitoring devices. Success of the project will be determined by the specificity and sensitivity of the proposed method in quantification of 1-OHP concentrations and its application to the overall aims of the ongoing project.

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NOMENCLATURE

ATSDR	Agency for Toxic Substances and Disease Registry
B(a)P	Benzo(a)pyrene
CDC	Center for Disease Control and Prevention
DI	Deionized
EPA	U.S. Environmental Protection Agency
GC/MS	Gas Chromatography with Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IRAC	International Agency for Research on Cancer
1-OHP	1-Hydroxypyrene
1-OHP-G	1-Hydroxypyrene-Glucuronide
OHNP	Hydroxynitropyrene
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
PAH	Polycyclic Aromatic Hydrocarbon

CHAPTER I

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of environmental containments that are produced through the incomplete combustion of organic matter, detected most abundantly in sources of diesel exhaust, cigarette smoke, and wood smoke.[1] Specific PAHs, such as benzo(a)pyrene and 1-nitropyrene, have been identified by the International Agency for Research on Cancer (IRAC) as known (Group 1) and probable (Group 2A) carcinogens, respectively.[2] Furthermore, PAHs have been listed as priority hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR),[3] and as priority pollutants by the U.S. Environmental Protection Agency (EPA).[4]

PAH exposure can occur through methods of inhalation, ingestion, and dermal absorption.[5] This exposure can be quantified through the analytical study of specific urinary PAH metabolites. For example, in a study conducted by the Center for Disease Control and Prevention (CDC), concentrations of phenolic metabolites of fluorine, phenathrene, naphthalene and pyrene (2,3,9-hydroxyfluorene, 1,2,3,4-hydroxyphenanthrene, 1,2-hydroxynaphthalene, and 1-hydroxypyrene, respectively) were measured in urine samples of smokers and nonsmokers. Concentrations of these metabolites were found to be higher among the population of smokers, supporting the correlation of increased PAH exposure with greater exposure to cigarette smoke.[1] Among the many PAH urinary metabolites, monohydroxylated PAHs have been commonly used as biomarkers in prior studies to assess the extent of PAH exposure in populations.[5] 1-hydroxypyrene (1-OHP) is frequently used as both a single biomarker and in a series of biomarkers, and is favored due to higher levels of detection in urine samples in comparison to

other metabolites. Such studies have achieved quantification of 1-OHP through analytical techniques including gas chromatography, liquid chromatography, and high-performance liquid chromatography (HPLC) with fluorescence detection, or more recently, HPLC separation followed by mass spectrometry.[6]

The aim of this project was to develop an analytical method to quantify 1-OHP concentrations in urine samples with high specificity and sensitivity using liquid chromatography with tandem mass spectrometry (LC-MS/MS), incorporating isotopic 1-OHP as an internal standard. This method was then applied to determine overall PAH exposure, based on the 1-OHP biomarker, in a population of pregnant women residing in South Texas.

Chemical properties of PAHs

PAHs are a large class of organic compounds composed of two or more fused aromatic rings, consisting of primarily carbon and hydrogen.[7] Compounds may contain various substituents, such as alkyl, amino, halogen, hydroxyl, or thiol groups. Heterocyclic derivatives of PAHs contain one or more heteroatoms, such as nitrogen, oxygen or sulfur, within the atomic structure.[8] PAH molecules are characterized by aromaticity, or an increased stability due to the resonance of delocalized π-electrons within the molecule.[9] Structural examples of various homocyclic PAHs are shown in Figure 1.

The physical properties of PAHs vary with both structure and molecular weight, but generally, PAHs are characterized as colored, crystalline solids at room temperature with high stability, low volatility, and low solubility in water.[7,10] Most notably, solubility and vapor pressure both decrease with increased molecular weight or number of rings.[11] As a result, low molecular weight compounds with less than 4 conjugated rings, such as naphthalene, have increased volatility and exist primarily in the gas phase.[7,12] An exception to this solubility trend

occurs in high molecular weight compounds (4 or more rings), in which compounds adopt a non-planar conformation to reduce steric strain. In these instances, polarity, and therefore solubility in water, is increased.[11]

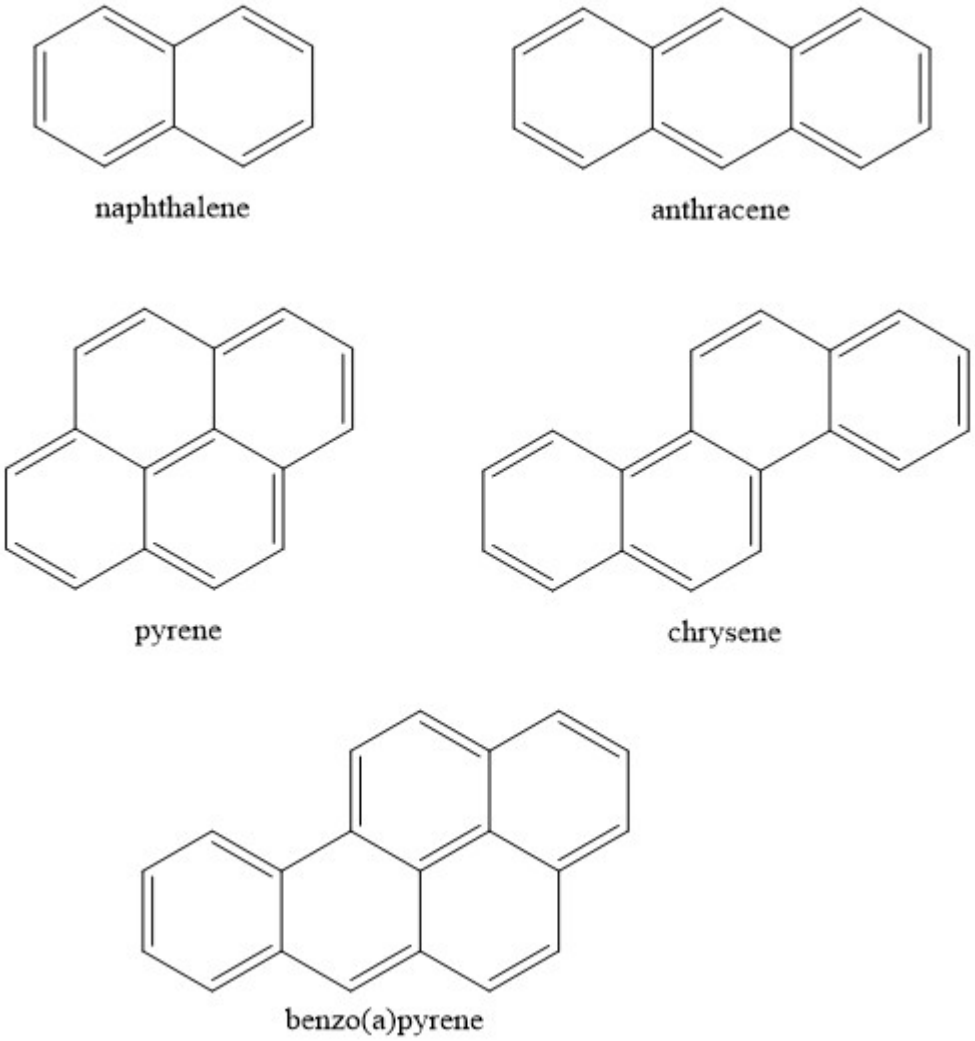
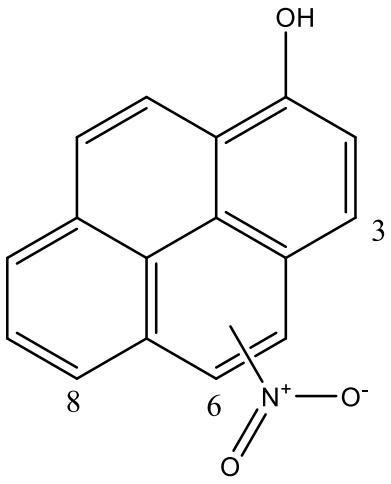


Figure 1. Chemical structures of homocyclic PAHs with varying molecular weights and arrangements.

Although lighter PAHs are considered to have weaker carcinogenic and mutagenic properties, they are likely to react with other atmospheric pollutions, such as nitrogen oxides, to form compounds with increased toxicity when in the gas phase.[7,12] Such substituted PAHs can be beneficial in tracking compounds from specific sources, such as the nitrogenation of 1-OHP in

the presence of nitrogen dioxide from diesel exhaust to form isomers of hydroxynitropyrene (OHNP), shown in Figure 2.[13]



3,6,8- hydroxynitropyrene

Figure 2. Chemical structure of 3, 6, and 8-OHNP.

Sources of PAHs

PAHs are emitted into the environment as air, soil, and water pollutants through the combustion of organic matter from both natural and anthropogenic sources. While natural origins such as forest fires and volcanic eruptions contribute to total environmental PAH levels, anthropogenic origins are considered to yield the largest contribution. Such sources include burning of wood or fossil fuels for heat or power generation, garbage incineration, cooking, and vehicular traffic emissions.[7,9,12] Although PAHs are present in the environment globally, they are found in the highest concentrations near urban centers. It should be noted that levels in urban environments vary depending on factors such as population density, types of emission sources, meteorological conditions, and temperature. Additionally, concentrations tend to be higher during the winter as a result of increased fuel combustion for residential heating.[9,12]

Exposure to PAHs

Major routes of PAH exposure to the general population include inhalation of ambient and indoor air pollution, smoking cigarettes or tobacco, and cooking and consumption of food.[li] Workers in industries such as transportation, manufacturing and oil refinery risk higher rates of PAH exposure, which has been correlated with increased occurrences of skin and lung cancers as well as other adverse health effects.[12,14] In non-smoking individuals, primary routes of exposure include cooking emissions and consumption of food, particularly if it has been grilled or fried.[7] Exposure in both humans and animals can also occur through ingestion of uncooked foods, such as lettuce, tomatoes, and kale, especially when were grown in proximity to a source of pollution, such as a highway. PAH content in such plants occurs due to a combination of particle-phase deposition of PAHs onto leaves and absorption of PAHs in contaminated soil. Concentrations vary depending on the extent of exposure, atmospheric conditions, and the biological properties of the plant.[12]

Health effects of PAHs

Adverse health effects due to PAH exposure vary depending on factors such as extent of exposure, PAH toxicity properties, and the route of exposure, such as inhalation, ingestion, or dermal absorption. Age and previously existing health conditions, such as asthma or coronary heart disease, may also affect the severity of health impact. Acute non-carcinogenic effects of exposure include eye and skin irritation, allergic reactions, nausea, and vomiting.[15,16] Chronic non-carcinogenic health effects, generally reported due to long-term occupational exposure, include kidney and liver damage and related diseases such as jaundice, cataracts, reproductive health complications, and breakdown of red blood cells.[7,12]

The carcinogenic effects of PAH exposure vary with the aforementioned factors as well as the extent of exposure to certain compounds. Specifically, eight PAH compounds have been identified by the EPA and other agencies as probable human carcinogens: benz(a)anthracene, benzo(a)pyrene, benzo(b)flyoranthene, benzo(k)fluoranthene, chrysene, dibenze(a,h)anthracene, indeno(1,2,3-c,d)pyrene, and benzo(ghi)perylene.[3,4,7] Of these, benzo(a)pyrene (B(a)P) is widely considered to be highly carcinogenic.[12,17] Reported carcinogenic effects of PAH exposure, generally occurring due to occupational exposure, include skin, lung, and stomach cancers.[7]

Biomarkers of PAHs

Following PAH exposure by inhalation, ingestion, or dermal absorption, PAHs can be biotransformed by either phase I or phase II metabolic enzymes to form hydroxylated derivatives and glucuronic acid or sulfate conjugates, respectively. Metabolized forms, such as 1-OHP, and PAH-DNA or protein adducts have increased aqueous solubility and are primarily excreted in urine. While PAH exposure can be assessed by methods such as external monitoring and blood analysis, urinary metabolites can be measured in a non-invasive manner with increased accessibility.[10] Such assessment is useful in identification of individuals with recent occupational or environmental exposure, determination of specific routes and sources of exposure, and correlation of extent of exposure with adverse health effects.[18]

1-OHP and 1-hydroxypyrene-glucuronide (1-OHP-G) are most commonly used as biomarkers to assess recent exposure to both general PAH mixtures and specific compounds of interest, such as B(a)P.[18,19] The wide success and applicability of 1-OHP as a biomarker is largely due to the high concentrations of pyrene (2-10%) of all environmental mixtures of PAHs,[7] and the occurrence of 1-OHP as the primary product of the metabolization of pyrene (90% likelihood of formation).[19] Molecular structures of pyrene and 1-OHP are given in Figure

3. Formation of 1-OHP-G is also highly favored (80% likelihood of formation) and is often favored to assess low exposure to PAHs, due to its high fluorescence.[10,18]

1-OHP as a major pyrene metabolite was first observed in pig urine by Keimig et al. in 1983, and first proposed as a biomarker for assessing PAH exposure by Jongeneelen et al. in 1987.[20-22] This method used HPLC with fluorescence detection to correlate concentrations of 1-OHP in the urine of rats following oral administration of pyrene in varying doses, and to assess occupational PAH exposure from creosote.[21] Since then, 1-OHP has been used in numerous studies to assess the extent PAH exposure in various populations using methods such as HPLC with fluorescence detection, gas chromatography with mass spectrometry (GC/MS), and more recently, LC-MS/MS.[10,17,19,22,23]

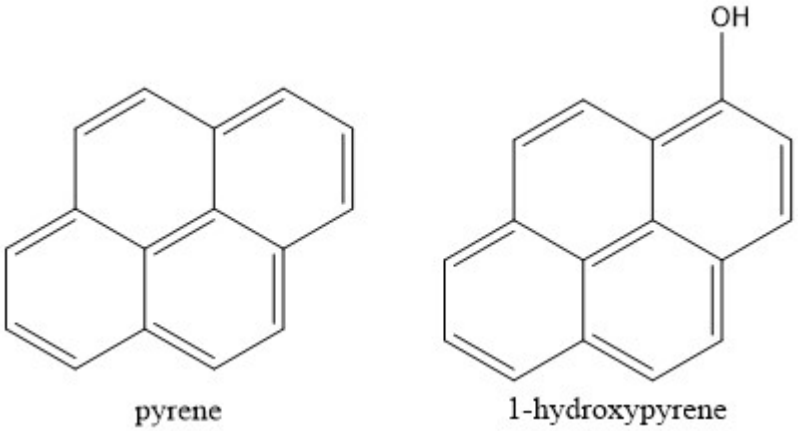


Figure 3. Chemical structures of pyrene and its primary urinary metabolite, 1-OHP.

CHAPTER II

METHODS

Materials

Authentic 1-hydroxypyrene (1-OHP) reference standard, pyrene-d_{10} and β-glucuronidase/aryl sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO). Blue rayon was purchased from Funakoshi (Tokyo, Japan). Solid phase extraction cartridges were purchased from Waters (Milford, MA). All of the experiments were done suing filtered and deionized (DI) water (18.2 MΩ cm) (Millipore, Milford, MA). All other chemicals and reagents were purchased commercially at the highest degree of purity available.

Synthesis

1-OHP-d_9 (for use as an internal standard) was synthesized according to a published method developed by Toriba et al.[13] The concentration of 1-OHP-d_9 was determined using UV-Visible spectrophotometry.

Urine sample preparation and extraction

1-OHP was extracted from human urine according to a method developed by Toriba et al. with modifications.[13] The collection of human urine and study population is described in detail below. Briefly, a 100 mL aliquot of each urine sample was thawed at room temperature. The pH was adjusted to 5.0 with 1.0 M HCl. 5 mL of 4 M acetate buffer (pH 5.0) and 75 μL β-glucuronidase was added to the sample prior to incubation (37 °C, 4 h) with gentle agitation. Following incubation, 100 mg of blue rayon was added to increase the sorption of 1-OHP, and the sample was incubated (1 h) at room temperature with agitation by shaker (60 rpm). The blue rayon was removed from the liquid phase, washed with 5 mL DI water, and allowed to air dry (3

min). The blue rayon was extracted with 20 mL MeOH:Ammonia (50/1, v/v) with aid of sonication (30 min). The blue rayon was removed from the sample and discarded. The liquid phase was dried under nitrogen and re-suspended in 5 mL MeOH:EtAc (1/1, v/v). The sample was applied to a Sep-Pak Alumina A cartridge, (Waters, Milford, MA) preconditioned with 50 mL MeOH:EtAC. The sample was eluted with 10 mL MeOH:EtAc. The elutant was dried under nitrogen and re-dissolved in 500 µL HPLC grade methanol. The sample was separated into 250 µL aliquots for 1-OHP and saved for later analysis of nitrated OHP metabolites. The samples designated for 1-OHP analyses were spiked with 20 µL of internal standard, 200 ng/mL 1-OHP-d_9.

Analysis of 1-OHP by LC-MS/MS

A Waters Acquity H-Class UPLC system coupled with a Thermo triple quadruple mas spectrometer was used for 1-OHP sample analyses. Separation was performed on a Zorbax SSHD Eclipse Plus-C_{18} column (3.0 x 50 mm, 1.8 µm; Agilent, Santa Clara, CA) with a guard column (2.1x 5 mm, 1.8 µm; Agilent, Santa Clara, CA) at 25 °C. A gradient elution of 0.1% ammonium acetate in water (eluent A) and HPLC grade methanol (eluent B) was used at a flow rate of 0.4 mL/min, based on methods reported by Ramsauer et al.[6] Sample volumes of 10 µL were injected for each analysis. The observed retention times and mass transitions are given in Table 1.

Table 1. Observed retention times and mass transitions of 1-OHP and 1-OHP-d_9

Analyte	RT (min)	Parent ion (m/z)	Daughter ion (m/z)
1-OHP	4.7	217	189
1-OHP-d_9	4.7	226	198

Study population and urine collection

1-OHP levels were measured as part of an ongoing study to assess exposure to air pollution in a population in Hidalgo County, shown to be at high risk for asthma development. This location was chosen for the study because it has been reported to have one of the highest rates of hospitalization due to childhood asthma, at 14.82%, in the state of Texas.[24] The overall goal of this study was to determine air pollution exposure in pregnancy since prenatal air pollution exposure is an emerging risk factor for childhood asthma development.[28,29]

Study population

The study population consisted of 17 women in their third trimester of pregnancy that received prenatal care from Rio Grande Regional Women's Clinic. And met all other inclusion criteria. All participants provided written and informed consent. All study procedures were approved by the Texas A&M University Institutional Review Board and access to participant identifiable data was restricted. Participants were between 21 and 35 years of age, had no history of preterm birth, were non-asthmatic and non-diabetic, lived in a non-smoking household, and had a singleton pregnancy. Specific demographic information is provided in Table 2.

Sample collection

Urine samples were obtained from the study population in three-week intervals between June 2015 and April 2016. Samples were collected at Rio Grande Regional Women's Clinic during the participant's regularly scheduled prenatal care appointments. Participants were instructed to collect their first void on the day of their appointment in a provided sterilized 120 mL plastic urine container. Samples were kept on ice during transportation. Upon laboratory receipt, urine volume was recorded and samples were diluted with deionized water to reach a final volume of 100 mL, if needed. A 5mL aliquot was designated from each sample for

creatinine analysis. The sample remainders were stored in polypropylene tubes at -80ºC until analysis.

Table 2. Population demographic information.[25]

Criterion	N	%
Ethnicity		
Hispanic	17	100
Education Level		
Less than 12 years	7	41
12 years	6	35
More than 12 years	1	6
Unknown	3	18
Smoking History		
Never	12	71
Before Pregnancy	2	12
Unknown	3	18
Type of Home		
Single Family Home - Detached	7	41
Single Family Home - Attached	1	6
Mobile Home	5	29
Unknown	4	24
Heating System		
Central Heating	4	24
Single Stoves/Heaters	3	18
Electric	7	41
Gas	0	0
Unknown	3	18
Cooling System		
Central Air Conditioning	5	29
Window Units	6	35
None	1	6
Unknown	5	29
Primary Cooking Method		
Electric	5	29
Gas	9	53
No Cooking at Home	1	6
Unknown	2	12
Employment		
Yes	2	12
No	15	88

During the third sampling period, hair follicle samples were collected to assess and confirm limited exposure to cigarette smoke by nicotine analysis. An isotope dilution GC/MS method developed by Kim et al. was used to determine nicotine concentrations.[26,27] Of the samples analyzed, 7 concentrations were found to be below the limit of detection, 0.0492 ng/mg, with the remaining 10 concentrations below 0.062 ng/mg. Low nicotine concentrations indicated that all of the study participants were not regularly exposed to second hand smoke and therefore, remained in the study.[25]

CHAPTER III

RESULTS AND DISCUSSION

Urinary concentrations of 1-OHP

Urinary concentrations of 1-OHP (ng/mL) were calculated from the generated calibration curve (Figure 4) using the obtained 1-OHP:1-OHP-d_9 peak ratios. To generate this standard curve, authentic human urine samples (with low 1-OHP background) were spiked with the 1-OHP and 1-OHP-d_9 standards and processed as described in the methods above.1-OHP standards were prepared in methanol and spiked into urine to yield the following concentrations: 0.0, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, and 50.0 ng/100 mL. 1-OHP concentrations were confirmed by UV-VIS spectroscopy absorbance at 242 nm with a molar absorptivity of 63096 L mol^{-1} cm^{-1}.[30] All samples were spiked with 20 µL of the internal standard (200 ng/mL 1-OHP-d_9).

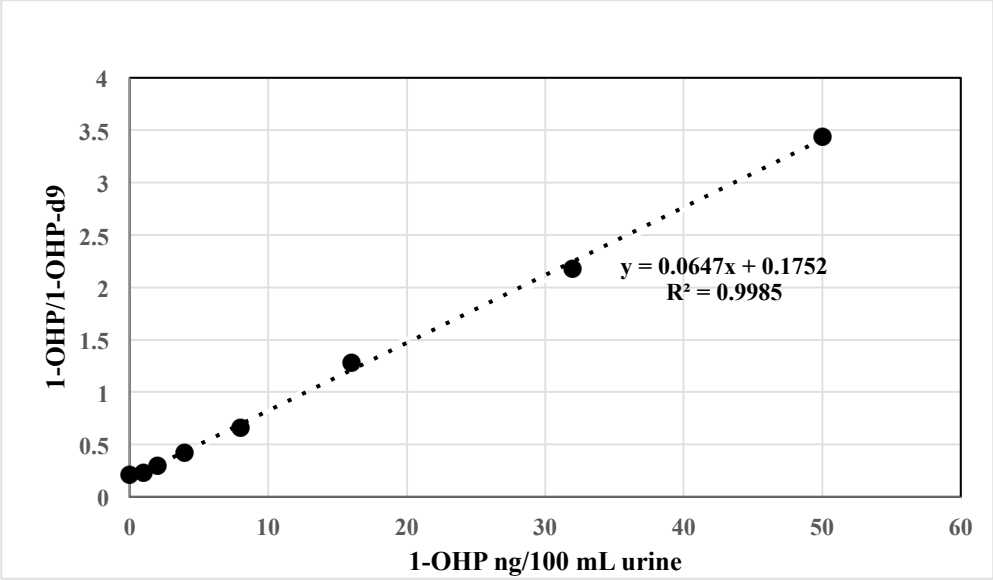


Figure 4. Calibration curve for calculation of 1-OHP concentration in urine samples.

1-OHP was detected in 88% of the samples at a limit of detection (LOD) of 0.01 ng/mL urine. Individual concentration values were normalized by respective creatinine concentrations (mg/dL) to determine the final urinary concentration of 1-OHP (ng/mg crt.). Concentration values are shown in Table 3. Values were in range of those in previously published studies.[6]

Table 3. Urinary concentrations of 1-OHP in study population.

1-OHP	Mean ± standard deviation, median (range)
ng/mL	0.079 ± 0.073, 0.054 (0.012-0.398)
ng/mg crt.	0.310 ± 0.395, 0.213 (0.036-2.082)

Chromatograms

Chromatograms for LC-MS/MS analysis of 1-OHP in blank and participant urine samples are given in Figures 5 and 6, respectively. Isotopic mass transitions for 1-OHP and 1-OHP-d_9 are shown on the graphs.

It should be noted that in a preliminary concentration analysis, the internal standard spiked prior to urine extraction was unable to be detected at our LOD. Extracted urine samples were therefore respiked with a higher concentration of 1-OHP-d_9, which was detected in 88% of the samples at the LOD. This method could lead to inaccuracy of the 1-OHP to 1-OHP-d_9 ratio, as the internal standard did not undergo the same work up procedure. In future applications of this method, it is suggested to spike the internal standard prior to sample incubation with blue rayon.

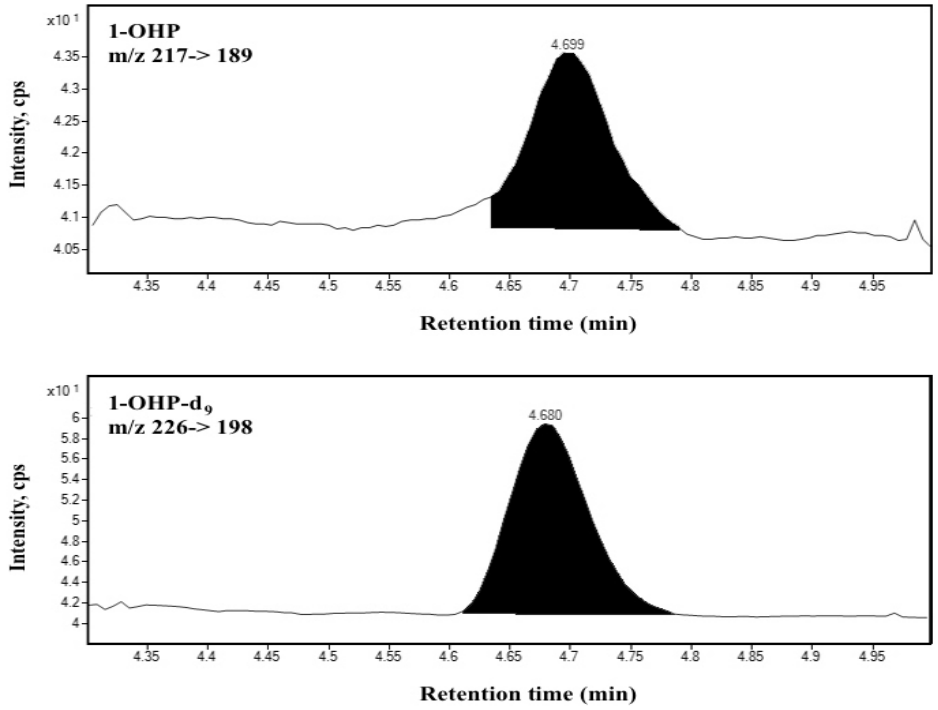


Figure 5. Chromatograms for 1-OHP and 1-OHP-d_9 spiked in a blank urine sample.

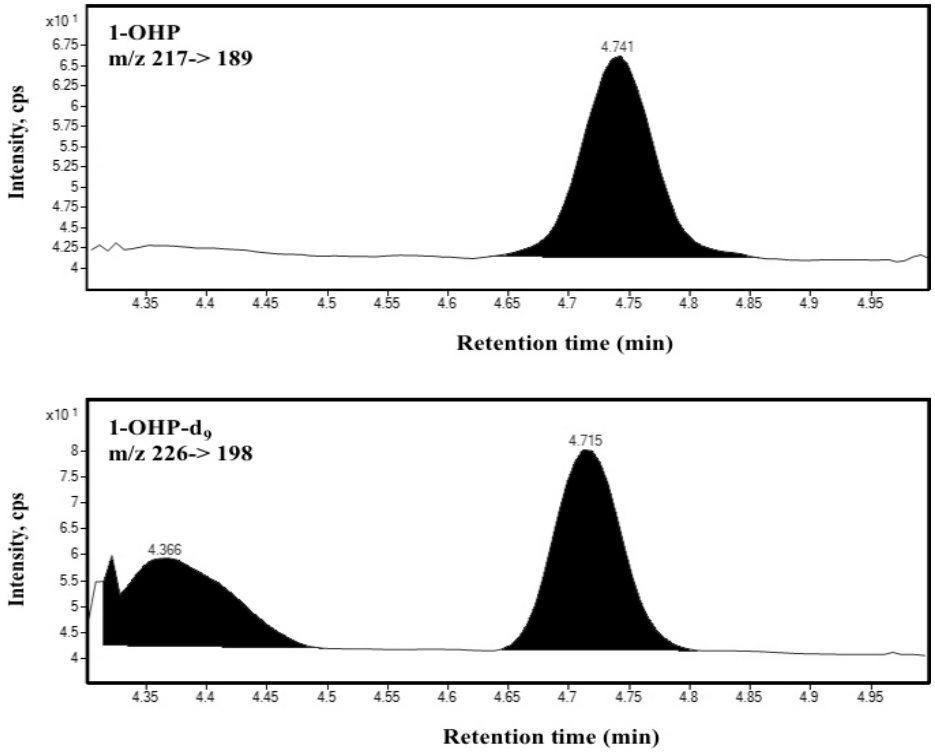


Figure 6. Chromatograms for 1-OHP and 1-OHP-d_9 in participant urine sample.

CHAPTER IV

CONCLUSION

Results illustrate that the majority of the study population had a measurable exposure to PAHs. 1-OHP levels measured in this population of non-smoking women were similar to those previously recorded for non-smoking individuals in numerous countries. For instance, the median 1-OHP level measured in non-smokers at baseline in our study population (0.054 ng/mL) is similar to 1-OHP concentrations measured in non-smokers from the U.S. (0.0505 ng/mL).[6] The relatively low levels of urinary 1-OHP measured in study participants suggest that inhabitants are exposed to background doses of PAHs likely from traffic or the indoor preparation of foods or ingestion of grilled or smoked foods.

In summary, the proposed method has sufficient sensibility for quantification of 1-hydroxypyrene in urine samples. This method and its results will be applied to other aspects of the ongoing population study, such as concentration of nitrated pyrene metabolites in urine samples as a benchmark of traffic related air pollution exposure.

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