**Distribution Agreement**

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Kanstantsin (Kostya) Kartavenka Date

# Development of Liquid Chromatographic Methods for Quantification of Malondialdehyde, 8-Oxo-2'-deoxyguanosine, and Hydroxylated Metabolites of Polycyclic Aromatic Hydrocarbons in Urine

By
Kanstantsin Kartavenka
Doctor of Philosophy
Environmental Health Sciences

_____
P. Barry Ryan, Ph.D.
Advisor

_____
Dana Boyd Barr, Ph.D.
Committee Member

_____
Jeremy Sarnat, ScD
Committee Member

_____
Parinya Panuwet, PhD
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

# Development of Liquid Chromatographic Methods for Quantification of Malondialdehyde, 8-Oxo-2'-deoxyguanosine, and Hydroxylated Metabolites of Polycyclic Aromatic Hydrocarbons in Urine

By

Kanstantsin (Kostya) Kartavenka
Specialist Degree, Belarusian State University, 2013

Advisor: P. Barry Ryan, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy
in Environmental Health Sciences
2018

## Abstract

Development of Liquid Chromatographic Methods for Quantification of Malondialdehyde, 8-Oxo-2'-deoxyguanosine, and Hydroxylated Metabolites of Polycyclic Aromatic Hydrocarbons in Urine

By Kanstantsin (Kostya) Kartavenka

### Introduction

Household air pollution as a result of use of biomass burning for cooking, heating and/or lightning was shown to be associated with numerous adverse health outcomes, including cancer and respiratory disorders, leading to 2.8 million deaths in 2015 worldwide. Despite global awareness, one third of the world population is still relying on biomass, predominantly from low- and middle-developed countries (LMDC). The incomplete combustion of this fuel results in a complex mixture, including polycyclic aromatic hydrocarbons (PAHs). Internal exposure dose to biomass burning is commonly assessed by measuring PAHs metabolites (OH-PAHs) in urine (biomarkers of exposure); however, no single precise biomarker was found for biomass burning exposure. Due to metabolism of PAHs it was suggested to use additionally oxidative stress biomarkers such as malondialdehyde (MDA) and a biomarker of oxidative DNA damage 8-Oxo-2'-deoxyguanosine (8-Oxo-dG) (biomarkers of effect). Most of existing methods are using either non-specific ultraviolet/visible or fluorescence detectors, or highly expensive tandem quadrupole mass detectors (MS/MS). Additionally, to characterize these three group of compounds, commonly three different methods are used. The complication of such analysis prevents the ability of LMDC to understand better exposure and quantify biomarkers of exposure and effect inside their countries. To achieve improvements in analytical capacities, I used liquid chromatographic (LC) system coupled with single quadrupole mass spectrometry (MS) (Aim 1, 2, 3) and due to limitation LC MS, I used LC MS/MS system to allow simultaneous analysis of all three groups of compounds (Aim 4).

### Methods

While majority of my work is dedicated to urinary measurements, initial method development started with exhaled breath (EBC) condensate with quantification of MDA using LC MS. To validate method applicability and robustness, 205 individual EBC samples were analyzed. Further analysis of MDA in urine samples using LC MS took into the account limitation that were observed in the Aim 1. To validate method for Aim 2, I used two set of pulled urine samples, pooled serum sample and unknown 138 urine samples given by Chaing Mai university. Kinetics of the reaction, autosampler storage stability were assessed. The method was tested with and without solid phase extraction. For Aim 3, we randomly selected sample previously analyzed as a part of cardiopulmonary outcomes and household air pollution trial. For validation of method for Aim 4, where OH-PAHs, MDA, and 8-Oxo-dG were quantified, urine samples collected from pregnant agricultural works in Northern Thailand in 2012 was used.

### Results

In Aim 1, we developed a sensitive analytical method with established reagent previously used in low-resolution, fluorescence methods. We adopted the method to mass spectrometry approach

which required advanced chromatography to achieve low limit of detection necessary for the specific matrix. In Aim 2, we take limitations from Aim 1 into the account, and developed a new approach to quantify MDA in urine and serum samples focusing on automatization of the protocol. In Aim 3, we developed a sensitive method that is capable for quantification of OH-PAHs in urine, which potentially allow low- and middle-income countries to understand exposure related to household air pollution widely presented there. For the Aim 4, the first analytical method for simultaneous quantification of MDA, 8-Oxo-dG, and OH-PAHs was developed.

## Conclusions

Overall, the present work describes a novel approach of using cost-effective single quadrupole mass spectrometry to quantify biomarkers of exposure and effect in urine samples. We showed that several compartments of the analytical instrument can be used more effectively to minimize cost of analysis. We demonstrated advanced application of sample preparation techniques including re-using of solid-phase extraction cartridges and combining analytes of different properties into one single analytical method via derivatization.

# Development of Liquid Chromatographic Methods for Quantification of Malondialdehyde, 8-Oxo-2'-deoxyguanosine, and Hydroxylated Metabolites of Polycyclic Aromatic Hydrocarbons in Urine

By

Kanstantsin (Kostya) Kartavenka
Specialist Degree, Belarusian State University, 2013

Advisor: P. Barry Ryan, Ph.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy
in Environmental Health Sciences
2018

# Acknowledgement

Firstly, I would like to thank my advisor Dr. P. Barry Ryan for taking the risks of inviting me for a PhD journey. I am deeply thankful for the research insights, mentorship, tips and enormous amount of conversations (scientific and not) we had throughout more than 5 years. I am also extremely thankful to Prinn Panuwet, the director of LEADER, for hot scientific conversations leading research and understanding into a reasonable direction. My research will never be completed without inputs, financial, scientific, and moral, from my two other committee members: Dr. Dana Boyd Barr and Dr. Jeremy Sarnat.

Secondly, the accomplished work will be hard to imagine without support from LEADER current and former staff (including Amanda, Cierra, Estefani, Erin, Savannah, Sierra, Priya, Perry, Tori) as well as people working at Bostik lab, specifically Nika and Rami. My friends and EHS students (Artyom, Aimee, Chloe, Debbie, Donghai, Jingxuan, King, Molly, Nick, Rong, Wenlu, Wolfgang, Yvonne) helped me in living and understanding the USA as well as support me during this journey.

Finally, I would like to thank my family, my parents, brother, grandmother, and my wife, Olya, for a great support, care, understanding and dealing with me through interesting and rough years of my PhD life.

# Table of Contents

# INTRODUCTION

Solid fuels (SF), often referred to as biomass burning, which include wood, branches, dung, coal and agricultural residues, are widely used in around the world as a means of food preparation, lighting, and heating. Despite growing general recognition of adverse health effect associated with biomass burning at household facilities (Bruce et al., 2015; Nagel et al., 2016; K. R. Smith et al., 2014; Steenland et al., 2018), approximately third of the world still rely on traditional solid biomass for food preparation (*Energy Access Outlook: From Poverty to Prosperity*, 2017). When biomass is burned using inefficient stoves significant amount of pollutants are released inside houses, commonly referred as household air pollution (K. R. Smith, Mehta, & Maeusezahl-Feuz, 2004). Due to the relative persistent of some components of HAP (e.g. polycyclic aromatic hydrocarbons (PAHs)), HAP poses a global threat. For example, PAHs were characterized in remote areas such as Arctic and North Pacific regions. Analysis of isomer ratios suggest biomass and coal burning suggesting long-distance transportation of HAP components from their potential sources (Ding et al., 2007). HAP was reported to be characterized as a single most important environmental health risk factor worldwide (Cheng et al., 2016; K. R. Smith et al., 2014) responsible for 2.8 million deaths in 2015 (Landrigan, 2017) and 85.6 million disability-adjusted life-years (Cohen et al., 2017). In addition, HAP contributes to ambient air pollution which was linked to additional 0.5 million deaths in 2010 (Bruce et al., 2015).

Exposure to HAP was reported to be associated with several respiratory responses, specifically acute lower respiratory tract infection (ALRI) in adults (Jary et al., 2016) and children (Dherani et al., 2008), chronic obstructive pulmonary disease (Assad, Balmes, Mehta, Cheema, & Sood, 2015), chronic bronchitis (Po, FitzGerald, & Carlsten, 2011), lung cancer (Raspanti et al., 2016; K. R. Smith et al., 2014), and upper aero-digestive cancers (Josyula et al., 2015). Non-respiratory responses include cataracts (K. R. Smith et al., 2014), cardiovascular disease (Fatmi & Coggon, 2016; K. H. Kim, Jahan, & Kabir, 2011), low birth weight and infant mortality (Epstein et al., 2013; Vyas, Vaghani, & Studies, 2018).

The incomplete combustion of SF leads to a mixture of components including, but not limited to, volatile organic compounds, fine particles (PM), carbonyls, carbon monoxide (CO), nitrogen oxides, formaldehyde, PAHs (Cheng et al., 2016; Humans, 2010; Kabir & Kim, 2011). Though exposure can be assessed via monitoring of household facilities, e.g. measuring products such as CO, PM, a more precise way of estimating exposure can be gained via measuring biomarkers of either exposure, or susceptibility, or effect. To our knowledge, there is no single, precise biomarker available for HAP; however, at a high exposure levels a single biomarker can be used, at more modest levels, a better exposure may be assessed via measuring multiple biomarkers (Rylance et al., 2013). One established technique to measure exposure to biomass burning is via measuring PAHs and their metabolites in human biological samples (Abdel-Shafy & Mansour, 2016; Rylance et al., 2013). PAHs can be defined as a class of compounds made of carbon and hydrogen having at least two fused benzene rings. Exposure to this group of compounds has been shown to be associated with breathing problems, inflammation, kidney and liver damages, immune suppression as well as carcinogenicity (Abdel-Shafy & Mansour, 2016; Rengarajan et al., 2015). Lipophilic properties of PAHs facilitate absorption via multiple routes of exposure such as inhalation, ingestion, and via dermal contact, predominantly distributing to organs rich in adipose tissue (Lawal, 2017).

Upon entering the organism, multiple enzymes are involved in the metabolism of PAHs. Detoxification of PAHs starts with oxidation to epoxides following enzymatic glucuronidation or sulfonation which can be characterized as true-detoxification pathways (Figure 1). On the other hand, hydroxylation may take place to form monohydroxylated (OH-PAHs) and polyhydroxylated PAHs, which can covalently bind to biologically important molecules like proteins and nucleic acids (Choi, Harrison, Komulainen, & Saborit, 2010; Ramesh et al., 2004; Xue & Warshawsky, 2005).

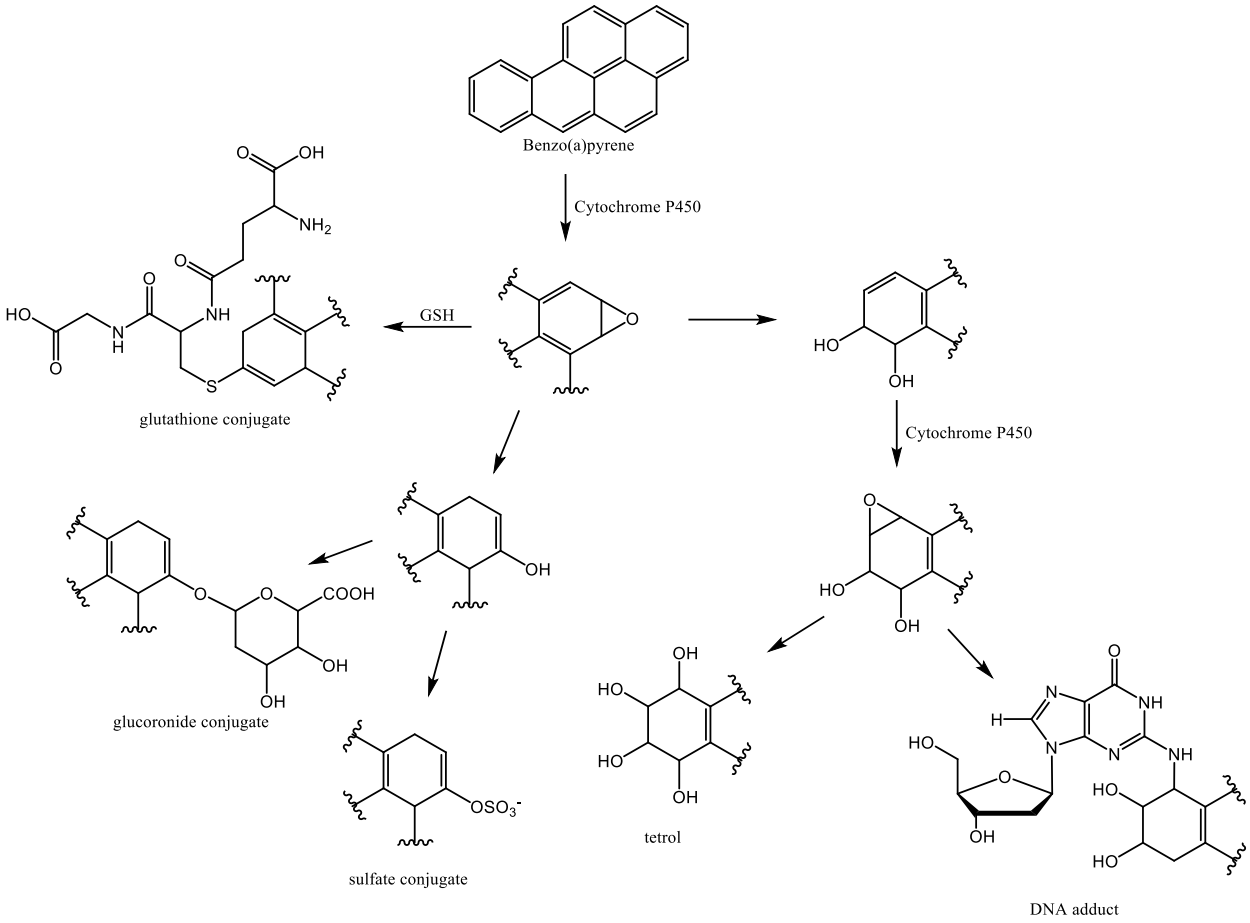



**Figure 1**. Detoxification and adducts formation (with 8-hydroxy-2′-deoxyguanosine) of polycyclic aromatic hydrocarbons (PAHs) using benzo(a)pyrene as example and one of the most extensively studied PAH (Baird, Hooven, & Mahadevan, 2005; Xue & Warshawsky, 2005)

In addition to direct binding to DNA, it was reported that detoxification mechanism also involves PAHs in redox cycle with oxygen generating reactive oxygen species (ROS) which are able to attack DNA (Figure 2) (Delfino, Staimer, Vaziri, & Health, 2011; Xue & Warshawsky, 2005). Therefore, the understanding PAHs intermediates are crucial to elucidating the fundamental toxicity of PAHs (Choi et al., 2010). As a result of increased ROS levels, it would be expected to monitor elevated levels of biomarker of oxidative stress in lipids such as malondialdehyde (MDA). For example, urinary levels of 1-hydroxypyrene (metabolite of pyrene) was associated with increased levels of MDA among kitchen

workers (C-H Pan, Chan, Huang, & Wu, 2008). Positive correlation between MDA and OH-PAHs was also reported among rural population from the North China Plain (Q. Yang et al., 2015).

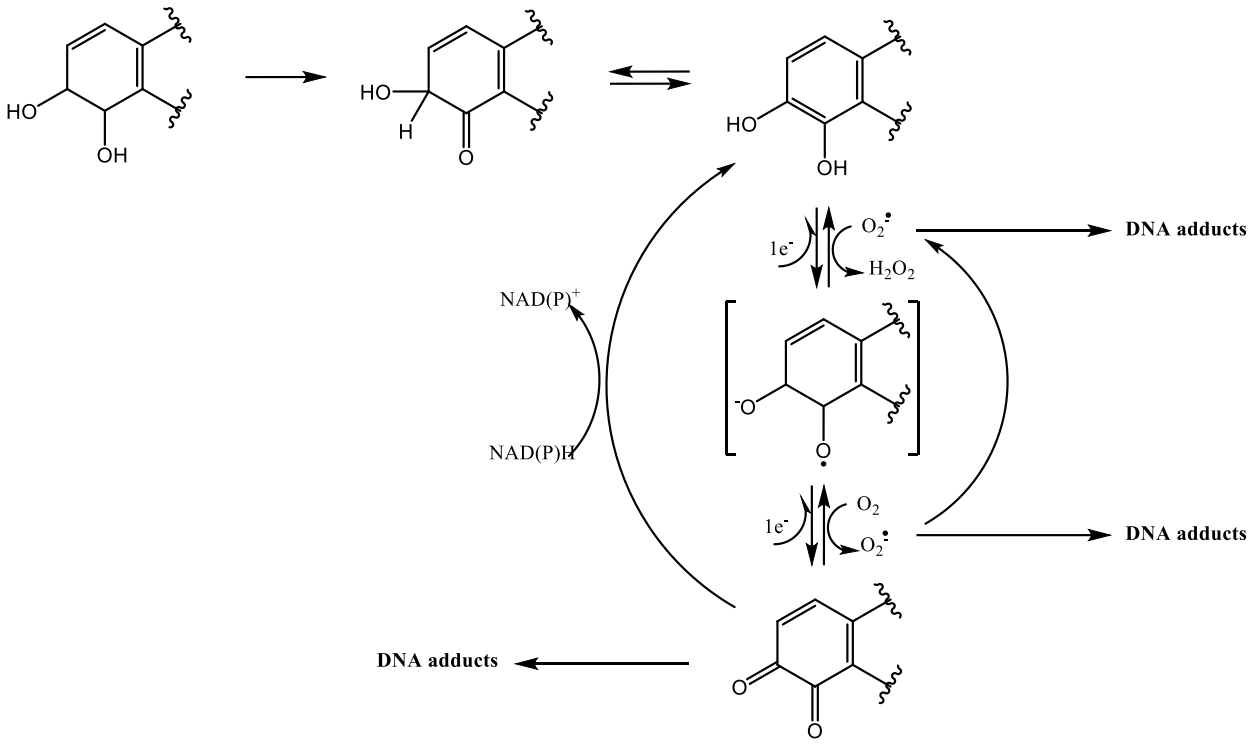



**Figure 2**. Formation of reactive oxygen species and DNA adducts (Xue & Warshawsky, 2005).

Another additional, common, and abundant biomarker of oxidative damage to DNA is 8-Oxo-2'-deoxyguanosine (8-Oxo-dG) (Valavanidis, Vlachogianni, & Fiotakis, 2009). It is formed as a result of interaction of hydroxyl radical with the nucleobase of the DNA strand, leading to the formation of multiple products including 8-hydroxy-2′-deoxyguanosine, which can be consequently transformed into 8-Oxo-dG (Figure 3). In a meta-analysis drawing upon data from 52 studies, it was concluded that exposure to combustion particles is consistently associated with elevated levels of both lipid peroxidation products (including MDA) and oxidatively damaged DNA products (including 8-Oxo-dG) in urine, plasma, EBC, and human blood cells, confirming both lipid peroxidation and oxidative DNA damage/carcinogenesis (Møller & Loft, 2010).

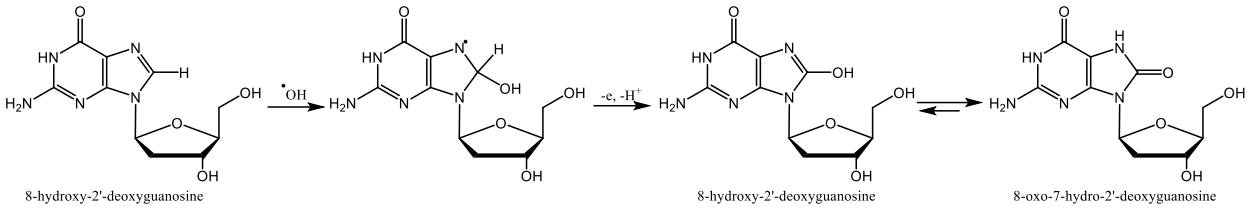



**Figure 3.** The formation of 8-Oxo-2'-deoxyguanosine as a result of oxidative damage to DNA, selected pathway (Valavanidis et al., 2009).

The importance of analysis of MDA, 8-Oxo-dG, and OH-PAHs dictates the demand for methods. Thus, majority of methods to quantify MDA in urine or exhaled breath condensate (EBC) and urine requires derivatization to enhance chromatographic separation, fluorescence or ionization properties of the adduct (M. Giera, H. Lingeman, & W. M. A. J. C. Niessen, 2012b; Khoubnasabjafari, Ansarin, & Jouyban, 2016). Despite known overestimation and high intra-laboratory variability of measured MDA due to harsh reaction conditions (95 ºC for 60 minutes, acidic pH (Ohkawa, Ohishi, & Yagi, 1979)) and associated artificial release of MDA, one of the most common derivatizing reagent is still thiobarbituric acid (Khoubnasabjafari et al., 2016). To avoid this issue, several derivatizing agents were proposed, including 2,4-dinitrophenyl hydrazine (Del Rio, Stewart, & Pellegrini, 2005; Khoubnasabjafari et al., 2016) and dansyl hydrazide (Lord, Rosenfeld, Volovich, Kumbhare, & Parkinson, 2009). Nevertheless, only a few methods were reported to quantify MDA in EBC; and to our knowledge no method was reported to quantify MDA in EBC or urine using liquid chromatography single quadrupole mass spectrometry (LC MS). That motivates us to develop isotope dilution LC MS methods for quantification of MDA in EBC and urine samples (**Aim 1 and 2**). Quantification of OH-PAHs is commonly accomplished using liquid chromatography (LC) either via low-selectivity LC coupled with fluorescence (Flu) detector or via highly selective and sensitive LC coupled with tandem quadrupole mass spectrometry (MS/MS). While exposure to HAP occurs in low- and middle-income countries, their ability to understand better exposure and quantify OH-PAHs are limited by the cost associated with established LC MS/MS methods. Typical LC single quadrupole MS is more affordable compared to LC MS/MS (1/2 to 1/3 of LC MS/MS cost), which motivates us to develop a method for that instrument (**Aim 3**). The absence of a single biomarker that can

characterize exposure to HAP or ambient air pollution leads to analysis of multiple biomarkers, such as 8-Oxo-dG, MDA, and OH-PAHs in the same sample. Usually this analysis is performed via executing three different methods, which commonly require three different purification and concentration procedures therefore significantly increasing cost of analysis (Rylance et al., 2013; Wu et al., 2017). The first and to our knowledge the only successfully published approach to analyze both 8-Oxo-dG and OH-PAHs was performed by Fan *et al*. (Fan, Wang, Ramage, & She, 2012) which found its further application in further exposure studies on association of air pollution and oxidative damage (J. Li, Fan, et al., 2015; J. Li, Lu, et al., 2015; Lu et al., 2016), however analysis of MDA was performed with a separate analytical technique. Our goal was to incorporate 8-Oxo-dG in the previously developed method (Aim 3) to be able to analyze biomarkers of effect (oxidative stress) and biomarkers of exposure (OH-PAHs) using one single method (**Aim 4**).

## DISSERTATION AIMS

**Overarching Aim**: Improve analytical methods for quantification of biomarkers of exposure and effect in biological samples. Ensure analytical capacities for countries with a scarcity of financial resources.

**Aim 1**: Quantification of malondialdehyde in exhaled breath condensate using pseudo two-dimensional ultra- performance liquid chromatography coupled with single quadrupole mass spectrometry.

**Aim 2**: Quantification of urinary malondialdehyde using ultra-performance liquid chromatography coupled with single quadrupole mass spectrometry.

**Aim 3**: Quantification of metabolites of polycyclic aromatic hydrocarbon (OH-PAH) in urine by ultra-performance liquid chromatography single quadrupole mass spectrometry.

**Aim 4**: Simultaneous quantification of biomarkers of exposure (metabolites of polycyclic aromatic hydrocarbons) and biomarker of effect (malondialdehyde and 8-Oxo-2'-deoxyguanosine) in urine using liquid chromatography tandem mass spectrometry.

# CHAPTER 1

**Quantification of malondialdehyde in exhaled breath condensate using pseudo two-dimensional ultra-performance liquid chromatography coupled with single quadrupole mass spectrometry**

Kostya Katavenka[1], Parinya Panuwet[2], Roby Greenwald[3], Karen H Ehret[1], Priya Esilda D'Souza[2], Dana Boyd Barr[1,2] and P. Barry Ryan[1,2]

1 Department of Environmental Health, Rollins School of Public Health, Emory University

2 Laboratory of Exposure Assessment and Development for Environmental Research (LEADER), Rollins School of Public Health, Emory University

3 Department of Environmental Health, School of Public Health, Georgia State University

## ABSTRACT

We developed a robust analytical method for quantification of malondialdehyde (MDA) in exhaled breath condensate via derivatization with 2,4-dinitrophenylhydrazine (DNPH). The target MDA-DNPH hydrazone was separated by ultra-performance liquid chromatography using two reversed-phase analytical columns ($C_{18}$ and phenyl-hexyl) inter-connected via a two-position, six-port switching valve to a single-quadrupole mass spectrometer. The target derivative was analyzed under positive electrospray ionization using single ion monitoring mode (m/z = 235 for the target derivative, and m/z = 237 for its labeled isotopic analog). This pseudo two-dimensional chromatographic separation provided optimum separation conditions for the target derivative resulting in the method's limit of detection (LOD) of 0.58 nM (or 36.2 pmol on-column), which is comparable to those reported previously using different techniques including tandem mass spectrometry. The method had a linear quantification range of 1.0 – 200 nM. The method showed good relative recoveries (92.2 – 102.0 %) and acceptable precisions (3.6 – 12.2 % for inter-day precision, and 4.3 – 12.4 % for intra-day precision for two quality control levels, 5 nM and 25 nM). The derivative was found to be stable at room temperature for 48 hours or during analysis. The method was used to analyze 205 exhaled breath condensate samples collected from individuals from the healthy population of student athletes. MDA was detected in approximately 95% of these samples with concentrations ranging from 1.16-149.63 nM. These data demonstrate that our method can be successfully used to measure MDA in population studies.

## KEYWORDS

Malondialdehyde, UPLC-MS, derivatization, exhaled breath condensate, pseudo two-dimensional chromatography

## INTRODUCTION

Malondialdehyde (MDA) is perhaps the most extensively studied end-product of polyunsaturated-lipid peroxidation under radical-induced oxidative stress conditions (Ayala et al., 2014a; Bartoli et al., 2011a; Del Rio et al., 2005; Gong, Zhu, et al., 2013b; Grob, Aytekin, & Dweik, 2008; Il'yasova, Scarbrough, & Spasojevic, 2012; Khoubnasabjafari, Ansarin, & Jouyban, 2015; Rahman & Kelly, 2003). MDA has been linked to oxidative stress-related health problems including chronic obstructive pulmonary disease (COPD), asthma, and cardiovascular diseases (Bartoli et al., 2011a; Khoubnasabjafari et al., 2016; Liang, Yeligar, & Brown, 2012). For example, increased levels of MDA in exhaled breath condensate (EBC), condensed exhalate from airway lining fluid, were observed in patients with asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, and cystic fibrosis compared to healthy non-smokers (Ahmadzai et al., 2013; Bartoli et al., 2011a; Liang et al., 2012). Concentrations of MDA in EBC and urine were shown to be correlated with air pollution levels (Gong, Zhu, et al., 2013b; Pelletier et al., 2017), while increased levels of MDA in EBC were associated with exposures to aerosols of nano-iron oxide (Pelclova et al., 2016) and nano-titanium dioxide (Pelclova et al., 2017) among workers. MDA concentrations were also associated with changes in lungs function and inflammatory markers of individuals exposed to traffic related pollutions (Romieu et al., 2008). In addition, treatment of asthma patients with anti-inflammation therapy revealed a decrease in MDA levels in EBC to those that are similar to non-asthmatics control subjects suggesting a direct link between MDA levels and oxidative stress (Massimo Corradi, Folesani, et al., 2003).

The majority of previous MDA measurement methods required binding of MDA with a derivatizing agent to facilitate liquid chromatographic separation and/or to allow detection using ultraviolet/visible absorption or fluorescence techniques because of the physicochemical properties of MDA (e.g., high reactivity, absence of fluorescence, and low molecular weight) (M. Giera, H. Lingeman, & W. M. A. Niessen, 2012a; Khoubnasabjafari et al., 2016). Thiobarbituric acid has been extensively used in the

analysis of MDA as a principal derivatizing agent (Khoubnasabjafari et al., 2016). However, the conditions of this derivatization are relatively harsh, resulting in an induction of lipid peroxidation that artificially releases more MDA into the sample's solution. Such processes contribute to overestimation of MDA levels, and therefore, complicates the interpretation and cross-validation of the results (Giera et al., 2012a; Khoubnasabjafari et al., 2016). To avoid this issue, other derivatizing agents requiring milder conditions have been introduced. These included 2,4-dinitrophenylhydrazine (DNPH) (Del Rio et al., 2005; Khoubnasabjafari et al., 2016), diaminonaphthalene, hydralazine (Rezaei, Jamshidzadeh, & Sanati, 2013), and dansylhydrazide (Lord et al., 2009). Yet, when derivatizing MDA with these derivatizing agents, it has often resulted in poor selectivity and sensitivity particularly with spectrophotometric and/or spectrofluorometric instruments. The limits of detection (LODs) of these methods were reported to be as high as 2.1 µM (Giera et al., 2012a).

To achieve desired the sensitivity and appropriate LODs for the analysis of trace amounts of endogenous MDA, use of highly sensitive and selective instruments such as tandem mass spectrometers have been preferred (Syslová et al., 2009). With tandem mass spectrometers, quantification of MDA could be conducted without derivatization (Syslová et al., 2009), nonetheless, derivatization is still commonly used. Chen *et al., 2011* quantified urinary MDA-DNPH derivative using liquid chromatography-tandem mass spectrometer, but noted a lack of reproducibility of their method due to the solid phase extraction approach (Chen, Huang, Pan, Hu, & Chao, 2011). Also, methods based on derivatization of MDA with pentafluorobenzyl bromide were proposed for quantification using gas chromatography coupled with single and tandem mass spectrometers with reported LODs as low as 2 amol (Tsikas et al., 2016). Only a few methods have been reported that analyze MDA in EBC.

Although tandem mass spectrometers are suitable for analysis of MDA in various biological matrices, lack of instrument availability, financial resources and/or operating expertise may limit their use, especially in low- and middle-income countries. This, in turn, makes the biological analysis of MDA at

low levels rather difficult. Therefore, we aimed to develop an alternative method using a single quadrupole mass spectrometer to analyze MDA in EBC samples. Although this instrument alone may not offer parallel selectivity to the tandem mass spectrometers, it surpasses the capabilities of ultraviolet/visible absorption or fluorescence instruments using an instrument much less expensive than a tandem mass spectrometer. To improve the sensitivity and selectivity of the single-quadrupole mass spectrometer, we derivatized MDA with DNPH and separated this derivative using pseudo two-dimensional liquid chromatography combining phenyl-hexyl and $C_{18}$ analytical columns via an existing two-position/six-port (2P/6P) valve of the mass spectrometer. To control for potential matrix effects (Panuwet et al., 2016), isotope dilution quantification was used. The method was validated using pooled EBC samples and tested for its suitability by analyzing 205 individual human EBC samples.

## MATERIAL AND METHODS

### Chemicals and reagents

We obtained acetonitrile (HPLC grade), formic acid, 88% (laboratory grade) and ethanol, 200 proof from Fisher Chemical (Waltham, MA, USA). Hydrochloric acid, 30% (for ultratrace analysis), malondialdehyde tetrabutylammonium salt, 96% (neat), 2,4-dinitrophenylhydrazine, 97% (reagent grade), and 3,5-Di-tert-4-butylhydroxytoluene (BHT), analytical standard were obtained from Sigma-Aldrich (Saint Louis, MO, USA). The internal standard, 1,1,3,3-Tetraethoxypropane-1,3,-$D_2$ ($D_2$-TEP) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Water was purified using an EMD Millipore Milli-Q Ultrapure water purification system (Billerica, MA, USA).

### Preparation of standard, quality control, and labeled internal standard solutions

High concentration standard stock and quality control solutions were prepared by weighing a known amount of neat standard then diluting with acetonitrile (Stock I solution). This stock solution was used to prepare an 800 nM stock solution in water weekly (Stock II solution). The stock I and II solutions were stored at -20 °C. Eight-point calibration solutions were prepared daily from corresponding stock II

solution in Milli-Q® water. The concentrations of the calibration solutions were as follows (1.0, 2.5, 5.0, 10, 25, 50, 100, 200 nM). Quality control solutions (prepared at concentrations of 5.0 and 25 nM) were prepared separately in Milli-Q® water from the corresponding stock II solution. Labeled internal standard (IS) stock solution was prepared by hydrolysis of $D_2$-TEP standard according to a previously published protocol (Pilz, Meineke, & Gleiter, 2000). This labeled internal standard (IS) stock solution was diluted with Milli-Q® water to yield a concentration of 250 nM.

**Preparation of blank, calibration, quality control, and unknown samples**

EBC samples were preserved with an anti-oxidizing agent, butylated hydroxytoluene (BHT), prepared via dilution of 25 mg of BHT in 10 mL ethanol. Sample vials were pre-loaded with 5 µL of the BHT solution, and then a 250 µL aliquot of EBC was added immediately following collection. Samples were then stored at -80 $^{o}$C prior to analysis. Each unknown sample was prepared according to the following procedure. First, 20 µL of labeled IS solution was added to a 250-µL glass vial insert what was placed inside an amber, 2.5-mL auto-sample vial. Second, 100 µL of unknown EBC sample was added to the same vial, followed by 20 µL of 10 mM DNPH in acetonitrile, and 20 µL of 1.5 M hydrochloric acid solution which was prepared in Milli-Q® water from 30% hydrochloric acid solution. Third, the vial was capped, vortex mixed, and then incubated at 37 $^{o}$C for 70 minutes.

In each analytical batch, an eight-point calibration curve, two quality control samples, a blank and 40 unknown samples were prepared altogether. For the calibration curve and quality control samples, 100 uL of the respective solutions was used *in lieu* of unknown sample. A blank sample was also prepared in a similar manner as unknown samples, except that 100 uL of Milli-Q® water was used instead. All other chemicals remained the same.

**Chromatographic separation and mass spectrometric conditions**

The sample analysis was carried out using an Agilent Infinity 1290 ultra-performance liquid chromatograph (UPLC) with a G4226A autosampler connected to an Agilent G6150B single quadrupole

mass spectrometer (MSD) (Santa Clara, CA, USA). The separation was performed on Agilent Eclipse Plus $C_{18}$ RRHD (1.8 µm 2.1x50 mm) and Agilent Eclipse Plus Phenyl-Hexyl (1.8 µm 2.1x50 mm) columns. The $C_{18}$ column temperature was set to 40 $^{o}$C, while the phenyl-hexyl column temperature was set to 25 $^{o}$C.

An injector program was used to facilitate injection of the samples. It is essential that the needle be washed with acetonitrile prior to sample draw. For analysis of an EBC sample, each of 20 µL sample volume was ejected, for five times, into the needle loop and mixed. With this procedure, a total of 100 µL of sample volume was injected into the columns. The binary mobile phase consists of A, Milli-Q® water with 0.05% v/v formic acid, and B, acetonitrile without additives. Separation was achieved via a gradient elution program (Table 1). The total program runtime was 14.5 minutes. Figure 2 illustrates the configuration of the chromatographic setup and the 2P/6P switching valve.

The target derivative including its labeled IS derivative were analyzed under a using positive electrospray ionization. The mass spectrometer was operated in single ion monitoring (SIM) mode. Capillary and nozzle voltages were set to 4000 V and 800 V, respectively. Nitrogen was used as a dry and sheath gas, their flow rates were set to 8.0 L/min and 12.0 L/min, respectively. Drying gas and sheath gas temperatures were set to 350 $^{o}$C and 360 $^{o}$C, respectively. The nebulizer pressure was set to 10 psi. The fragmentor voltage was set to 120 V, while the dwell time was set to 444 msec. The MDA-DNPH derivative was monitored at m/z of 235, while the $D_2$-MDA-DNPH derivative was monitored at m/z of 237. The peak of the target derivative was selected based on its retention time (RT), relative retention time to labeled IS peak, peak shape, and signal-to-nose ratio.

**Determination of limit of blank (LOB), LOD, selection lowest calibration point**

LOB and LOD were assessed based on a method described by Armbruster and Pry (Armbruster & Pry, 2008). Briefly, the LOB was estimated through the measurement of five replicates of blank samples on five different days (total of 25 samples). The LOB was calculated using the equation (1) below.

$$LOB = Mean_{Blank} + 1.645 \times SD_{Blank} \quad (1)$$

To calculate the LOD, a set of non-replicative measurements of the lowest calibration level (1.0 nM) from 17 days was used. Standard deviation was calculated from this set of data. The LOD was then calculated according to the equation (2) below.

$$LOD = LOB + 1.645 \times SD_{Cal1} \quad (2)$$

**Method validation**

The method precision was determined in according to protocol described by Chesher (Chesher, 2008) using two quality control levels (QCL and QCH). Replicate samples (5 samples/day at each concentration level) were prepared and analyzed daily during a 5-day period to determine inter-day and intra-day precision. The precisions were expressed as relative standard deviation (%RSD) values.

The method relative recoveries (RR) were determined, according to FDA guideline using a set of replicates, quality control materials (QCL and QCH) prepared at concentrations of 5.0 and 25 nM (N = 25). The method RR were calculated by dividing the average, quantified values of the quality control materials with the respective, expected concentration values for both levels. The method RR were expressed as percentage values (Health & Services, 2001).

A study of autosampler storage stability was performed via a duplicate injection of QCL, QCH, and pooled EBC sample, every 12 hours for 48 hours after derivatization. Samples were stored in the autosampler at room temperature (approximately 22 $^{o}$C) during the intervening times.

Matrix effects were evaluated by comparing mean responses (peak area) of labeled IS presented in matrix-free samples (calibration curve and QC) and in the EBC samples, across different analytical batches. To control for and manage matrix effect, isotope dilution quantification was used (Panuwet et al., 2016).

The method was used to analyze 205 exhaled breath condensate samples to assess the suitability of the method. EBC samples were collected from 138 unique adolescent subjects who were participants in after-school sports programs and agreed to participate in the Study of Air Pollution and Physical Activity

(SAPPA). SAPPA was approved by the Institutional Review Boards of Emory and Georgia State Universities. Informed consent was given by the adult participant or a parent or guardian of the minor participants; minor participants gave assent to be in the study. The mean (SD) age of subjects was 16.6 (1.3) with a range of 14-19 years. Most participants were male (97 male and 41 female) and African American (134 black, 4 Hispanic, and none of other races or ethnicities). EBC samples were collected on-location using the R-tube® (Respiratory Research, Charlottesville, VA). Condensation was achieved using aluminum sleeves chilled with dry ice (-68°C). Participants exhaled through the R-tube® until 1 mL of condensate was produced, typically 6-10 minutes. Samples were immediately aliquoted and frozen in dry ice.

## RESULTS

### Chromatographic separation condition

The optimum chromatographic separation conditions were achieved using a pseudo two-dimensional chromatographic configuration combining Agilent Eclipse Plus $C_{18}$ RRHD and Agilent Eclipse Plus Phenyl-Hexyl analytical columns. Fig. 2 shows examples of chromatograms of the target and labeled IS peaks that were extracted from EBC, lowest calibration level (matrix-free), low-level quality control (matrix-free), and blank (matrix-free) samples.

### Limit of blank and limit of detection

The LOB for EBC samples was 0.18 nM and the LOD was 0.58 nM (36.2 pmol on-column amount). The lowest calibration level was 1.0 nM.

### Method validation

The method precisions and accuracies are summarized in Table 2. The accuracies ranged 92%-102%. The inter-day precisions ranged 4.3%-12% while the intra-day precisions were 3.6%-12%. The target and labeled IS derivatives were stable at room temperature up to 48 hours after derivatization. Figure xx shows the plots of relative response ratios (the peak area of target derivative divided by the peak area

of labeled IS derivative) obtained from replicates of QCL and pooled EBC samples analyzed between 0-48 hours post derivatization. For the matrix effect study, the result (Figure 5) shows that the average peak area responses of labeled IS derivative detected in EBC and matrix-free samples were similar.

Of 205 individual EBC samples analyzed, approximately 95% had detectible amounts of MDA at a concentration range of 1.2-150 nM. About 4% of the analyzed samples had MDA levels below the LOD. About 1% of the analyzed samples had levels higher than the LOD but lower than the lowest calibration level. For all the EBC samples that had MDA concentrations above 1.0 nM, the median was 6.82 [IQR 4.08 – 9.88] nM.

## DISCUSSION

We successfully developed a sensitive and selective method capable of quantifying levels of MDA in EBC samples collected from human subjects in an epidemiologic investigation. Our method employs ultra-high-performance liquid chromatography coupled with single quadrupole mass spectrometer (UPLC-MS). Our method is the first reported to be capable of measuring MDA using this type of instrument. While HPLC-MS instrumentation has been less used for measurement of trace levels of low molecular weight compounds in biological samples because their mass-to-charge ratios lie in the mass range of great chemical noise, single quadrupole instruments are typically 1/3 to ½ the cost of triple quadrupoles making them more accessible to low budget laboratories. Although triple quadrupole mass spectrometry is usually used to analyze these samples, we showed that the combination and optimization of separation mechanisms may solve the problem in a more financially affordable way.

Our method deployed chemical derivatization using DNPH, mainly to enable effective, reversed-phase, chromatographic separation of the target MDA derivative. The optimum separation of the derivatives against the background noise was achieved through a pseudo two-dimensional chromatographic separation condition, combining Agilent Eclipse Plus $C_{18}$ RRHD and Agilent Eclipse Plus Phenyl-Hexyl analytical columns together. This configuration was executed via the pre-existing 2P/6P-

valve located on the mass spectrometers. This valve is typically used to divert the HPLC flow to either a waste reservoir or to the nebulizer for electrospray ionization. Use of this pre-existing switching valve added no extra cost into the current instrument setup. In addition, the method does not require any extraction, further reducing any costs associated with sample preparation.

The method was validated and demonstrated satisfactory performance. The method accuracies and precisions are shown to be in compliance with the FDA guidance (Health & Services, 2001). The method has minimal matrix effects (Figure 5). The derivatives were found to be stable inside the autosampler at room temperature for at least 48 hours post derivatization (Figure 6). Minor increase in MDA level over time may result from release of bound form of MDA (Cui et al., 2018). The developed method has the LOD of 0.58 nM and is able to cover a quantification range of 1.0-200 nM. During the method development, it was found that using either a $C_{18}$ column or a Phenyl-Hexyl column alone provided poor separation of the target derivative against its background noise. As such, it contributed to the LOD that is not relevant to the biological concentrations of MDA found in EBC samples. Figure 4 shows the chromatograms of the MDA derivatives separated using each of the two analytical columns employed.

We also tested a different, pseudo two-dimensional chromatographic configuration, where a Phenyl-Hexyl column was placed before a $C_{18}$ column. This configuration failed to achieve optimum separation, even under a matrix-free condition. In addition, with this configuration, peaks of the derivatives were broad, resulting in an increased LOD and thus reducing the applicability of such approach to the analysis of typical human EBC samples (data not shown). The current order of analytical columns, however, provides a sharper peak of derivatives and lower LOD than the other order. Combining two different reversed-phase analytical columns has enabled the introduction of differential π-π interaction mechanisms that results in a better isolation of the target and labeled IS derivative from interfering compounds.

Because of the low LOD achieved from the current method setting, it is sufficient to analyze EBC samples collected from humans. As noted, the method was able to detect MDA in about 96% of the analyzed samples. According to Table 3, where a brief overview of the previous studies on MDA concentrations measured in EBC samples is provided, our method was able to produce the comparable values. Our LOD is as low as those reported from methods using a more sophisticated, and costly, instrumentation such as tandem mass spectrometry.

Chemical derivatization of MDA has its limitations. The use of strong acids or bases during derivatization can induce further release of MDA from biological components found in the samples (Cui et al., 2018). This results in a higher MDA concentration than the biologically produced concentration following endogenous lipid peroxidation processes. Because of this limitation, it is impractical to compare the results across individuals or from studies using different chemical derivatization conditions. In addition, we suggest that chemical derivatization of MDA only be used in epidemiological studies seeking to investigate the change in individual MDA production. These include any studies employing a pair-wise sample collection or longitudinal sample collection across multiple time from each individual. With such study designs, the artificial release of MDA during derivatization would be unlikely to bias the overall outcomes.

When collecting EBC samples, it is important to note the collection conditions. Some researchers recommend that the measured MDA concentrations be corrected for total EBC volume and condensation temperature to enable an accurate comparison between studies (Goldoni et al., 2005). However, these concerns are reduced when assessing an acute within-subject change in MDA concentration from EBC samples collected under identical conditions and analyzed concurrently.

Also, because the current m/z ratios of the derivatives we generate in our study still lie within the noise region of the mass spectrometer, we observe a relatively high intra-day variation of MDA concentration in blank samples. At this region, the sensitivity of the target derivatives is likely

suppressed, resulting in a higher degree of signal variation across different samples. As such, the LOD for each sample will vary. If derivatizing agents with higher molecular weight are used, the m/z ratios of the derivatives will shift to a higher mass region that is known to have less chemical noise likely increasing the sensitivity of the method. Thus, other potential derivatizing reagents should be tested in future studies.

## CONCLUSION

We developed a sensitive, inexpensive, pseudo two-dimensional UPLC analytical method that is capable of quantifying biological concentrations of endogenous MDA in EBC samples. The developed method is validated, and its performance complies with the FDA guideline. The method was implemented on 205 unknown EBC samples, overall more than 500 injection were performed on a single configuration of columns, which confirmed method robustness and applicability to epidemiological studies. The sensitivity of the method (Table 3) is improved over fluorescence-based methods and is comparable to other mass spectrometric based-methods, including those using expensive tandem mass spectrometric technique.

## FUNDING

This work was financially supported by NIH grants 5UM1HL134590-03, P50ES026071, 83615301, P30ES019776, K25ES020355 and the Laney Graduate School of Emory University.

## References


1. Ayala, A., et al., *Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal.* Oxidative Medicine and Cellular Longevity, 2014. **2014**: p. 31.
2. Khoubnasabjafari, M., K. Ansarin, and A. Jouyban, *Reliability of malondialdehyde as a biomarker of oxidative stress in psychological disorders.* BioImpacts : BI, 2015. **5**(3): p. 123-127.
3. Del Rio, D., A.J. Stewart, and N. Pellegrini, *A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress.* Nutrition, Metabolism and Cardiovascular Diseases, 2005. **15**(4): p. 316-328.
4. Rahman, I. and F. Kelly, *ReviewBiomarkers in Breath Condensate: A promising New Non-invasive Technique in Free Radical Research.* Free Radical Research, 2003. **37**(12): p. 1253-1266.
5. Gong, J., et al., *Malondialdehyde in exhaled breath condensate and urine as a biomarker of air pollution induced oxidative stress.* Journal of exposure science & environmental epidemiology, 2013. **23**(3): p. 322-327.
6. Bartoli, M.L., et al., *Malondialdehyde in Exhaled Breath Condensate as a Marker of Oxidative Stress in Different Pulmonary Diseases.* Mediators of Inflammation, 2011. **2011**: p. 7.
7. Grob, N., M. Aytekin, and R. Dweik, *Biomarkers in exhaled breath condensate: a review of collection, processing and analysis.* Journal of breath research, 2008. **2**(3): p. 037004.
8. Il'yasova, D., P. Scarbrough, and I. Spasojevic, *Urinary biomarkers of oxidative status.* Clinica Chimica Acta, 2012. **413**(19): p. 1446-1453.
9. Khoubnasabjafari, M., K. Ansarin, and A. Jouyban, *Critical Review of Malondialdehyde Analysis in Biological Samples.* Current Pharmaceutical Analysis, 2016. **12**(1): p. 4-17.
10. Liang, Y., S.M. Yeligar, and L.A.S. Brown, *Exhaled Breath Condensate: A Promising Source for Biomarkers of Lung Disease.* The Scientific World Journal, 2012. **2012**: p. 7.
11. Ahmadzai, H., et al., *Exhaled breath condensate: a comprehensive update*, in *Clinical Chemistry and Laboratory Medicine*. 2013. p. 1343.
12. Pelletier, G., et al., *Associations between urinary biomarkers of oxidative stress and air pollutants observed in a randomized crossover exposure to steel mill emissions.* International Journal of Hygiene and Environmental Health, 2017. **220**(2, Part B): p. 387-394.
13. Pelclova, D., et al., *Oxidative stress markers are elevated in exhaled breath condensate of workers exposed to nanoparticles during iron oxide pigment production.* Journal of breath research, 2016. **10**(1): p. 016004.
14. Pelclova, D., et al., *Markers of lipid oxidative damage in the exhaled breath condensate of nano TiO2 production workers.* Nanotoxicology, 2017. **11**(1): p. 52-63.
15. Romieu, I., et al., *Exhaled breath malondialdehyde as a marker of effect of exposure to air pollution in children with asthma.* Journal of Allergy and Clinical Immunology, 2008. **121**(4): p. 903-909.e6.
16. Corradi, M., et al., *Aldehydes and Glutathione in Exhaled Breath Condensate of Children with Asthma Exacerbation.* American Journal of Respiratory and Critical Care Medicine, 2003. **167**(3): p. 395-399.
17. Giera, M., H. Lingeman, and W.M.A. Niessen, *Recent Advancements in the LC- and GC-Based Analysis of Malondialdehyde (MDA): A Brief Overview.* Chromatographia, 2012. **75**(9): p. 433-440.
18. Rezaei, Z., A. Jamshidzadeh, and E. Sanati, *A rapid and sensitive method for the determination of malondialdehyde as its hydralazine derivative in human plasma using high performance liquid chromatography.* Analytical Methods, 2013. **5**(12): p. 2995-2999.
19. Lord, H.L., et al., *Determination of malondialdehyde in human plasma by fully automated solid phase analytical derivatization.* Journal of Chromatography B, 2009. **877**(13): p. 1292-1298.

20. Syslová, K., et al., *Rapid and easy method for monitoring oxidative stress markers in body fluids of patients with asbestos or silica-induced lung diseases.* Journal of Chromatography B, 2009. **877**(24): p. 2477-2486.
21. Chen, J.-L., et al., *Determination of urinary malondialdehyde by isotope dilution LC-MS/MS with automated solid-phase extraction: A cautionary note on derivatization optimization.* Free Radical Biology and Medicine, 2011. **51**(9): p. 1823-1829.
22. Tsikas, D., et al., *Development, validation and biomedical applications of stable-isotope dilution GC–MS and GC–MS/MS techniques for circulating malondialdehyde (MDA) after pentafluorobenzyl bromide derivatization: MDA as a biomarker of oxidative stress and its relation to 15(S)-8-iso-prostaglandin F2α and nitric oxide (NO).* Journal of Chromatography B, 2016. **1019**: p. 95-111.
23. Panuwet, P., et al., *Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring.* Critical Reviews in Analytical Chemistry, 2016. **46**(2): p. 93-105.
24. Pilz, J., I. Meineke, and C.H. Gleiter, *Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative.* Journal of Chromatography B: Biomedical Sciences and Applications, 2000. **742**(2): p. 315-325.
25. Armbruster, D.A. and T. Pry, *Limit of Blank, Limit of Detection and Limit of Quantitation.* The Clinical Biochemist Reviews, 2008. **29**(Suppl 1): p. S49-S52.
26. Chesher, D., *Evaluating Assay Precision.* The Clinical Biochemist Reviews, 2008. **29**(Suppl 1): p. S23-S26.
27. Health, U.D.o. and H. Services, *Guidance for industry, bioanalytical method validation.* http://www. fda. gov/cder/guidance/index. htm, 2001.
28. Cui, X., et al., *Relationship between free and total malondialdehyde, a well-established marker of oxidative stress, in various types of human biospecimens.* Journal of Thoracic Disease, 2018. **10**(5).
29. Goldoni, M., et al., *Influence of condensation temperature on selected exhaled breath parameters.* BMC Pulmonary Medicine, 2005. **5**(1): p. 10.
30. Corradi, M., et al., *Aldehydes in Exhaled Breath Condensate of Patients with Chronic Obstructive Pulmonary Disease.* American Journal of Respiratory and Critical Care Medicine, 2003. **167**(10): p. 1380-1386.
31. Lee, J.S., et al., *Health surveillance study of workers who manufacture multi-walled carbon nanotubes.* Nanotoxicology, 2015. **9**(6): p. 802-811.
32. Roberta, A., et al., *Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry.* Rapid Communications in Mass Spectrometry, 2003. **17**(7): p. 637-645.
33. Corradi, M., et al., *Comparison between exhaled and sputum oxidative stress biomarkers in chronic airway inflammation.* European Respiratory Journal, 2004. **24**(6): p. 1011-1017.
34. Casimirri, E., et al., *Biomarkers of oxidative-stress and inflammation in exhaled breath condensate from hospital cleaners.* Biomarkers, 2016. **21**(2): p. 115-122.
35. Balazs, A., et al., *Monitoring oxidative stress during chronic obstructive pulmonary disease exacerbations using malondialdehyde.* Respirology, 2014. **19**(1): p. 74-79.
36. Gong, J., et al., *Malondialdehyde in exhaled breath condensate and urine as a biomarker of air pollution induced oxidative stress.* Journal of Exposure Science and Environmental Epidemiology, 2013. **23**(3): p. 322.

**Table 1.** Graduate elution liquid chromatography program for analysis of malondialdehyde-2,4-dinitrophenylhydrazine in exhaled breath condensate samples. Where A and B stands for binary mobile phase composition, A – Milli-Q® water with 0.05% formic acid v/v, B – 100% acetonitrile without additives.

| Time, min | B composition | Flow rate, ml/min | Valve Position | Time, min | B composition | Flow rate, ml/min | Valve Position |
|---|---|---|---|---|---|---|---|
| 0.0 | 3.0% | 0.5 | To waste | 10.7 | 100% | 0.3 | To MSD |
| 4.5 | 27.0% | 0.5 | To waste | 11.0 | 100% | 0.3 | To MSD |
| 5.0 | 29.6% | 0.4 | To waste | 11.7 | 100% | 0.5 | To MSD |
| 6.2 | 36.0% | 0.37 | To MSD | 12.9 | 100% | 0.5 | To MSD |
| 7.2 | 37.6% | 0.35 | To MSD | 13.0 | 51% | 0.5 | To MSD |
| 7.5 | 38.0% | 0.3 | To MSD | 13.1 | 3.0% | 0.4 | To MSD |
| 10.5 | 42.7% | 0.3 | To MSD | 14.0 | 3.0% | 0.4 | To waste |

**Table 2.** Relative recoveries and precisions of the method calculated from replicate samples prepared at two different concentrations.

| Expected concentration level | Relative recovery, % | Precision, % | |
|---|---|---|---|
| | | Within-day | Between-day |
| 5.0 nM | 92 | 12 | 12 |
| 25 nM | 102 | 3.6 | 4.3 |

**Table 3.** Summary of the previous studies with reported concentrations of malondialdehyde in exhaled breath condensate samples. Where APCI+ stands for atmospheric pressure chemical ionization operated in positive ionization mode, MS/MS – tandem mass spectrometer, ESI- - electrospray ionization operated in negative ionization mode, TBA - thiobarbituric acid, DPNH – 2,4-dinitrophenylhydrazine.

| LOD [nM] | Value [nM] | Derivatizing agent | Detector/Mass Analyzer | Subject characteristics for showed value | Reference |
|---|---|---|---|---|---|
| 1.07 | 19.4 ± 1.9 | DNPH | APCI+ MS/MS | Healthy non-smoking children | Corradi *et al* (Massimo Corradi, Folesani, et al., 2003) |
| 1.07 | 12.1 ± 1.8 | DNPH | APCI+ MS/MS | Healthy non-smoking children | Corradi *et al* (Massimo Corradi, Rubinstein, et al., 2003) |
| N/A | 4.11 ± 1.29 | DNPH | APCI+ MS/MS | Workers who manufacture multi-walled carbon nanotubes | Lee *et al* (Lee et al., 2015) |
| 1.00 | 11.2 (1.07-23.4) | DNPH | APCI+ MS/MS | Healthy controls | Andreoli *et al* (Roberta, Paola, Massimo, Antonio, & A., 2003) |
| 1.00 | 24 (11.8-32.6) | DNPH | APCI+ MS/MS | Chronic obstructive pulmonary disease (COPD) patients | Corradi *et al* (M. Corradi et al., 2004) |
| 0.1 | 1.57 (1.17-2.9) | DNPH | APCI+ MS/MS | Cleaners chronically exposed to chlorinated compounds | Casimirri *et al* (Casimirri et al., 2016) |
| 4.1 | 15.2 (4-79) | TBA | Fluorescence | Patients with idiopathic pulmonary fibrosis | Bartoli *et al* (Bartoli et al., 2011a) |
| 1.8 | 15.2 (12-15.5) | TBA | Fluorescence | Non-smoking adults aged 45 to 88 years old | Cui *et al* (Cui et al., 2018) |
| 10 | 96.1 ± 11.6 | TBA | Fluorescence | Acute exacerbation of COPD patients | Antus *et al* (Balazs, Gabriella, Orsolya, & Imre, 2014) |
| 1.8 | 16.0 ± 1.9 | TBA | Fluorescence | Non-smoking participants during Olympics in Beijing | Gong *et al* (Gong, Zhu, Kipen, Wang, Hu, Ohman-Strickland, Lu, Zhang, Wang, & Zhu, 2013) |
| 0.29 | N/A* | None | ESI- MS/MS | N/A | Syslova *et al* (Syslová et al., 2009) |

Note: Data are mean ± SD or median (IQRs: 25th–75th percentile).

* Due to typographical errors that we observe in result section, we exclude this study from reporting values

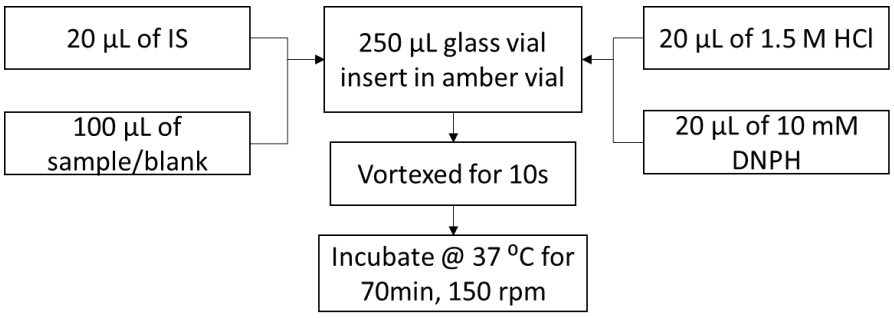



**Figure 1**. Sample preparation diagram.

Note: IS – internal standard, HCl – hydrochloric acid, DNPH – 2,4-dinitrophenylhydrazine.

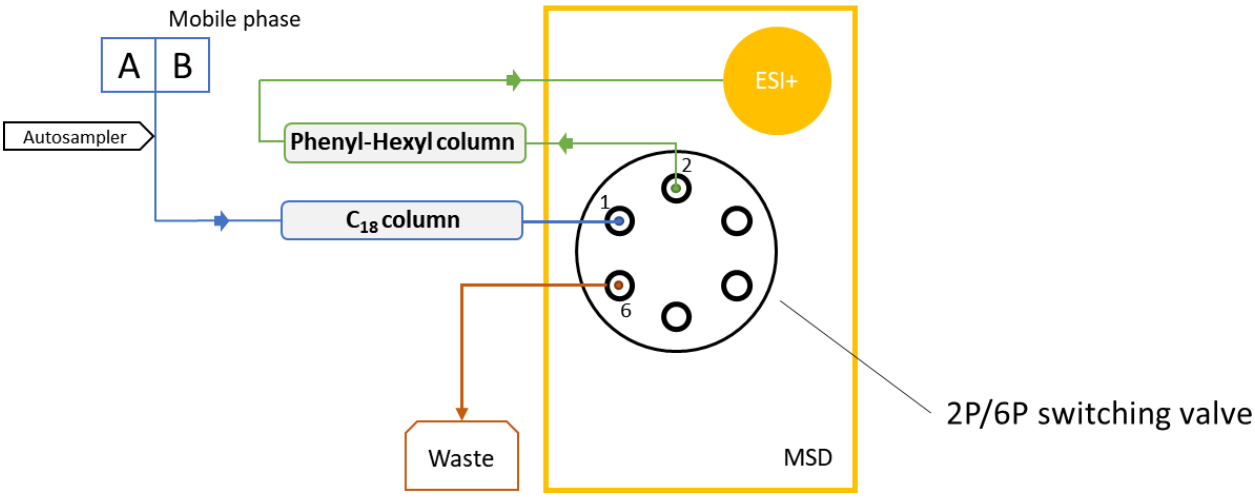



**Figure 2.** Column switching configuration using an existing 2-position/6-port (2P/6P) switching valve located on a mass spectrometer (MSD). Where A and B represent binary mobile phase composition, A – Milli-Q® water with 0.05% v/v formic acid, B – 100% acetonitrile without additives. Initial separation of malondialdehyde (MDA) from interference compounds is performed on C18 column, where 2P/6P valve is switched to waste position (1 to 6). At the approximate time of elution of MDA from C18 column, 2P/6P valve is switched to MS position (1 to 2) to facilitate separation of MDA on phenyl-hexyl column and further detection in MSD.

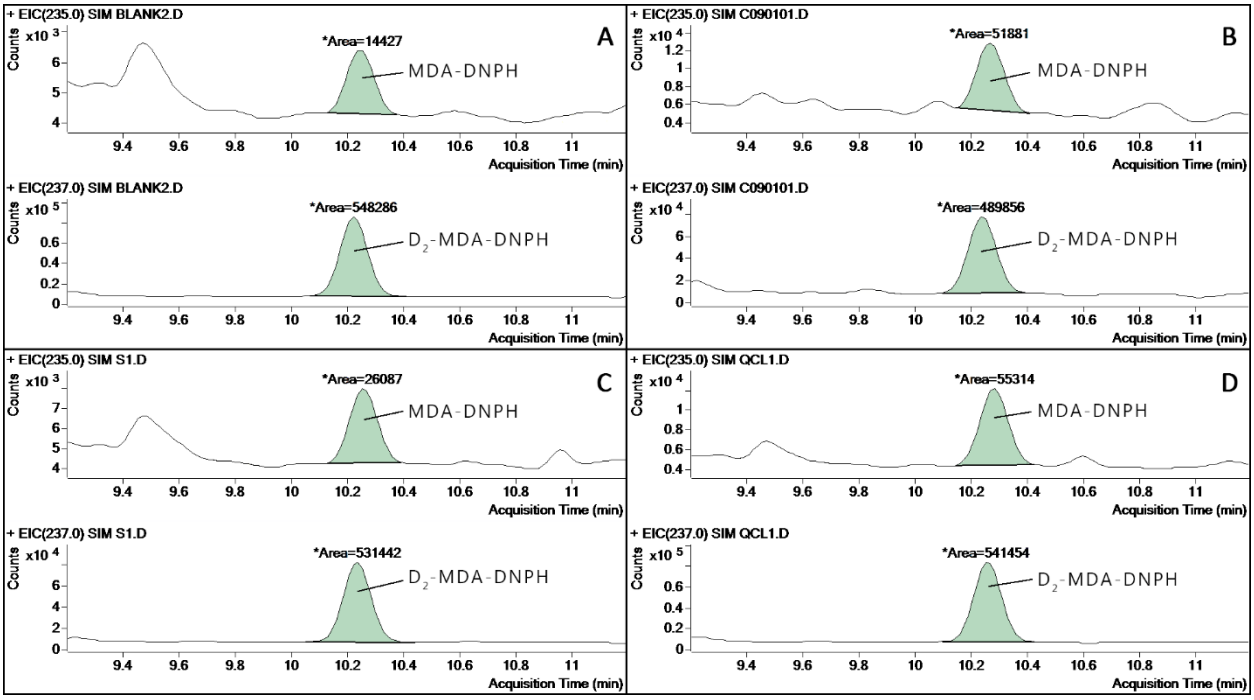



**Figure 3.** Chromatograms of malondialdehydr-2,4-dinitrophenylhydrazine [MDA-DNPH (m/z = 235)] and deuterated internal standard $D_2$-MDA-DNPH (m/z = 237) derivatives.

Note: A – blank, B –EBC sample, 6.02 nM, C – lowest calibration level, 1.0 nM, D – low-level quality control, 5.0 nM.

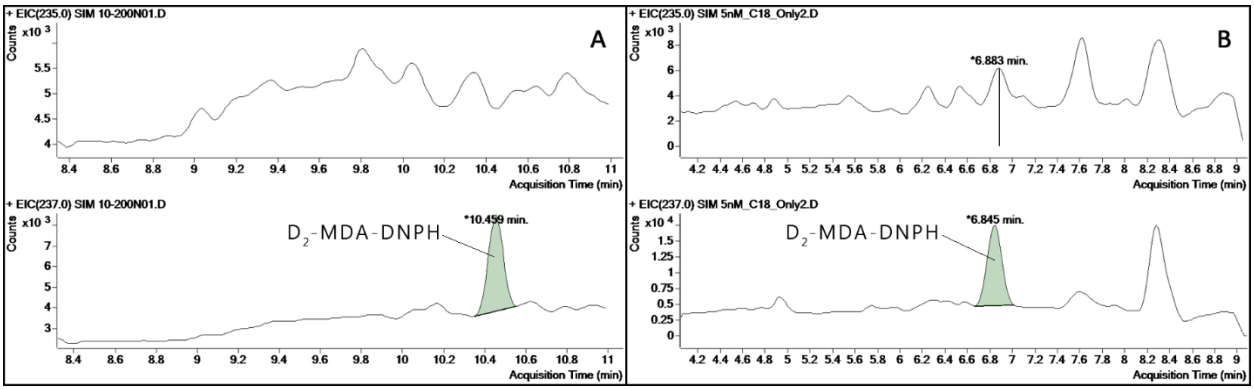



**Figure 4.** Chromatograms of MDA derivatives found in 10 (A) and 5 (B) nM matrix-free standard under the single column separation. A: using $C_6$-Phenyl column, B: $C_{18}$ column.

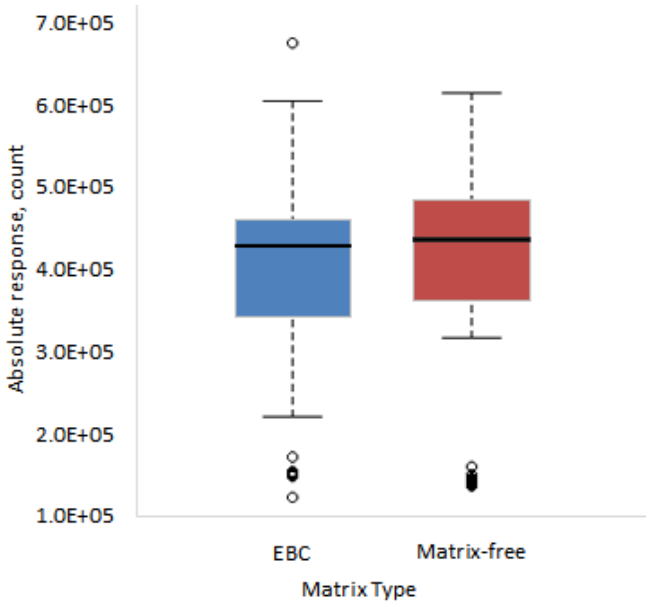



**Figure 5.** Comparison of the internal standard response between exhaled breath condensate (EBC) samples and matrix-free samples (blank, calibration curve, quality control samples).

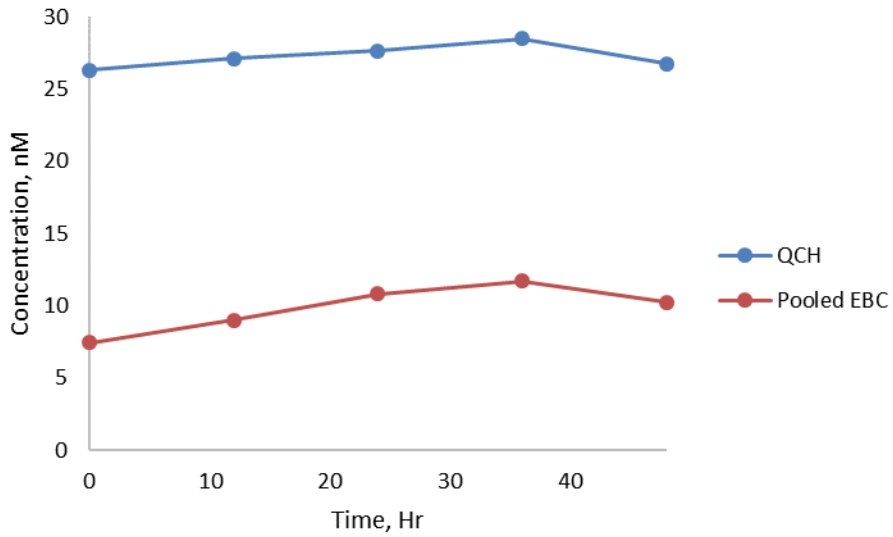



**Figure 6.** Stability of malondialdehyde-2,4-dinitrophenylhydrazine derivatives in autosampler under room temperature.

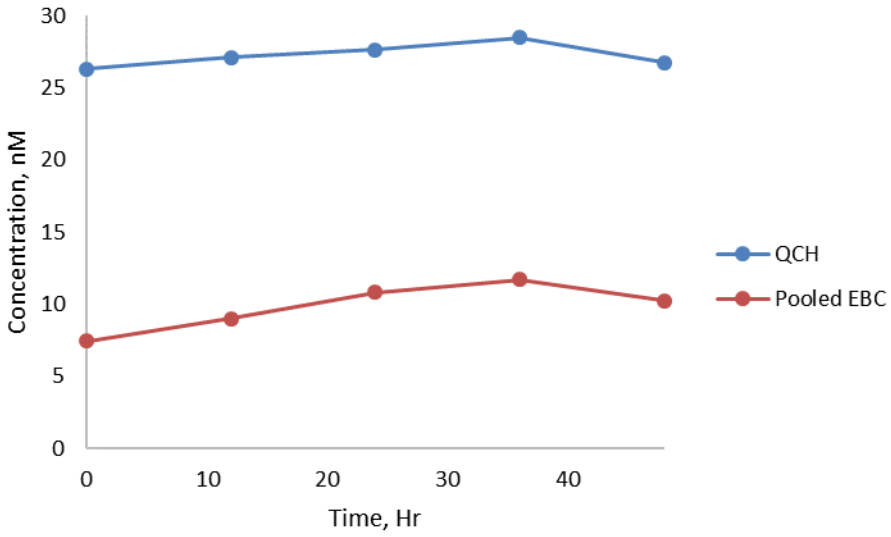



**Figure 6.** Stability of malondialdehyde-2,4-dinitrophenylhydrazine derivatives in autosampler under room temperature.

# CHAPTER 2

**Quantification of malondialdehyde in urine by ultra-performance liquid chromatography coupled with single quadrupole mass spectrometry using dansylhydrazide as a derivatizing agent**

Kostya Kartavenka[1], Parinya Panuwet[2], Volha Yakimavets[2], Dana Boyd Barr[1,2] and P. Barry Ryan[1,2]

1 Department of Environmental Health, Rollins School of Public Health, Emory University

2 Laboratory of Exposure Assessment and Development of Environmental Research (LEADER), Rollins School of Public Health, Emory University

## ABSTRACT

We developed a robust quantitative analytical method for the analysis of malondialdehyde (MDA) in urine samples using dansylhydrazide (DH) as a derivatizing reagent. The derivatization procedure was carried out, in part, using an autosampler injection program to minimize errors associated with addition of reagents and was optimized to yield a stable, pyrazole derivative of MDA and its labeled D2-MDA (for up to 96 hours post derivatization). The target MDA-DH derivatives were separated on an Agilent Zorbax Eclipse Plus Phenyl-Hexyl (3.0x100 mm, 3.5 μm) column located inside a temperature-controlled column compartment of an ultra-performance liquid chromatographer. The mass-to-charge ratios (m/z) of the target derivatives (m/z of 302 and 304 for MDA-DH and D2-MDA-DH, respectively) were analyzed in single ion monitoring mode using a single quadrupole mass spectrometer operated under positive electrospray ionization. Following the optimization of chromatographic and mass spectrometric parameters, the method lowest limit of quantification in sample was set to 9.0 nM (or 28.1 fmol on-column amount). The method displayed a quadratic quantification range of 9.0 – 800 nM in sample. The method showed good relative recoveries (100 – 101 %) and acceptable precisions (1.9 – 4.9 % for inter-day precision, and 4.0 – 6.8 % for intra-day precision) as observed from the analysis of replicates of two quality control samples prepared at 50 nM and 200 nM, respectively. The method was implemented onto a set of urine samples (n=138) recently collected from Thai farmers who were exposed to a mixture of pesticides during filed application. The data demonstrate that this method is capable of measuring urinary levels of MDA, leading to its application in epidemiologic investigations of the associations between MDA formation and pesticide exposures.

## KEYWORDS

Malondialdehyde, UPLC MS, derivatization, dansylhydrazide

## INTRODUCTION

Malondialdehyde (MDA) is one of the most common biomarkers of oxidative stress studied extensively in the recent years. MDA is constantly formed as a byproduct during synthesis of prostaglandins under stressed conditions (Del Rio et al., 2005; Frankel & Neff, 1983; Hecker & Ullrich, 1989) as well as characterizes the end-product of lipid peroxidation (Ayala et al., 2014b; Chen et al., 2011; Del Rio et al., 2005; Khoubnasabjafari et al., 2016) [Aim 1]. MDA was intensively studied in various biological fluids including serum (Karatas, Karatepe, & Baysar, 2002; Tsikas, 2017), plasma (Moselhy, Reid, Yousef, & Boyle, 2013; Sobsey et al., 2016), urine (Chen et al., 2011), exhaled breath condensate (EBC) (Bartoli et al., 2011b; Gong, Zhu, et al., 2013a), and tissues (Mateos & Bravo, 2007) linking to various pathologies reviewed elsewhere(Bartoli et al., 2011b; Tsikas, 2017; Zorawar, Karthigesu, Pramjit, & Rupinder, 2014), proving its importance in epidemiological and medical research. In addition to MDA role as a biomarker of oxidative stress, it can bind to DNA bases like guanine, adenine, and cytosine (Marnett, 1999; Plastaras, Guengerich, Nebert, & Marnett, 2000), resulting in increased rate of mutations (Marnett, 1999); the formation of interstrand cross links (Niedernhofer, Daniels, Rouzer, Greene, & Marnett, 2003) and protein-DNA cross-links were reported (Voitkun & Zhitkovich, 1999) were reported *in vitro*. Besides genotoxic activities, MDA was reported to bind to primary amines of lipoproteins and creating cross-links (Uchida, 2000), therefore likely contributing to atherogenesis; thus elevated levels of serum MDA was associated with accelerated rates of cardiovascular disease (Boaz et al., 1999; Lorente et al., 2015).

Liquid chromatographic methods for quantification of MDA are commonly accomplished via binding MDA to thiobarbituric acid (TBA) despite known issues associated with relatively harsh conditions of the derivatization (Del Rio et al., 2005) that can lead to overestimation of MDA due to artificial release from the matrix and non-specificity of TBA assay (Frankel & Neff, 1983). For example, the same urinary MDA levels quantified with TBA assay were ten-fold times higher than those quantified with 2,4-dinitrophenylhydrazine (DNPH) at a relatively mild conditions (Korchazhkina, Exley, & Andrew Spencer, 2003). As a result, alternative derivatization reagents have been proposed including DNPH (B.

Kim, Jung, & Kho, 2017; Korchazhkina et al., 2003), dansylhydrazide (DH) (Hogard, Lunte, & Lunte, 2017; Lord, Rosenfeld, Raha, & Hamadeh, 2008; Lord et al., 2009), rhodamine B hydrazide (P. Li et al., 2013), and other (Giera et al., 2011; Oh & Shin, 2017; Stalikas & Konidari, 2001; Steghens, van Kappel, Denis, & Collombel, 2001) were developed to overcome limitations of TBA-based method. The detection and quantification of these derivatives are commonly performed using ultraviolet/visible detection (B. Kim et al., 2017; Steghens et al., 2001), fluorescence detection (Ivica, Skoumalová, Topinková, & Wilhelm, 2011), single-quadrupole mass selective detector (MSD) [Aim 1], and tandem-quadrupole multiple reaction monitoring mass spectrometry (MS/MS) (Oh & Shin, 2017; Sobsey et al., 2016). It should be noted that MS/MS quantification, under some conditions, does not require derivatization (Syslová et al., 2009). Gas chromatographic methods are also available. These methods require derivatization and quantification is commonly performed using electron capture detection, nitrogen-phosphorus detection, and MSD (Giera et al., 2012b; Yeo, Helbock, Chyu, & Ames, 1994).

The purpose of this study to develop simple and robust analytical procedure for determination of MDA in human urine using liquid chromatography with single quadrupole mass spectrometry (LC MS). In our previous work [Aim 1], we developed pseudo two-dimensional LC MS method that was able to quantify MDA in EBC samples. However, we experienced several limitations forcing us to search for a different derivatizing reagents, settling specifically on DH. To control for potential matrix effects (Panuwet et al., 2016), isotope-dilution quantification was used. The method was validated in according to FDA guidance for bioanalytical method validation (*Guidance for industry, bioanalytical method validation*, 2018). The method was tested for its suitable by analyzing 138 individual human urine and a pooled serum sample.

## MATERIAL AND METHODS

### Chemical and reagents

We obtained acetonitrile (HPLC grade) from Fisher Chemical (Waltham, MA, USA). Hydrochloric acid, 30% (for ultratrace analysis), malondialdehyde tetrabutylammonium salt, 96% (neat) were obtained

from Sigma-Aldrich (Saint Louis, MO, USA), dansylhydrazine[1] (DH), 97% (reagent grade) from TCI America (Portland, OR, USA), ammonia hydroxide, 14.8 M from Ward's Science (Rochester, NY, USA). The internal standard, 1,1,3,3-Tetraethoxypropane-1,3,-$D_2$ ($D_2$-TEP) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Water was purified using a Milli-Q Ultrapure water purification system (EMD Millipore, Billerica, MA). Strata™-X 33u solid phase extraction (SPE) cartridges were purchased from Phenomenex (Torrance, CA, USA).

**Preparation of standard, quality control, and labeled internal standard solutions**

High concentration standard stock and quality control solutions were prepared by weighing a known amount of neat standard then diluting with acetonitrile (Stock I solution). This stock solution was used to prepare an 800 nM stock solution in water weekly (Stock II solution). The stock I and II solutions were stored at -20 °C. Eight-point calibration solutions were prepared daily from corresponding stock II solution in Milli-Q water. The concentrations of the calibration solutions were as follows (9, 27, 45, 75, 100, 200, 400, 800 nM). Two-point Quality control solutions (QC) (50 and 200 nM) were prepared separately in Milli-Q water from corresponding stock II solution. Labeled internal standard (IS) stock solution was prepared via hydrolysis of $D_2$-TEP (Pilz et al., 2000) to yield $D_2$-MDA of approximate concentration of 0.01 mM. This labeled IS solution was diluted with acetonitrile to yield concentration of 10 μM stock solution. This labeled IS stock solution was used to prepare 500 nM labeled IS in acetone and 100 nM labeled IS in Milli-Q by dissolving in corresponding solvent. A DH solution was prepared via dissolving 23 mg of DH in 10 mL acetonitrile. The DH derivatizing solution (DHDS) was prepared in an amber autosampler vial via mixing 1:1:2 3 M hydrochloric acid solution in Milli-Q:DH solution:Milli-Q. Ammonia hydroxide solution (AHS) was prepared via diluting ammonia hydroxide solution in Milli-Q to yield a concentration of 4 M.

[1] While vendors list the derivatizing agent under dansylhydrazine name, in according to IUPAC it should be named as dansylhydrazide

**Preparation of blank, calibration, quality control, and unknown samples**

Prior to analysis, each urine sample was stored at 4 ºC. We performed two approaches for sample preparation: A) autosampler injection program (AIP) assisted sample preparation and B) manual sample preparation with SPE. Blank, calibration curve, and QCs were prepared in the same manner via spiking of corresponding solution into appropriate vial insert using either of approach.

**Sample preparation assisted by the AIP**

Sample preparation include dilution of 50 µL of urine with 100 µL Milli-Q, 25 µL were transferred directly into the 250 µL insert in autosampler vial, capped and placed into the autosampler (Figure 1A). The AIP performed spiking of each sample with 5 µL (500 nM labeled IS) following by 5 µL of DHDS. Prior and post spiking, the needle was washed in the washing port with 1:1 acetonitrile:Milli-Q solution (Figure 1B). Internally, the needle was washed for 0.1 minute between each spiking via pushing mobile phase (5% acetonitrile in water) at 1 ml/min flow rate. After completion of spiking of all vials, each vial was manually vortexed and the autosampler rack was placed into the incubator for 2 hours at 45 ºC at a constant vortex of 200 RPM. After the incubation, the rack was placed back into LC autosampler. Another sequence was executed to spike each vial with 5 µL of AHS (Figure 1B), following by manual vortexing.

**Manual sample preparation with sample clean-up and extraction using SPE**

For this approach, 100 µL of urine sample was transferred to 15 mL centrifuge tube, 0.9 mL of Milli-Q was added and vortexed, 200 µL was transferred to a separate vial. To prepare serum samples, 50 µL of serum was transferred into centrifuge tube and spiked with 200 µL of methanol for protein precipitation. Serum samples were briefly vortexed and centrifuged for 10 minutes at 2500 RPM. The sample was spiked with 50 µL of 100 nM labeled IS, 25 µL 1.5M hydrochloric acid, and 25 µL DH solutions. Samples were capped and placed into the incubator for 2 hours at 45 ºC at a constant vortex rate of 200 RPM. After the incubation samples were transferred into Strata-X SPE cartridge (pre-conditioned with 2 mL ACN following by 2.5 mL Milli-Q). Each cartridge was washed with 2 mL Milli-Q and

2.5 mL 3:4 methanol:Milli-Q solution, dried with vacuum for 5 minutes and eluted with 2.5 mL 100 % acetonitrile. Samples were evaporated with gentle nitrogen flow at 15 psi in TurboVap® (Figure 1C).

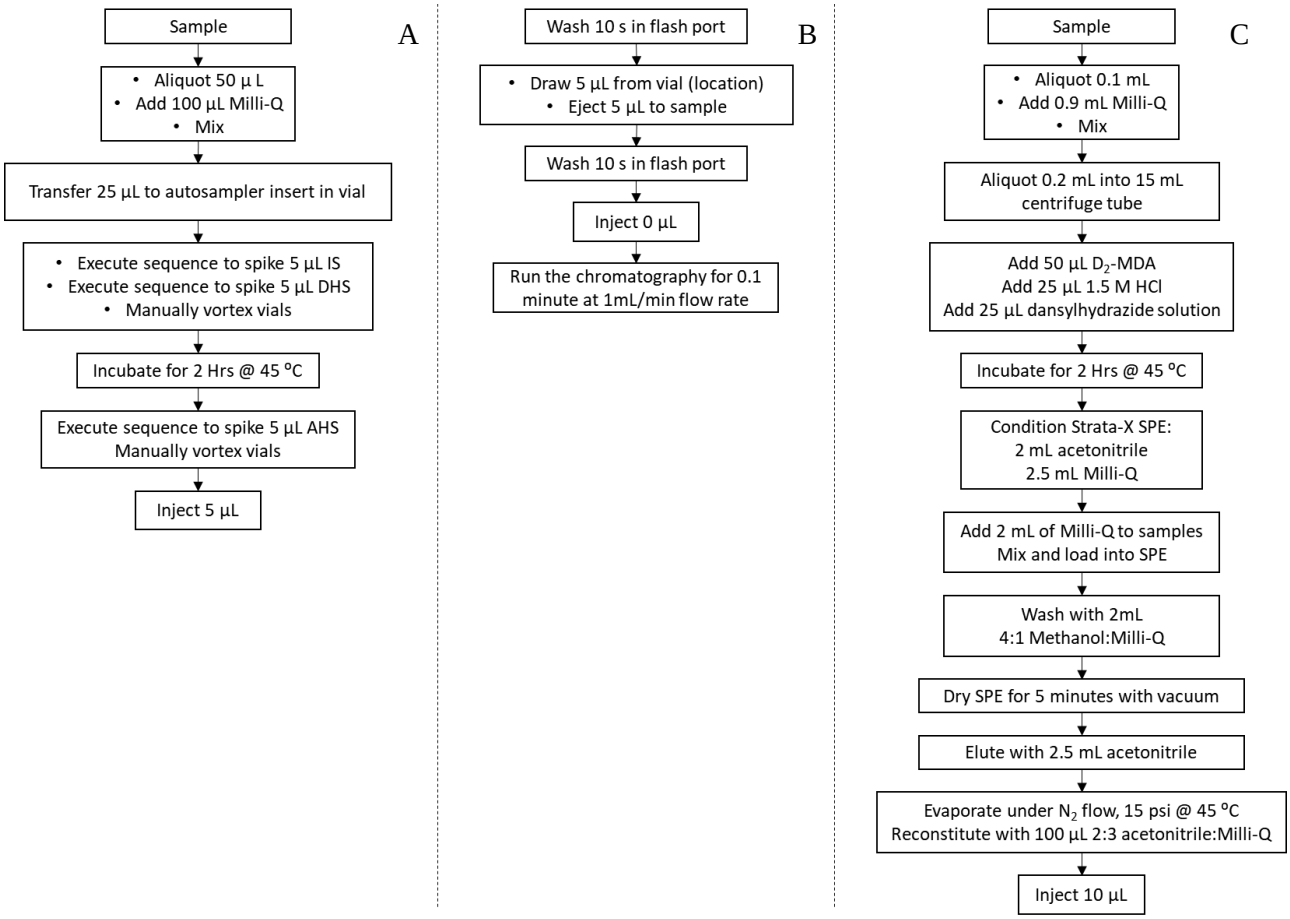



**Figure 1**. Sample preparation flow charts: A) assisted by the autosampler injection program (AIP), B – a typical AIP used for in-vial sample preparation, C) sample preparation using manual derivatization procedure with solid phase extraction on Strata-X 33u cartridge.

### 2.4 Chromatographic separation and mass spectrometric conditions

The sample analysis was carried out using an Agilent Infinity 1290 ultra-performance liquid chromatograph (UPLC) with a G4226A autosampler connected to an Agilent G6150B single quadrupole mass spectrometer (MSD) (Santa Clara, CA, USA). The separation was performed on Agilent Zorbax Eclipse Plus Phenyl-Hexyl (3.5 µm 3.0x100 mm) columns. The column temperature was set to 45 ºC. For each analysis, 5 µL was injected with needle wash. The binary mobile phase consists of A: 100% acetonitrile, B: 100% Milli-Q. The separation was facilitated by a gradient elution program (Table 1).

**Table 1.** Graduate elution liquid chromatography program for analysis of malondialdehyde-danylhydrazide adduct in urine. A – Milli-Q®, B – 100% acetonitrile. Both solvents were not spiked with any additional additives.

| Time, min | B composition | Flow rate, ml/min | Time, min | B composition | Flow rate, ml/min |
|---|---|---|---|---|---|
| 0.0 | 5.0% | 0.5 | 7.3 | 43.0% | 0.4 |
| 0.01 | 5.0% | 1.0 | 7.5 | 100% | 0.4 |
| 0.5 | 5.0% | 1.0 | 7.7 | 100% | 1.0 |
| 2.5 | 43.0% | 1.0 | 9.5 | 100% | 1.0 |
| 6.0 | 43.0% | 1.0 | 9.7 | 5.0% | 1.0 |
| 6.5 | 43.0% | 0.4 | 12.0 | End of LC program | |

The target pyrazole, including its deuterated IS, were analyzed using positive electrospray ionization. The mass spectrometry was operated in single ion monitoring (SIM) mode from 6.0 to 7.6 minutes. Capillary and nozzle voltages were set to 1800 V and 2000 V, respectively. Nitrogen was used as a dry and sheath gas, their flow rates were set to 5.0 L/min and 12.0 L/min, respectively. Drying gas and sheath gas temperatures were set to 350 ºC and 360 ºC, respectively. The nebulizer pressure was set to 25 psi. The fragmentor voltage was set to 120 V, while the dwell time was set to 204 msec. The MDA-DH derivative (Figure 2A) was monitored at m/z of 302, while the $D_2$-MDA-DH derivative was monitored at m/z of 304. The peak of the target derivative was selected based on its retention time (RT), relative retention time to labeled IS peak, peak shape, and signal-to-nose ratio. For kinetics investigation, we were monitoring linear form of malondialdehyde-dansylhydrazide adduct (Figure 2B) and malondialdehyde bound to 2 molecules of DH (Figure 2C).

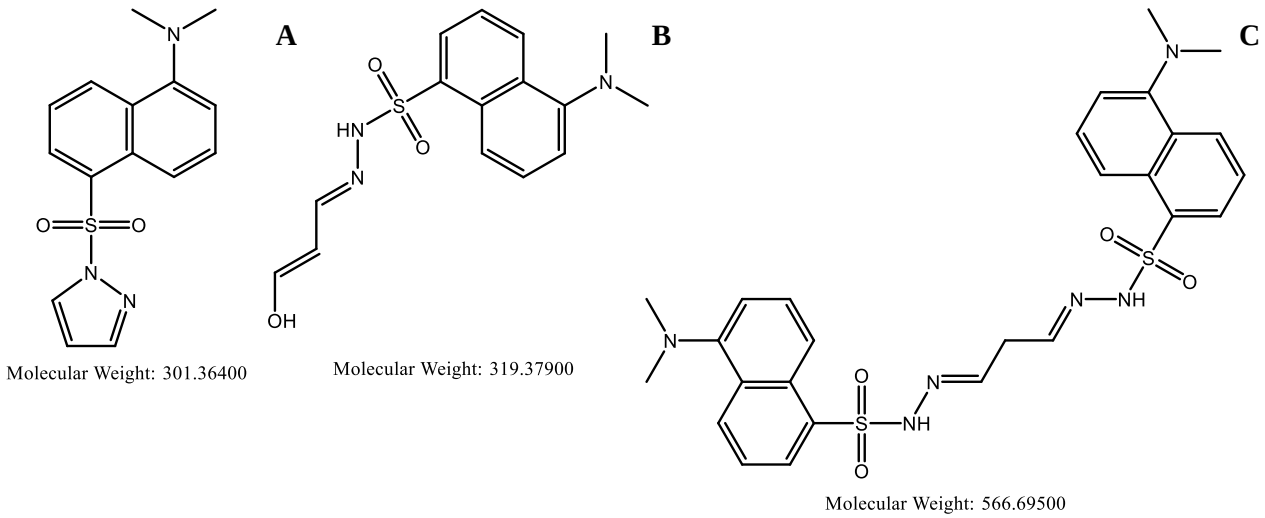



**Figure 2**. Chemical structures of hydrazones that were investigated in the study.

Note: A – a cyclic form of malondialdehyde-dansylhydrazide adduct (pyrazole), B – a linear form of malondialdehyde-dansylhydrazide adduct (monohydrazone), C – malondialdehyde bound to two molecules of dansylhydrazide (bihydrazone).

**Method Validation**

The method precision was determined in according to protocol described by Chesher (Chesher, 2008) using two quality control levels (QCL and QCH). Replicate samples (5 samples/day at each concentration level) were prepared and analyzed daily during a 5-day period to determine inter-day and intra-day precision. The precisions were expressed as relative standard deviation (%RSD) values.

The method relative recoveries (RR) were determined, according to FDA guideline (*Guidance for industry, bioanalytical method validation*, 2018) using a set of replicates, quality control materials (QCL and QCH) prepared at concentrations of 50 and 200 nM (N = 25). The method RR were calculated by dividing the average, quantified values of the quality control materials with the respective, expected concentration values for both levels. The method RR were expressed as percentage values.

A study of autosampler storage stability was performed via a quintuplicate injection of QCL, QCH and five randomly selected individual sample were injected immediately and in 13, 38, 67, 95 hours after the

derivatization. Samples were stored in the autosampler at a room temperature (approximately 22 ºC) during the intervening times. For replicate injection of urine samples, immediate injection (Time = 0 hours) was selected as the expected concentrations. Relative changes (RC) of concentration was calculated with the following equation and expressed in percentage. Stability was assessed as ±15 of expected concentration. Stability of the hydrazone itself was assessed via relative response loss/gain in the same manner.

$$RC = \frac{Concentration_{Time} - Nominal\ concentration}{Nominal\ concentration} \times 100 \qquad \text{N} \qquad [1]$$

Matrix effects were evaluated by comparing mean responses (peak area) of labeled IS presented in matrix-free samples (QC) and in the urine samples. To control for and manage matrix effect, isotope dilution quantification was used (Panuwet et al., 2016).

Since the reaction of MDA with DH lead to formation of various products, the kinetics of formation of each derivative was assessed via injection of derivative in 5, 10, 13, 21, 33, 60, 90, 120 minutes after incubation.

The method was used to analyze 138 urine samples to assess the suitability of the method. The sample collection protocol and subject recruitment procedure were reviewed and approved by the Institutional Review Board of Chiang Mai University.

## RESULTS

### Chromatographic separation condition

The optimum chromatographic conditions were achieved with Agilent Plus Phenyl-Hexyl (3.5 μm 3.0x100 mm) column. Chromatograms of blank, point of the lowest limit of quantification (LLOQ), and an individual urine sample are shown on Figure 3; a typical calibration curve prepared via AIP is shown on Figure 4. A shorter column may be used (e.g. Agilent Eclipse Plus Phenyl-Hexyl RRHD (1.8 μm 2.1x50 mm)), however through our experience  larger pore size ensures longer stability of pressure in HPLC system.

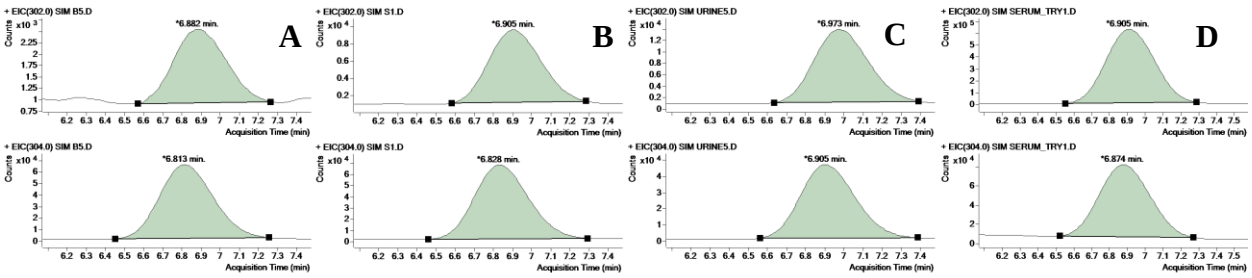



**Figure 3**. A typical chromatogram of A – blank sample, B – lowest quantification level (9 nM), C – individual urine sample 23.5 nM, D – pooled serum sample (concentration is above highest level of calibration). The top chromatograms represent responses of unlabeled malondialdehyde-dansylhydrazide adduct (m/z = 302), the bottom chromatograms show labeled hydrazone.

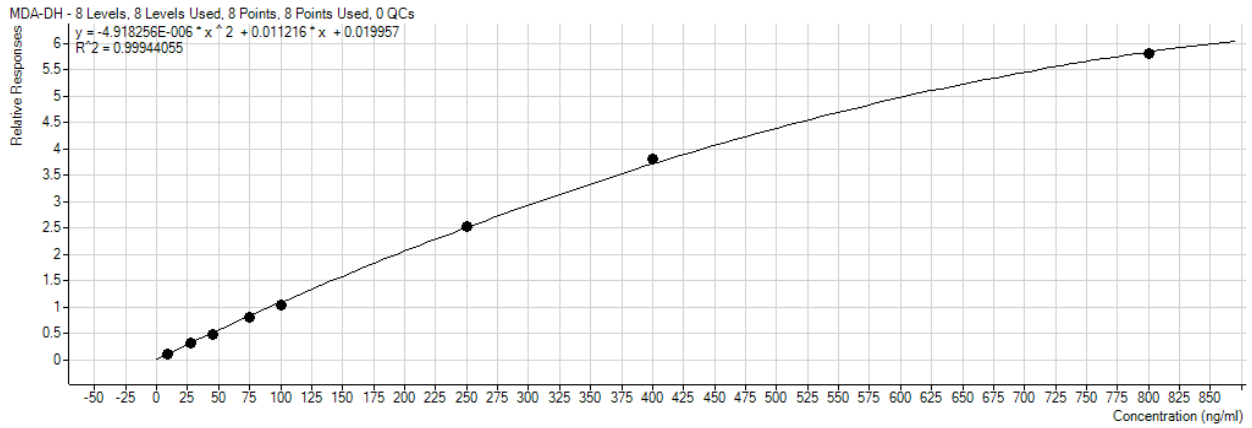



**Figure 4**. A typical calibration curve (sample concentration 9 nM – 800 nM) prepared by the autosampler injection program.

**Limit of quantification**

LLOQ was selected by examining additional 3 and 6 nM concentrations levels. Calibration level of 9 nM (28.1 fmol on-column amount) was selected being in 80-120% relative recovery range during the five days of study validation. Method limit of detection was not assessed since abundance of MDA in urine allow wide range of dilution factors. In our study we selected an 18-fold dilution factor for urine, expecting majority of urinary MDA values to be in QCL-QCH range. Since at the high end of the

calibration curve saturation was observed, it important to dilute urine to a linear part of the calibration (9 – 250 nM). Additionally, the dilution of urine is essential to decrease matrix effects when non-SPE approach is used.

**Method validation**

The method precision and accuracies for both approaches: AIP to assist sample preparation (Table 2) and using manual sample preparation followed by SPE (Table 3) are shown. The RR for both approaches is within 100-105 % range, and inter- and intra-day precision are in 1.9-11 % range.

**Table 2.** Relative recoveries and precisions of the method that assists sample preparation with autosampler injection program, without extraction.

| Expected concentration level | Relative recovery, % | Precision, % | |
|---|---|---|---|
| | | Within-day | Between-day |
| 50 nM | 100 | 4.9 | 6.8 |
| 200 nM | 101 | 1.9 | 4.0 |

**Table 3**. Relative recoveries and precisions of the method with manual hydrazone derivatives preparation, followed by solid phase extraction.

| Expected concentration level | Relative recovery, % | Precision, % | |
|---|---|---|---|
| | | Within-day | Between-day |
| 50 nM | 105 | 4.8 | 11 |
| 200 nM | 102 | 2.0 | 8.8 |

The formation of the cyclic form (Figure 2A) of MDA-DH pyrazole (m/z = 302), monohydrazone (m/z = 320), and bihydrazone (m/z = 567) are shown on Figure 5A. The relative response (a ratio between peak abundance of unlabeled to labeled ions) from which concentration sample is quantified for isotopically-diluted method is presented on Figure 5B. By observing significant changes in relative response (Figure 5B) at early time of hydrazones formation, it is likely that there are differences in the kinetics of formation of unlabeled and deuterated hydrazones, suggesting, that either the time of reaction

should be fixed by parameters (temperature, pH, duration) or a region with relative response changes reaching to its plateau should be selected.

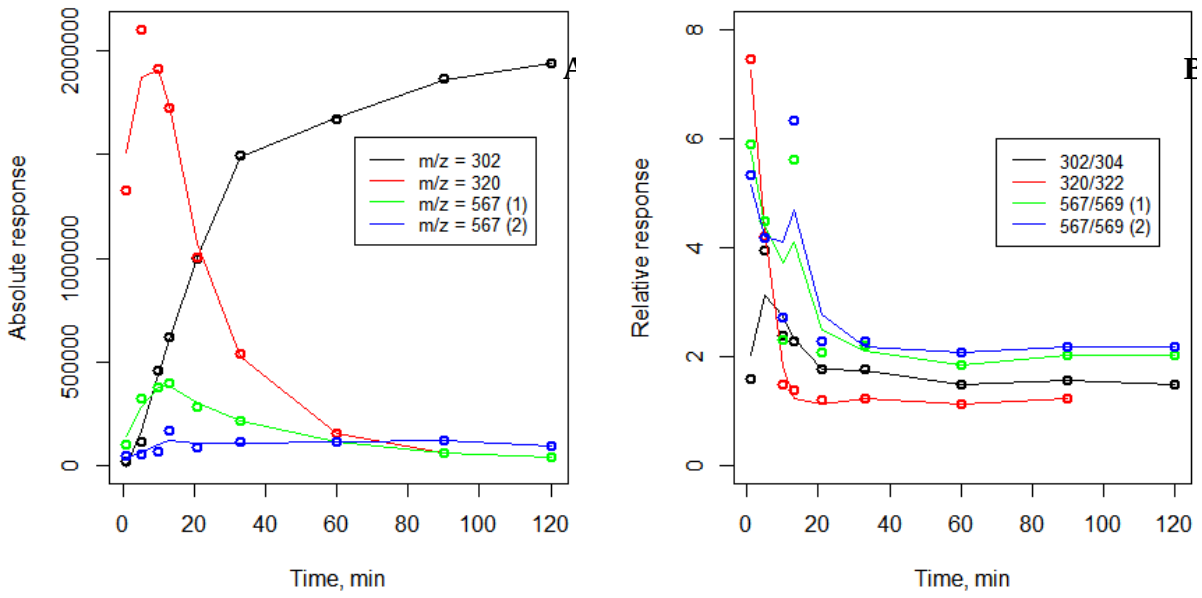



**Figure 5**. Formation/degradation of different hydrazone products after incubation at 45 ºC.

Autosampler storage stability (at approximately 22 ºC) is presented on Figure 6 for urine-free samples. Samples treated with AHS after the derivatization (which shifts pH from approximately 1 to approximately 9) showed better stability over time. Samples which were extracted using SPE were at approximately neutral pH and showed similar stability to samples treated with AHS (data not shown). At QCL level, samples treated with AHS were all within the ± 15% accuracy criterion suggested by FDA (*Guidance for industry, bioanalytical method validation*, 2018); at QCH level, the majority of samples treated with AHS were within ± 15% accuracy criterion. We also examined relative gain/loss in labeled IS response associated with treatment with AHS (Figure 7). It is likely that hydrazone is less stable in the base environment compared to acid conditions (Binding et al., 1998), on average 40-80% of hydrazone is decomposed in a base environment in 4 days stored at 22 ºC *ca*. The relative changes in concentration of

five randomly selected samples was assessed over the same period of time (Figure 8A). The relative change in labeled IS response count is shown on Figure 8B. Similar loss in labeled IS response is observed for urine samples compared to matrix-free (water samples). Matrix effects associated with urine are shown in Figure 8C. From a two-sample Wilcoxon test (p-value < 0.0001) we can conclude that there is a significance matrix effect associated with urine sample matrix.

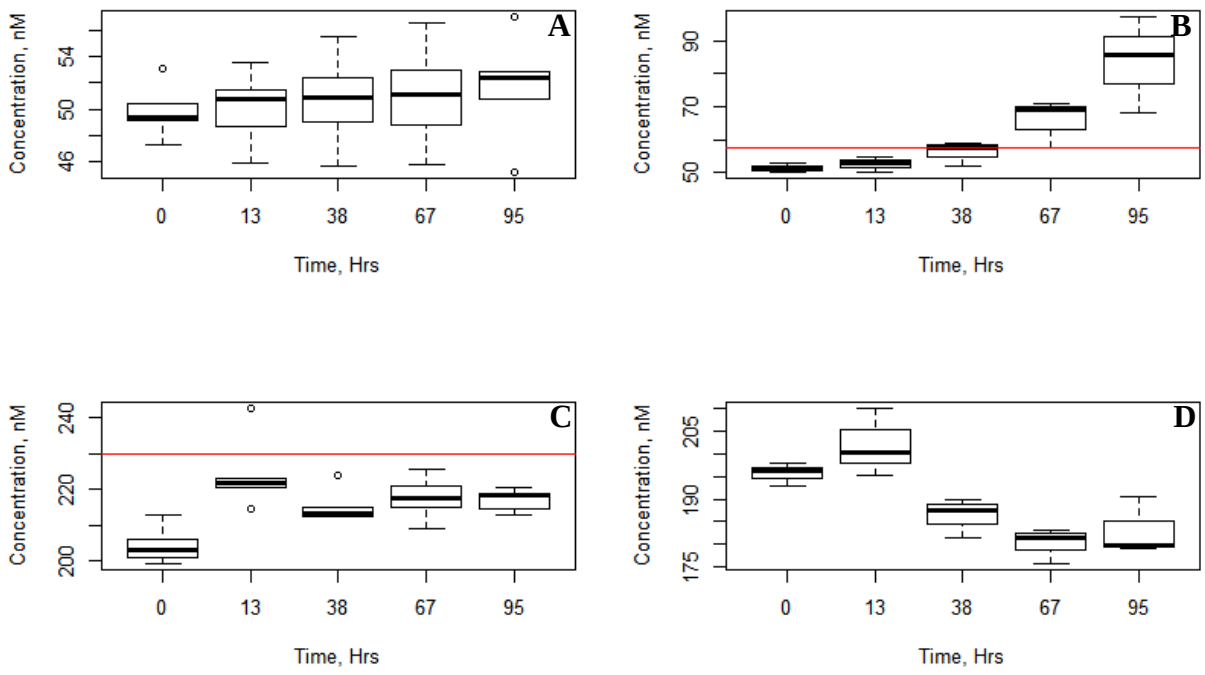



**Figure 6**. Autosampler storage stability of **A** – quality control low (QCL) at concentration of 50 nM treated with ammonia hydroxide solution after the completion of the derivatization (quintuplicate injections), **B** – QCL prepared without treatment after the derivatization (triplicate injections), **C** – quality control high (QCH) at concentration of 200 nM treated with ammonia hydroxide solution after the completion of the derivatization (quintuplicate injections), **D** – QCH prepared without treatment after the derivatization (triplicate injection). Red line (if presented) represents accuracies of ± 15% to expected concentration.

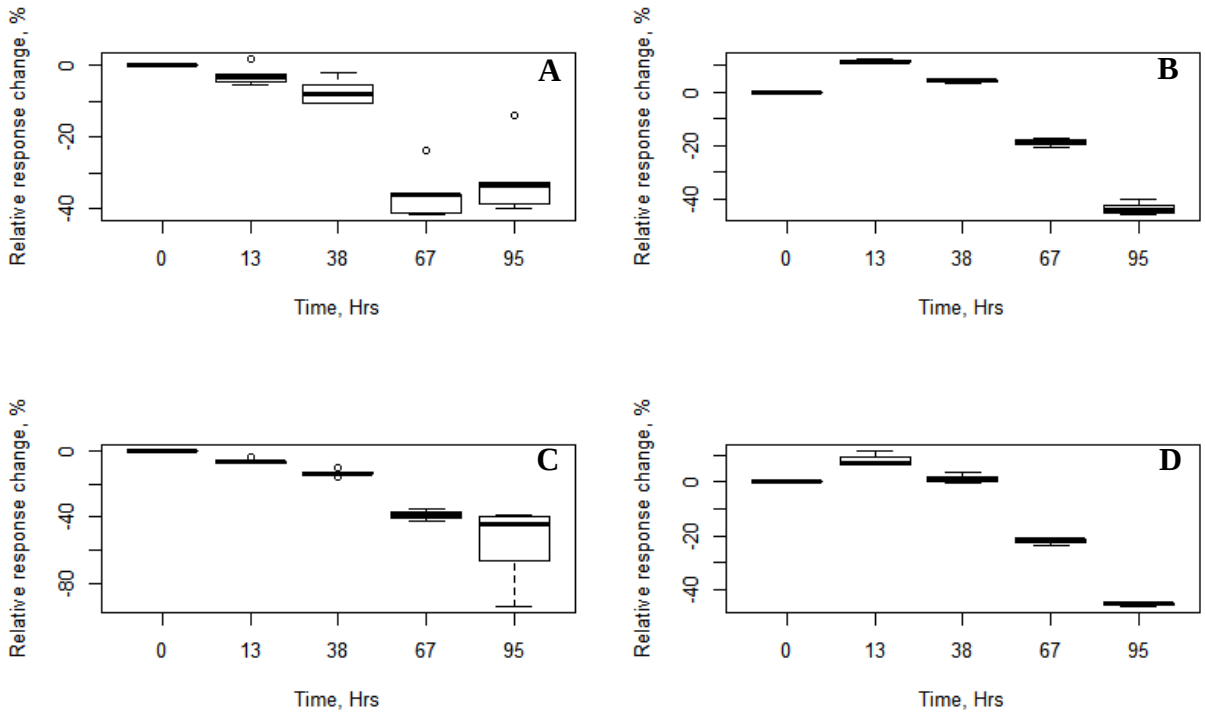



**Figure 7**. Relative response gain/loss of isotopically labeled malondialdehyde-dansylhydrazide derivative ($D_2$-MDA-DH) in a cyclic form. **A** – $D_2$-MDA-DH in quality control low (QCL) at concentration of 50 nM treated with ammonia hydroxide solution after the completion of the derivatization (quintuplicate injections), **B** – $D_2$-MDA-DH in QCL prepared without treatment after the derivatization (triplicate injections), **C** – $D_2$-MDA-DH in quality control high (QCH) at concentration of 200 nM treated with ammonia hydroxide solution after the completion of the derivatization (quintuplicate injections), **D** – $D_2$-MDA-DH in QCH prepared without treatment after the derivatization (triplicate injection)

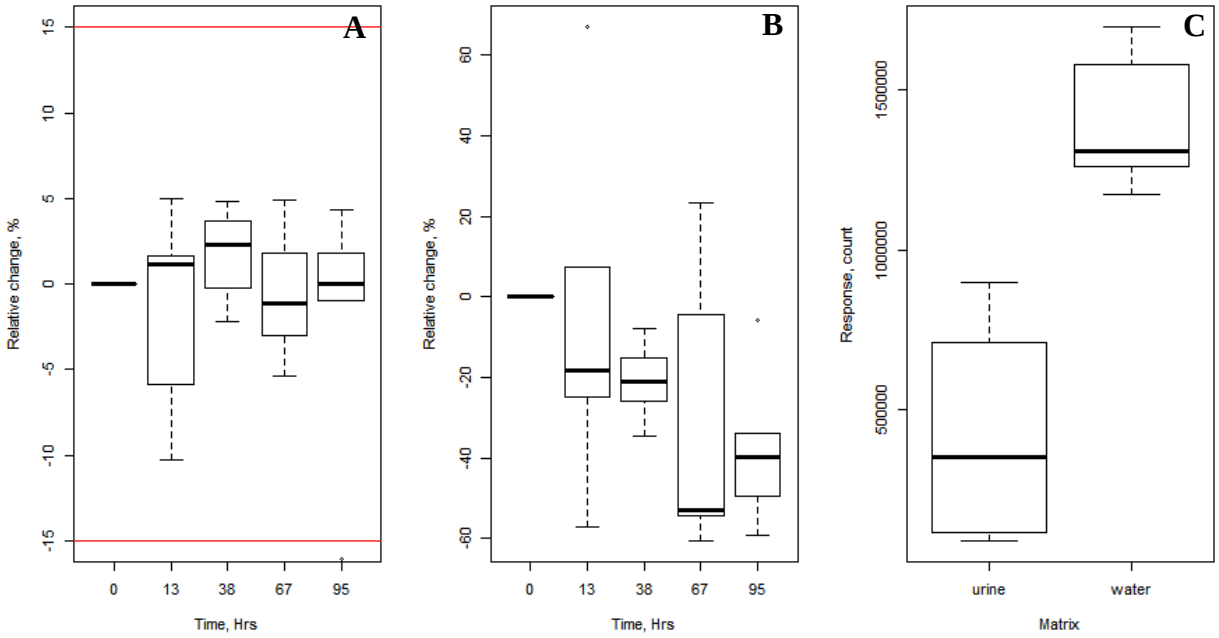



**Figure 8**. A – relative changes to urine samples (5 randomly selected individual samples) over time as a part of autosampler storage stability procedure. B – relative changes in labeled derivates over the course of assessment. C – matrix effect associated with the presence of urine matrix. All samples in A, B, C we treated with ammonia hydroxide solution after the derivatization.

## DISCUSSION

We developed a sensitive, selective, and in-expensive method capable of quantifying urinary levels of MDA in its derivative form (as a pyrazole) collected from human subjects. Our method is the first reported to be capable of measuring urinary MDA using LC single quadrupole mass spectrometry. The method was also shown to be applicable to a more sophisticated matrices such as serum (Figure 3D) that require additional clean-up procedure like SPE. We optimized our method to reduce usage of more expensive IS. Based on our estimates, AIP spends approximately 2 minutes for sample preparation (including loading of IS, DHS, and AHS), which allow to increase throughput of the method to up to 90 samples per day. During method development, we were trying increase autonomy of sample preparation

by reducing manual vortexing of samples, however even though the software allows a “mix” function in AIP, it was not able to mix samples. To achieve sufficient homogenization of components, sampling air (via offsetting needle) and bubbling reaction medium with a different ejection speed was proposed and showed its effectiveness (data not shown). However, several drawbacks were observed including excessive usage of autosampler metering device and increase of sample preparation by 5 minutes since approximately 15 repeats should be performed in order to homogenize the sample. Overall, the AIP can significantly reduce cost associated with usage of labeled IS and decrease contamination of the instrument by the derivatizing reagent.

The use of DH as a derivatizing agent substantially changes the properties of MDA., Specifically, it increases its retention properties in reversed-phase chromatographic separation and SPE extraction. For examples, without derivatization, MDA was not retaining in Strata-X column, while after the derivatization it allows to use 80 % methanol solution to wash the cartridge, which allow us to analyze more complicated samples, like serum. By varying sample preparation conditions (Steghens et al., 2001), the method can be applied for measuring both total and free MDA in serum, however dilution factors should be estimated.

Several hydrazones were studied as a result of the interaction of DH with MDA (Hirayama, Yamada, Nohara, & Fukui, 1984; Lord et al., 2008; Lord et al., 2009). With the exception of the cyclic form (pyrazole), MDA hydrazones can exist in diastereomer forms which may be separated using non-chiral columns. During our assessment of kinetics, we anticipated observing (Figure 5) formation of such diastereomers for the bihydrazone. However, additional examinations should be performed (e.g. high-resolution mass spectrometry of products formation under collision-induced dissociation) to confirm their isomerism. The formation of monohydrazone adduct was rapid (Figure 5A) and it was applied in several LC/fluorescence methods previously with automated solid phase analytical derivatization (Lord et al., 2008; Lord et al., 2009). Additionally, namely monohydrazone shows the largest sensitivity, however the relative ratio of labeled to unlabeled standard (which determines concentration) is rapidly changing in that period (Figure 5B), together with potential existence of diastereomers (Figure 9) will likely dramatically

affect the method's reproducibility. We proposed the following diagram of hydrazone products formation and degradation (Figure 9). Based on our study the true relationship between isomers cannot be established. The pyrazole appears to be the final and most stable product that is presented in a high abundancy, having only one isomer. We did not assess further transformation pathways of pyrazole.

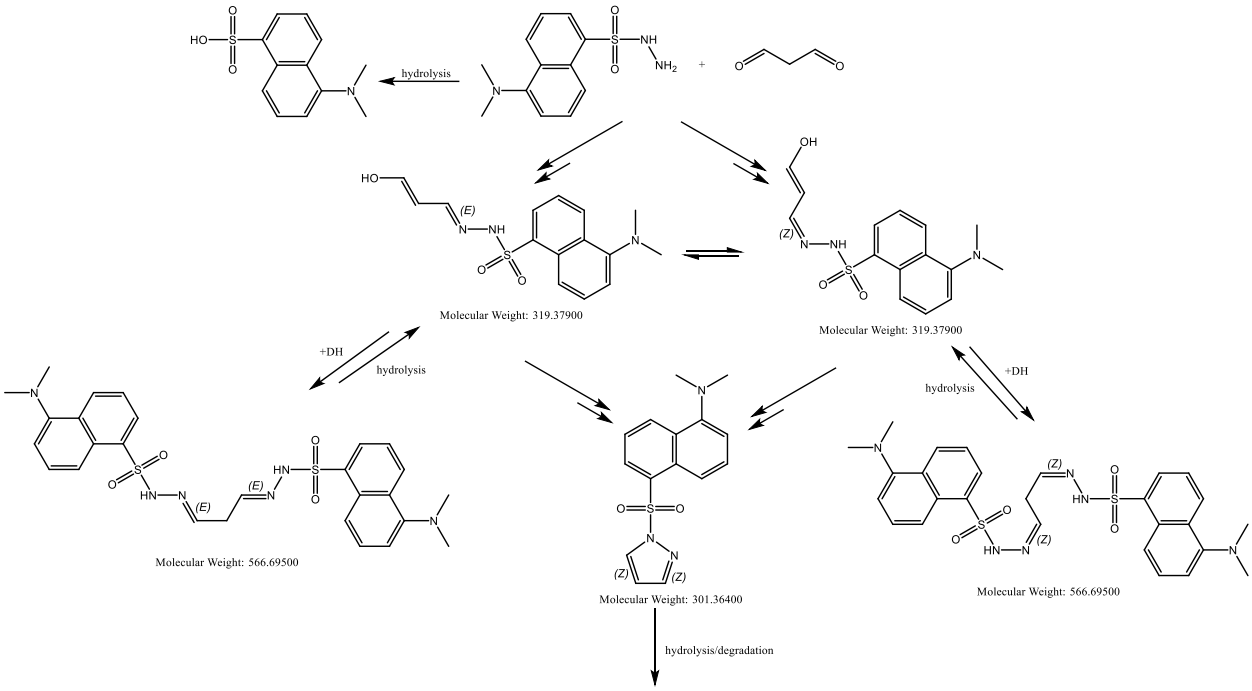



**Figure 9**. Anticipated diagram of malondialdehyde hydrazones and pyrazole formation/degradation.

The previous study (Binding et al., 1998) showed an importance in balancing between water and acid content for degradation of hydrazones of various aldehyde. In our work we observe similar patterns, however we observed, that degradation of the pyrazole is higher in basic pH. Despite this observation, the stability of the ratio between labeled and unlabeled standards is higher in basic or neutral pH compared to acidic pH, likely due to suppression of deuterium-hydrogen exchange, suggesting its necessity to obtain reproducibly method and increase autosampler storage stability.

## CONCLUSION

We developed sensitive and selective analytical method that is capable of quantifying urinary MDA levels using LC MS. We determined that pyrazole is the final most stable product of chemical interaction

between MDA and DH, monohydrazone can be still used in a controlled environment. We showed that, due to dramatic changes in MDA properties due to derivatization, the method can be combined with more advanced purification techniques like SPE and can be used for analysis of MDA in matrices such as serum. The method's performance was evaluated in according to FDA criteria. Our work shows that relatively simple autosampler can be used to optimize sample preparation, decrease usage of high-cost reagents like IS, and exposure of the instrument to excess amount of derivatizing reagent.

## ACKNOWLEDGEMENT

This work was financially supported by NIH grants 5UM1HL134590-03, P50ES026071, EPA 83615301, P30ES019776 and the Laney Graduate School of Emory University.

## REFERENCES


1. Del Rio, D., A.J. Stewart, and N. Pellegrini, *A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress.* Nutrition, Metabolism and Cardiovascular Diseases, 2005. **15**(4): p. 316-328.
2. Frankel, E.N. and W.E. Neff, *Formation of malonaldehyde from lipid oxidation products.* Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 1983. **754**(3): p. 264-270.
3. Hecker, M. and V. Ullrich, *On the mechanism of prostacyclin and thromboxane A2 biosynthesis.* 1989. **264**(1): p. 141-150.
4. Khoubnasabjafari, M., K. Ansarin, and A. Jouyban, *Critical Review of Malondialdehyde Analysis in Biological Samples.* Current Pharmaceutical Analysis, 2016. **12**(1): p. 4-17.
5. Chen, J.-L., et al., *Determination of urinary malondialdehyde by isotope dilution LC-MS/MS with automated solid-phase extraction: A cautionary note on derivatization optimization.* Free Radical Biology and Medicine, 2011. **51**(9): p. 1823-1829.
6. Ayala, A., et al., *Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal %J Oxidative Medicine and Cellular Longevity.* 2014. **2014**: p. 31.
7. Karatas, F., M. Karatepe, and A. Baysar, *Determination of free malondialdehyde in human serum by high-performance liquid chromatography.* Analytical Biochemistry, 2002. **311**(1): p. 76-79.
8. Tsikas, D., *Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges.* Analytical Biochemistry, 2017. **524**: p. 13-30.
9. Moselhy, H.F., et al., *A specific, accurate, and sensitive measure of total plasma malondialdehyde by HPLC.* 2013. **54**(3): p. 852-858.
10. Sobsey, C.A., et al., *Development and evaluation of a liquid chromatography–mass spectrometry method for rapid, accurate quantitation of malondialdehyde in human plasma.* Journal of Chromatography B, 2016. **1029-1030**: p. 205-212.
11. Bartoli, M.L., et al., *Malondialdehyde in Exhaled Breath Condensate as a Marker of Oxidative Stress in Different Pulmonary Diseases %J Mediators of Inflammation.* 2011. **2011**: p. 7.
12. Gong, J., et al., *Malondialdehyde in exhaled breath condensate and urine as a biomarker of air pollution induced oxidative stress.* Journal Of Exposure Science And Environmental Epidemiology, 2013. **23**: p. 322.
13. Mateos, R. and L. Bravo, *Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins).* Journal of Separation Science, 2007. **30**(2): p. 175-191.
14. Zorawar, S., et al., *Use of malondialdehyde as a biomarker for assessing oxidative stress in different disease pathologies: a review.* Iranian Journal of Public Health, 2014. **43**(3 Suppl.): p. 7-16.
15. Marnett, L.J., *Lipid peroxidation—DNA damage by malondialdehyde.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1999. **424**(1): p. 83-95.
16. Plastaras, J.P., et al., *Xenobiotic-metabolizing Cytochromes P450 Convert Prostaglandin Endoperoxide to Hydroxyheptadecatrienoic Acid and the Mutagen, Malondialdehyde.* 2000. **275**(16): p. 11784-11790.
17. Niedernhofer, L.J., et al., *Malondialdehyde, a Product of Lipid Peroxidation, Is Mutagenic in Human Cells.* 2003. **278**(33): p. 31426-31433.
18. Voitkun, V. and A. Zhitkovich, *Analysis of DNA–protein crosslinking activity of malondialdehyde in vitro.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1999. **424**(1): p. 97-106.
19. Uchida, K., *Role of reactive aldehyde in cardiovascular diseases.* Free Radical Biology and Medicine, 2000. **28**(12): p. 1685-1696.

20. Boaz, M., et al., *Serum malondialdehyde and prevalent cardiovascular disease in hemodialysis.* Kidney International, 1999. **56**(3): p. 1078-1083.
21. Lorente, L., et al., *Serum Malondialdehyde Levels in Patients with Malignant Middle Cerebral Artery Infarction Are Associated with Mortality.* PLOS ONE, 2015. **10**(5): p. e0125893.
22. Korchazhkina, O., C. Exley, and S. Andrew Spencer, *Measurement by reversed-phase high-performance liquid chromatography of malondialdehyde in normal human urine following derivatisation with 2,4-dinitrophenylhydrazine.* Journal of Chromatography B, 2003. **794**(2): p. 353-362.
23. Kim, B., W. Jung, and Y. Kho, *Quantification of Malondialdehyde in Human Urine by HPLC-DAD and Derivatization with 2,4-Dinitrophenylhydrazine.* Bulletin of the Korean Chemical Society, 2017. **38**(6): p. 642-645.
24. Hogard, M.L., C.E. Lunte, and S.M. Lunte, *Detection of reactive aldehyde biomarkers in biological samples using solid-phase extraction pre-concentration and liquid chromatography with fluorescence detection.* Analytical Methods, 2017. **9**(12): p. 1848-1854.
25. Lord, H.L., et al., *Automated derivatization and analysis of malondialdehyde using column switching sample preparation HPLC with fluorescence detection.* 2008. **31**(2): p. 387-401.
26. Lord, H.L., et al., *Determination of malondialdehyde in human plasma by fully automated solid phase analytical derivatization.* Journal of Chromatography B, 2009. **877**(13): p. 1292-1298.
27. Li, P., et al., *Determination of malondialdehyde in biological fluids by high-performance liquid chromatography using rhodamine B hydrazide as the derivatization reagent.* Free Radical Biology and Medicine, 2013. **65**: p. 224-231.
28. Steghens, J.-P., et al., *Diaminonaphtalene, a new highly specific regent for HPLC-UV measurement of total and free malondialdehyde in human plasma or serum.* Free Radical Biology and Medicine, 2001. **31**(2): p. 242-249.
29. Stalikas, C.D. and C.N. Konidari, *Analysis of Malondialdehyde in Biological Matrices by Capillary Gas Chromatography with Electron-Capture Detection and Mass Spectrometry.* Analytical Biochemistry, 2001. **290**(1): p. 108-115.
30. Oh, J.-A. and H.-S. Shin, *Simple and sensitive determination of malondialdehyde in human urine and saliva using UHPLC–MS/MS after derivatization with 3,4-diaminobenzophenone.* Journal of Separation Science, 2017. **40**(20): p. 3958-3968.
31. Giera, M., et al., *Mild and selective labeling of malondialdehyde with 2-aminoacridone: assessment of urinary malondialdehyde levels.* Analyst, 2011. **136**(13): p. 2763-2769.
32. Ivica, J., et al., *HPLC Separation of Fluorescent Products of Lipid Peroxidation in Erythrocytes and Mitochondria.* 2011. **73**(1): p. 67-73.
33. Syslová, K., et al., *Rapid and easy method for monitoring oxidative stress markers in body fluids of patients with asbestos or silica-induced lung diseases.* Journal of Chromatography B, 2009. **877**(24): p. 2477-2486.
34. Yeo, H.C., et al., *Assay of Malondialdehyde in Biological Fluids by Gas Chromatography-Mass Spectrometry.* Analytical Biochemistry, 1994. **220**(2): p. 391-396.
35. Giera, M., H. Lingeman, and W.M.A.J.C. Niessen, *Recent Advancements in the LC- and GC-Based Analysis of Malondialdehyde (MDA): A Brief Overview.* 2012. **75**(9): p. 433-440.
36. Panuwet, P., et al., *Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring.* Critical Reviews in Analytical Chemistry, 2016. **46**(2): p. 93-105.
37. *Guidance for industry, bioanalytical method validation*, U.D.o.H.H. Services, Editor. 2018.
38. Pilz, J., I. Meineke, and C.H. Gleiter, *Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative.* Journal of Chromatography B: Biomedical Sciences and Applications, 2000. **742**(2): p. 315-325.
39. Chesher, D., *Evaluating Assay Precision.* The Clinical Biochemist Reviews, 2008. **29**(Suppl 1): p. S23-S26.

40. Binding, N., et al., *Analytical reliability of carbonyl compound determination using 1,5-dansylhydrazine-derivatization.* 1998. **362**(3): p. 270-273.
41. Hirayama, T., et al., *The high performance liquid chromatographic determination of total malondialdehyde in vegetable oil with dansyl hydrazine.* 1984. **35**(3): p. 338-344.

# CHAPTER 3

**Quantification of metabolites of polycyclic aromatic hydrocarbon (OH-PAH) in urine by ultra-performance liquid chromatography single quadrupole mass spectrometry**

Kostya Kartavenka[1], Savannah D. Gupton[2], Parinya Panuwet[2], Erin E. Williams[2], Cierra L. Johnson [2], Dana Boyd Barr[1,2] and P. Barry Ryan[1,2]

1 Department of Environmental Health, Rollins School of Public Health, Emory University

2 Laboratory of Exposure Assessment and Development of Environmental Research (LEADER), Rollins School of Public Health, Emory University

## ABSTRACT

A robust quantitative analytical method was developed and validated for the analysis of urinary polycyclic aromatic hydrocarbons monohydroxylated metabolites using 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride as a derivatizing reagent. Sample cleanup, derivatization, and post-derivatization cleanup were performed on a single solid phase extraction cartridge. The separation was achieved using ultra high-performance liquid chromatographic system coupled with single quadrupole mass selective detector. Reproducibility of measurements was shown to be valid for up to 9 days after the derivatization. Derivatives were characterized via collision induced dissociation using tandem quadrupole mass spectrometry (MS/MS). The presented method was cross-validated to the established liquid chromatography (LC) tandem mass spectrometry approach showing unbiased agreement of data for 2-hydroxynaphthalene, 2-/3-hydroxyphenanthrene, while for 1-hydroxynaphthalene, 1-/4-hydroxyphenanthrene and 1-hydroxypyrene equation to convert to LC MS/MS method were established. Cross-validation for 2-/3-hydroxyfluorene was not performed since peaks were not separable using LC MS/MS approach. Method relative recoveries (RR) and precision was assessed using a set of two quality control levels, showing acceptable values of 100-109% for RR and 2.3-4.4% for inter-day and 2.1-14% for intra-day precisions for all compounds. Overall, the method can be used as an alternative technique to the established highly expensive LC MS/MS in epidemiological studies.

## KEYWORDS

OH-PAHs, UPLC MS, derivatization

## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) represent group of compounds containing fused benzene rings resulting from incomplete, low-temperature combustion of organic materials (Haritash & Kaushik, 2009). Most common anthropogenic sources of PAHs include incomplete burning of fuels, plant materials, cigarettes, and industrial activities. Routes of exposure to PAHs include inhalation of ambient air, consumption of food prepared via grilling and flame-broiling (K.-H. Kim, Jahan, Kabir, & Brown, 2013), or exposed from other human activities such as e-waste burning sites (Y. Wang et al., 2012), and through skin from materials containing PAHs (Yebra-Pimentel, Fernández-González, Martínez-Carballo, Simal-Gándara, & nutrition, 2015). Occupational exposure to PAHs have been linked to acute responses such as eye irritation, nausea, vomiting, diarrhea (Unwin, Cocker, Scobbie, & Chambers, 2006). Long-term effects of exposure to PAHs may indicate a higher threat to human health (Abdel-Shafy & Mansour, 2016). PAHs were associated with several respiratory responses including acute lower respiratory tract infection (Dherani et al., 2008; Jary et al., 2016), chronic obstructive pulmonary disease (Assad et al., 2015), chronic bronchitis (Po et al., 2011), lung cancer (Raspanti et al., 2016; K. R. Smith et al., 2014), and upper aero-digestive cancers (Josyula et al., 2015). Non-respiratory responses include cataracts (K. R. Smith et al., 2014), cardiovascular disease (Fatmi & Coggon, 2016; K. H. Kim et al., 2011), low birth weight (Steenland et al., 2018), and infant mortality (Epstein et al., 2013; Vyas et al., 2018).

A common way of studying internal exposure to PAHs is through measuring their monohydroxylated metabolites (OH-PAHs) levels in urine (Bortey-Sam et al., 2016; Hansen, Mathiesen, Pedersen, & Knudsen, 2008; Urbancova et al., 2017). All analytical methods for analysis of OH-PAHs in urine require de-glucuronidation. Due to matrix effects (Lankova et al., 2016; Lawal, 2017; Panuwet et al., 2016; B.-C. Yang et al., 2017), sample preparation generally requires one of the following extraction: liquid-liquid extraction (Bortey-Sam et al., 2016; Z. Li et al., 2006), solid-phase extraction (SPE) (Chetiyanukornkul et al., 2006; Fan, Wang, Ramage, et al., 2012; Jongeneelen et al., 1988; Onyemauwa et al., 2009; Ramsauer et al., 2011), solid-phase micro extraction (C. J. Smith et al., 2002; B.-C. Yang et al., 2017), or other extraction (Itoh, Tao, & Ibusuki, 2005; Zhao et al., 2013) following by analysis using liquid

chromatography (LC) using a fluorescence detector (FD) (Chetiyanukornkul et al., 2006; Jongeneelen et al., 1988), or with tandem quadrupole mass spectrometry detector (MS/MS) (Bortey-Sam et al., 2016; Fan, Wang, Ramage, et al., 2012; Itoh et al., 2005; Onyemauwa et al., 2009; Ramsauer et al., 2011). Analysis has also been performed using gas chromatography coupled with high-resolution mass spectrometry (Z. Li et al., 2006; C. J. Smith et al., 2002), single-quadrupole mass detector (Motorykin et al., 2015), or MS/MS (Grova, Salquèbre, Schroeder, & Appenzeller, 2011). Derivatization is often required to ensure chromatographic separation, sensitivity of analysis, volatilization, or ionization (Z. Li et al., 2006; Motorykin et al., 2015); methods that do not require derivatization exist for LC MS/MS (Bortey-Sam et al., 2016; Fan, Wang, Ramage, et al., 2012; Onyemauwa et al., 2009) and LC FD, however LC FD methods commonly require large volume size of 10 mL (Chetiyanukornkul et al., 2006; Jongeneelen et al., 1988).

Among LC methods capable of analyzing OH-PAHs, only LC MS/MS methods give the best results for specificity in analysis of biological samples. However, analysis with LC MS/MS is associated with high-cost, limiting instrument availability especially in the low- and middle-income countries (LMIC). At the same time, approximately one-third of the world, predominantly from LMIC, is relying on biomass burning, which is leading to indoor and outdoor exposures to PAHs (Bruce et al., 2015; K. R. Smith et al., 2014; Steenland et al., 2018). It has been noted that, exposure to PAHs was responsible for over 2.8 million deaths in 2015 (Landrigan, 2017) and 85.6 million disability adjusted life years (Cohen et al., 2017). Absence of a reliable cost-efficient analytical technique may hinder their ability to understand better exposure and quantify OH-PAHs. Therefore, we aimed to develop LC single quadrupole mass spectrometry method capable to quantify urinary OH-PAHs. To improve selectivity and sensitivity, we use derivatizing agent, 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride (1,2-DMI-5-SC[2]). A similar derivatizing agent, 1,2-dimethyl-1H-imidazole-4-sulfonyl chloride (1,2-DMI-4-SC) was described by Xu, *et al.*, towards application on 1-hydroxypyrene using LC MS/MS (Xu & Spink, 2007), while 1,2-DMI-5-

[2] While derivatizing reagent itself is shortened as 1,2-DMI-5-SC, the adduct which it forms is 1,2-DMI-5-S, the same abbreviation pattern applies to 1,2-DMI-4-SC.

SC is widely used to measure of estrogen using HPLC MS/MS (Keski-Rahkonen, Desai, Jimenez, Harwood, & Handelsman, 2015; Q. Wang, Mesaros, & Blair, 2016). To control for loss during sample preparation and potential matrix effects, (Panuwet et al., 2016), isotope-dilution quantification was used. The method was validated and cross-validated on 28 samples which were analyzed using LC MS/MS.

## MATERIAL AND METHODS

### Chemical and reagents

We obtained acetonitrile (HPLC grade), methanol (HPLC grade), iso-octane (HPLC grade), ethyl-acetate (HPLC grade), sodium acetate from Fisher Chemical (Waltham, MA, USA), pentane (HPLC grade) from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ, USA), acetone (HPLC grade) from Sigma-Aldrich (Darmstadt, Germany). β-Glucuronidase from *Helix pomatia* and 1,2-dimethyl-1H-imidazole-5-sulphonyl chloride (Aldrich$^{CPR}$) were purchased from Sigma-Aldrich, ammonia hydroxide, 14.8 M from Ward's Science (Rochester, NY, USA), sodium hydroxide 0.1N standardized solution was obtained from Alfa Aesar (Haverhill, MA, USA), sodium bicarbonate, ACS was obtained from VWR (Radnor, PA, USA). All unlabeled OH-PAHs standards (1-naphtol (1-NAP) (98%), 2-naphtol (2-NAP) (98%), 2-hydroxyfluorene (2-FLU) (98%), 3-hydroxyfluorene (3-FLU) (98%), 1-hydroxyphenanthrene (1-PHE) (98%), 2-hydroxyphenanthrene (2-PHE) (98%), 3-hydroxyphenanthrene (3-PHE) (98%), 4-hydroxyphenanthrene (4-PHE) (98%), 1-hydroxypyrene (1-PYR) (98%), 6-hydroxychrysene (6-CHR) (98%)) and their labeled standards (1-naphtol (13C6, 99%), 2-naphtol (13C6, 99%), 2-hydroxyfluorene (13C6, 99%), 3-hydroxyfluorene (13C6, 99%), 1-hydroxyphenanthrene (13C4, 99%), 2-hydroxyphenanthrene (13C6, 99%), 3-hydroxyphenanthrene (131C6, 98%), 4-hydroxyphenanthrene (13C4, 99%), 1-hydroxypyrene (13C6, 99%), 6-hydroxychrysene(13C6, 98%)) were obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA) as a stock standard of 50 ug/mL in toluene.

1,2-DMI-5-SC derivatizing solution was prepared by diluting neat compound in acetone to yield concentration of 0.5 μg/ml. Water was purified using a Milli-Q® Ultrapure water purification system (EMD Millipore, Billerica, MA).

**Preparation of standard, quality control, and labeled internal standard solutions**

We prepared a high concentration stock solution of OH-PAHs via mixing of known amount of each stock standard solutions in amber glass volumetric flask in methanol. A set of ten calibration solutions were prepared via serial dilution (Table 1). Labeled internal standard (IS) stock solution was prepared in methanol via mixing all 10 labeled standards (Table 1). Two levels (Table 1) quality control solutions were prepared in amber glass volumetric flask in methanol via mixing of known amount of each stock standard solutions, prepared in a separate day from calibration solutions. All solutions were stored at -20 ºC prior to use.

**Table 1**. Summary of levels for calibration, quality control, and labeled internal standard solutions used in the method. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene.

| Purpose | Concentration in Storage Vial, ng/ml | Concentration in Sample (after spiking), ng/ml | Compounds |
|---|---|---|---|
| **Calibration** | 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 4000 | 0.125, 0.25, 0.5, 1.25, 2.5, 5, 12.5, 25, 50, 100 | 1-NAP, 2-NAP |
| | 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 400 | 0.0125, 0.025, 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, 5, 10 | 2-/3-FLU, 1-/2-/3-/4-PHE, 1-PYR, 6-CHR |
| **Quality control, low** | 50 | 1.25 | 1-NAP |
| | 100 | 2.5 | 2-NAP |
| | 20 | 0.5 | 2-/3-FLU, 1-/2-/3-/4-PHE, 1-PYR, 6-CHR |
| **Quality control, high** | 250 | 6.25 | 1-NAP |
| | 500 | 12.5 | 2-NAP |
| | 100 | 2.5 | 2-/3-FLU, 1-/2-/3-/4-PHE, 1-PYR, 6-CHR |
| **Labeled internal standard** | 500 | 25 | 1-/2-NAP |
| | 100 | 2.5 | 2-/3-FLU, 1-/2-/3-/4-PHE, 1-PYR, 6-CHR |

**Preparation of blank, calibration, quality control, and unknown samples**

To account for matrix effects (*Guidance for industry, bioanalytical method validation*, 2018; Panuwet et al., 2016), OH-PAHs calibration solutions were prepared in ten-fold diluted pooled urine: 2 mL of

diluted urine was transferred into test tube and spiked with 50 μL standard of respective level (Table 1). Blank [3]samples were prepared with the same matrices as calibration solutions. Clean-up, derivatization and purification steps for calibration, QC and blanks solutions were performed in the same manner as unknown samples described below (Figure 1).

**Sample preparation for analysis as a derivative**

Prior to analysis, each urine sample was stored at -20 ºC. On the day of analysis, urine was brought to room temperature, 2 mL of urine was transferred to 15 mL centrifuge tube, spiked with 50 μL labeled IS and spiked with 2 mL of β-glucuronidase for deconjugation at 37 ºC for 16 hours (Figure 1). Upon completion of the deconjugation, content of each tube was loaded into Strata X 33u cartridge (Phenomenex (Torrance, CA, USA)), conditioned with 1.5 mL acetonitrile and 2.5 mL Milli-Q. Cartridges were washed with 2.5 mL Milli-Q, 1 mL 1:4 methanol:Milli-Q, and 2 mL 1:1 acetone:Milli-Q solutions. Then cartridges were dried under vacuum for 5 minutes, new set of test tubes were installed and cartridges were eluted with 0.5 mL 9:1 acetone:methanol and 2 mL 100% acetone, followed by vacuum to assure full elution of the eluent. Eluent was removed in the evaporator at 55 ºC under 10 psi nitrogen flow. During evaporation of the eluent, cartridges were conditioned with 2.5 mL Milli-Q followed by 0.5 mL of bicarbonate-sodium hydroxide buffer solution (pH 10.2). When samples were evaporated, 0.2 mL of the same buffer was added to each sample, then spiked with 0.2 mL of 1,2-DMI-5-SC solution (0.1 mg/mL), vortexed and transferred to corresponding cartridge. Derivatization was carried out on the SPE cartridge at room temperature for 30 minutes. Salts were removed with 2.5 mL Milli-Q and the SPE cartridge was washed further with 2.5 mL 4:1 methanol:Milli-Q solution. Cartridges were dried under vacuum applied for 5 minutes and derivatives were eluted with 5 mL acetonitrile. The solvent was then removed in the evaporator at 55 ºC under nitrogen flow at 10 psi. Samples were reconstituted with 100 μL of 2:3 acetonitrile:Milli-Q solution.

[3] FDA uses term “zero calibrator” instead of blank

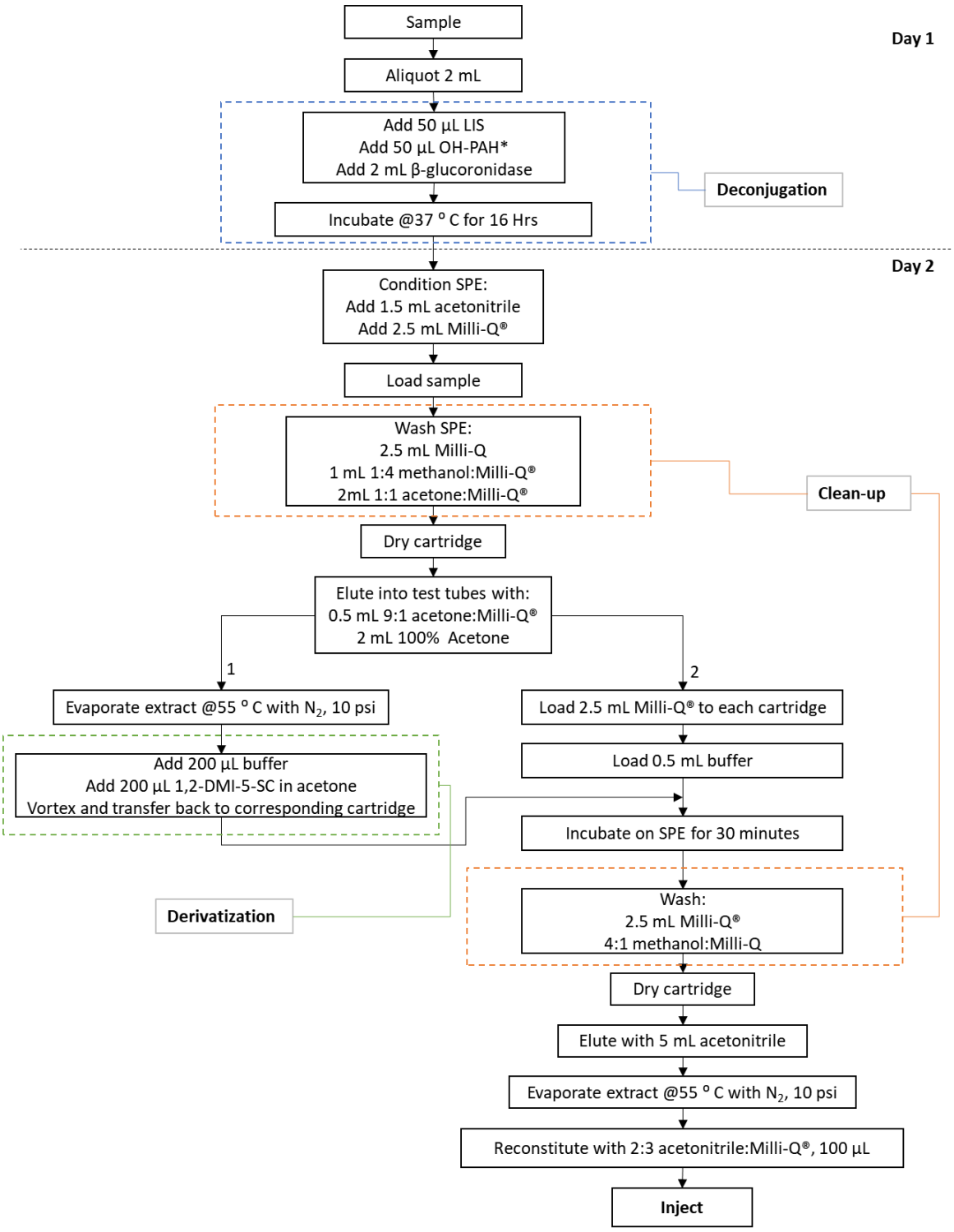



**Figure 1**. Sample preparation flow chart. Abbreviations: 1,2-DMI-5-SC is a derivatizing agent, 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride, LIS – labeled internal standard, * (asterisk) referred to calibration solutions preparation, where unlabeled standard is spiked into the matrix 10X diluted urine,

SPE – stands for Strata-X 33u solid phase extraction cartridge, “buffer” stands for sodium bicarbonate-sodium hydroxide buffer solution adjusted to pH 10.2.

**Sample preparation for analysis in their free form**

For cross-validation purpose, tandem-quadrupole mass spectrometry analysis was performed. For this analysis sample volume, spiking amount, and deconjugation steps were the same (Figure 1, day 1). OH-PAHs were extracted sequentially with 4 mL penthane:ethyl acetate 7:3 and 4 mL iso-octane:ethyl acetate 7:3 solutions. Both fractions were combined in 15 mL centrifuge test tube and evaporated under nitrogen flow (15 psi) at 45 ºC. Samples were reconstituted with 50 µL Milli-Q:methanol 1:1 solution.

**Chromatographic separation and mass spectrometric conditions**

**Analysis of OH-PAHs in the derivatized forms**

The sample analysis was carried out using an Agilent Infinity 1290 ultra-performance liquid chromatograph (UPLC) with a G4226A autosampler connected to an Agilent G6150B single quadrupole mass spectrometer (MSD) (Santa Clara, CA, USA). The separation was performed on Agilent Eclipse Plus Phenyl-Hexyl RRHD (1.8 µm 2.1x150 mm). The column temperature was set to 45 ºC. The binary mobile phase consists of A, 100% Milli-Q®, and B, 100% acetonitrile. Prior to injection of 7 µL, needle wash was performed in acetonitrile:Milli-Q 4:1 for 5 seconds, separation was achieved via a gradient elution program (Table 2). The total program runtime was 35.0 minutes.

**Table 2.** Gradient elution liquid chromatography program for analysis of monohydroxylated polycyclic aromatic hydrocarbons in the form of derivatives in urine samples. Where A and B stands for binary mobile phase composition expressed in percentage, A –Milli-Q® without additives, B – 100% acetonitrile.

| Time, min | B composition | Flow rate, ml/min | Time, min | B composition | Flow rate, ml/min |
|---|---|---|---|---|---|
| 0.0 | 33.0 % | 0.6 | 26.6 | 53.0 % | 0.3 |
| 7.7 | 33.0 % | 0.6 | 29.0 | 53.0 % | 0.3 |
| 8.0 | 33.0 % | 0.3 | 29.2 | 80.0 % | 0.3 |
| 10.3 | 33.0 % | 0.3 | 30.0 | 80.0 % | 0.3 |
| 10.7 | 33.0 % | 0.6 | 30.5 | 100.0 % | 0.3 |
| 18.7 | 33.0 % | 0.6 | 31.0 | 100.0 % | 0.3 |
| 19.1 | 36.0 % | 0.6 | 31.3 | 100.0 % | 0.7 |
| 19.5 | 36.0 % | 0.6 | 32.7 | 100.0 % | 0.7 |
| 19.7 | 36.0 % | 0.3 | 33.0 | 33.0 % | 0.7 |
| 22.0 | 36.0 % | 0.3 | 33.4 | 33.0 % | 0.7 |
| 22.2 | 41.0 % | 0.3 | 33.7 | 33.0 % | 0.6 |
| 26.5 | 41.0 % | 0.3 | | | |

The target derivatives including their labeled IS derivatives were analyzed using positive electrospray ionization. The mass spectrometer was operated in a single ion monitoring (SIM) mode: time segments, monitored m/z, dwell time, and gain are summarized in Table 3. The peak of the target derivatives was selected based on their retention time (RT), relative retention time to labeled IS peak, peak shape, and signal-to-nose ratio.

**Table 3**. Time segments and select ion monitoring (SIM) mode parameters.

| Time segment, min | Compound | m/z | Gain factor | Dwell time, ms |
|---|---|---|---|---|
| 7 – 11.4 | 1-/2-Hydroxynapthalene | 303 | 5 | 204 |
| | 1-/2-Hydroxynaphthalene, $^{13}$C6 | 309 | | 204 |
| 19.6 – 23.9 | 4-Hydroxyphenanthrene | 353 | 5 | 101 |
| | 4-Hydroxyphenanthrene, $^{13}$C4 | 357 | | 101 |
| | 2-/3-Hydroxyfluorene | 341 | | 101 |
| | 2-/3-Hydroxyfluorene, $^{13}$C6 | 347 | | 101 |
| 24.0 – 28.4 | 1-/2-/3-Hydroxyphenanthrene | 353 | 5 | 135 |
| | 1-Hydroxyphenanthere, $^{13}$C4 | 357 | | 135 |
| | 2-/3-Hydroxyphenanthere, $^{13}$C6 | 359 | | 135 |
| 28.5 – 29.9 | 1-Hydroxypyrene | 377 | 5 | 204 |
| | 1-Hydroxypyrene, $^{13}$C6 | 383 | | 204 |
| 30.0 – 31.0 | 6-Hydroxychrysene | 403 | 5 | 204 |
| | 6-Hydroxychrysene, $^{13}$C6 | 409 | | 204 |

Across all time segments the following parameters were constant: nozzle voltage was set to 2000 V, nitrogen was used as a dry and sheath gas, with flow rates set to 5.7 L/min and 12.0 L/min, respectively, drying gas and sheath gas temperatures were set to 350 ºC and 360 ºC, respectively. The nebulizer pressure was set to 30 psig and the fragmentor voltage was set to 150 V. The capillary voltage was adjusted to maximize instrument response for target analytes eluted at a different organic content (Table 4).

**Table 4**. Single quadrupole capillary voltage values.

| Time segment, min | Value, kV | Time segment, min | Value, kV |
|---|---|---|---|
| 0.0 – 18.0 | 1.8 | 23.5 – 31.0 | 2.0 |
| 19.0 – 23.0 | 1.7 | 31.0 – 35.0 | 1.8 |

**Analysis of OH-PAHs in a free form**

For method cross-validation purpose, sample analysis was performed on an Agilent Infinity 1260 high-performance liquid chromatograph (HPLC) with a G1367E autosampler connected to an Agilent G6460A tandem quadrupole mass spectrometer (Santa Clara, CA, USA). The method was adopted from Lankova, *et al.*, (Lankova et al., 2016) with a minor changes. The separation was achieved using Phenomenex Kinetex F5 (5 μm 3x100 mm) column. The column temperature was set to 50 ºC. The binary mobile phase consists of A, Milli-Q adjusted to pH 8.5 with ammonia hydroxide, B, methanol without additives.

5 μL was injected with needle wash in 100% methanol for 5 seconds, separation was achieved via LC gradient elution program (Table 5). The total program runtime was 20.2 minutes.

**Table 5.** Graduate elution liquid chromatography program for analysis of monohydroxylated polycyclic aromatic hydrocarbons in urine samples. Where A and B stands for binary mobile phase composition expressed in percentage, A –Milli-Q® adjusted to pH 8.5 with ammonia hydroxide, B – 100% methanol.

| Time, min | B composition | Flow rate, ml/min | Time, min | B composition | Flow rate, ml/min |
|---|---|---|---|---|---|
| 0.0 | 30.0 % | 0.4 | 12.7 | 65.5 % | 0.7 |
| 0.1 | 30.0 % | 0.4 | 13.0 | 70.0 % | 0.7 |
| 1.0 | 40.0 % | 0.65 | 13.2 | 71.0 % | 0.7 |
| 5.0 | 40.0 % | 0.65 | 13.4 | 72.0 % | 0.4 |
| 5.3 | 40.0 % | 0.4 | 14.0 | 75.0 % | 0.4 |
| 6.0 | 40.0 % | 0.4 | 15.0 | 100.0 % | 0.4 |
| 8.0 | 45.0 % | 0.4 | 16.7 | 100.0 % | 0.45 |
| 8.2 | 45.5 % | 0.7 | 16.71 | 100.0 % | 0.65 |
| 10.4 | 51.0 % | 0.7 | 17.0 | 100.0 % | 0.65 |
| 10.7 | 51.7 % | 0.4 | 17.5 | 30.0 % | 0.65 |
| 12.0 | 55.0 % | 0.4 | 18.0 | 30.0 % | 0.65 |
| 12.5 | 62.5 % | 0.4 | 18.01 | 30.0 % | 0.4 |

The target analytes including their labeled IS were ionized using negative electrospray ionization. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode (Table 6). Across all time segments/analytes the following parameters were constant: capillary and nozzle voltages were set to 3000 V and 1000 V respectively, nitrogen was used as a dry and sheath gas, their flow rates were set to 5.0 L/min and 12.0 L/min, respectively, drying gas and sheath gas temperatures were set to 330 ºC and 350 ºC, respectively, the nebulizer pressure was set to 45 psig, the fragmentor voltage was set to 150 V, cell accelerated voltage was set to 5 V. Fragmentation occurs in collision induced dissociation mode.

**Table 6**. Multiple reaction monitoring (MRM) mode parameters for quantification of monohydroxylated polycyclic aromatic hydrocarbons metabolites. Cell accelerated voltage was set to 5 V and fragmentor voltage across all transitions. Abbreviations: CE – collision energy, NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene

| Time segment, min | Analyte | MRM Transition | CE, V | Delta EMV | Dwell time, ms |
|---|---|---|---|---|---|
| 5 – 9 | 1- and 2-NAP | 143 -> 115 | 28 | 1000 | 350 |
| | 1- and 2-NAP, $^{13}$C6 | 149 -> 121 | 28 | | 350 |
| 9 – 13 | 2- and 3-FLU | 181 -> 180 | 25 | 800 | 150 |
| | 2- and 3-FLU, $^{13}$C6 | 187 -> 186 | 25 | | 150 |
| 13 – 15.3 | 1-, 2-, 3- and 4-PHE | 193 -> 165 | 36 | 1000 | 250 |
| | 1- and 4- PHE, $^{13}$C4 | 197 -> 168 | 36 | | 250 |
| | 2- and 3- PHE, $^{13}$C6 | 199 -> 171 | 36 | | 250 |
| 15.3 – 18 | 1-PYR | 217 -> 189 | 38 | 500 | 250 |
| | 1-PYR, $^{13}$C6 | 223 -> 195 | 38 | | 250 |
| | 6-CHR | 243 -> 215 | 38 | | 250 |
| | 6-CHR, $^{13}$C6 | 249 -> 221 | 38 | | 250 |

To assess instrument response at lowest limit of quantification (LLOQ) for non-derivatized OH-PAHs in negative ESI SIM mode, MRM program for HPLC MS/MS was converted into SIM mode (using second quadrupole (MS2)) without any additional changes of either chromatography or other MS parameters.

**Method validation**

Method validation was performed only for UPLC MS as that is the purpose of this paper. The method precision was determined in according to protocol described by Chesher (Chesher, 2008) using two quality control levels (QCL and QCH, Table 1). Replicate samples (5 samples/day at each concentration level) were prepared and analyzed daily during a 5-day period to determine inter-day and intra-day precision. The precisions were expressed as relative standard deviation (%RSD) values.

The method relative recoveries (RR) were determined, according to FDA guideline(*Guidance for industry, bioanalytical method validation*, 2018) using a set of replicates, quality control materials (QCL

and QCH) prepared at concentrations listed in Table 1 (N = 25). The method RR were calculated by dividing the average, quantified values of the quality control materials with the respective, expected concentration values for both levels. The method RR were expressed as percentage values. Lowest calibration level was selected in according to FDA criteria for bioanalytical method validation(*Guidance for industry, bioanalytical method validation*, 2018). Limit of detection was selected as a lowest calibration level.

A study of autosampler storage stability was performed via a triplicate injection of QCL and QCH after 50, 160, 200, and 230 hours after the derivatization. Samples were stored in the autosampler at room temperature (approximately 22 ºC) during the intervening times. Stability was assessed via relative change of both concentration and labeled IS response to its mean value at time 0 (the first injection). FDA guidance recommends ±15% for precision, we expressed this value as a relative change of concentration to its mean value for the first injection (reference value). Additionally, we performed a correlation test for relative loss/gain in between compounds to assess if degradation is universal for all tested OH-PAHs.

$$Relative\ change = \frac{Response_{Time\ i,replicate\ j}}{Mean\ (Response_{Time=0})} - 1$$

SPE method recovery was evaluated using one-level quality control solution by calculating relative ratio of unlabeled and labeled peak areas for sample spiked prior to load into SPE and sample spiked after elution from SPE by measuring OH-PAHs in their free form using HPLC MS/MS method. To assess SPE recoveries of OH-PAHs in the derivative form, the same approach was used, however instead of direct injection into the instrument, samples were derivatized following by the established protocol.

Matrix effects were evaluated by comparing mean responses (peak area) of labeled IS presented in ten-times diluted, across different analytical batches.

As part of validation and cross-validation between UPLC MS and LC MS/MS methods, sample from previously analyzed study (Fandiño-Del-Rio et al., 2017) was used. Samples were picked randomly from the freezer and analyzed without any information with respect to sample identification. Therefore, the aim of the present study is purely method development and cross-validation.

### Statistical analysis

All statistical analysis was conducted in RStudio (Version 1.0.14) using R (Version x64 3.5.1). The following packages were used to accomplish data transformation and analysis: blandr (Ver. 0.5.1), data.table (Ver. 1.11.4), ICC (Ver. 2.3.0), shiny (Ver. 1.1.0), dplyr (Ver. 0.7.6), ggplot2 (Ver. 3.0.0).

## RESULTS AND DISCUSSION

### Derivatization and chromatographic separation condition

Initially, we performed successful derivatization of OH-PAHs using dansylchloride (DC) and 1,2-DMI-4-SC. Both reagents were previously described for quantification of 1-hydroxypyrene (Y. Li et al., 2005; Xu & Spink, 2007) and 3-hydroxybenzo[a]pyrene (Yao et al., 2014). However, derivatization with DC compromises chromatographic separation of OH-PAHs derivatives (data not shown). 1,2-DMI-4-SC improves separation; however, several compounds were not been able to be separated (Figure 2). The following columns (Agilent (C18 and Phenyl-Hexyl), Phenomenex (Fusion-RP, Bisphenyl, Hydro-RP, F5)) were tested to ensure separation of OH-PAHs-1,2-DMI-4-S adduct, however the best separation was achieved with Agilent Phenyl-Hexyl that we initially selected. Several other derivatizing agents, which we expect to have similar reaction mechanism were tested, including 1-acetylpiperidine-4-sulfonyl chloride, 1-cyanopropane-1-sulfonyl chloride, and 3-methylpiperidine-1-sulfonyl chloride (purchased from Sigma-Aldrich). We were not able to register ions useful for quantification, other than for 1,2-DMI-5-SC. OH-PAHs-1,2-DMI-5-S adducts had slightly lower instrument response compared to OH-PAHs-1,2-DMI-4-S, however complete separation was achieved (Figure 3).

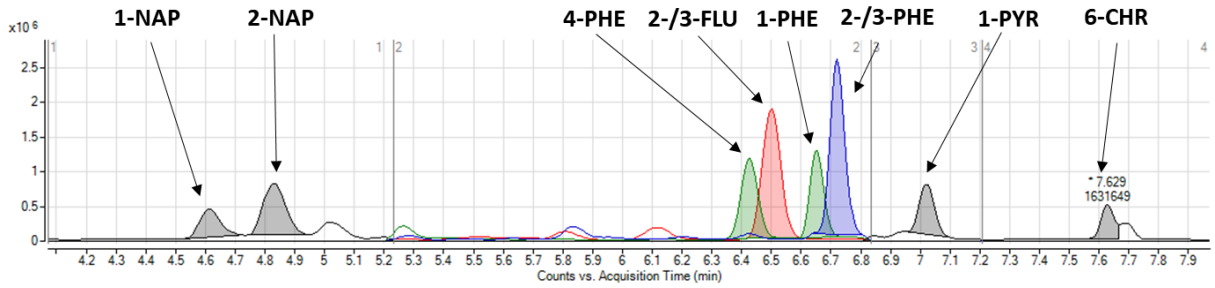



**Figure 2**. Separation of monohydroxylated polycyclic aromatic hydrocarbon derivatized with 1,2-dimethyl-1H-imidazole-4-sulfonyl chloride on Agilent Eclipse Plus Phenyl-Hexyl RRHD (1.8 µm 2.1x50 mm). 2-/3-FLU and 2-/3-PHE were completely non-separable. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene.

Derivatization conditions were originally taken from the previously published manuscripts on DC and 1,2-DMI-4-SC: derivatization for 10 minutes at 60 ºC and using liquid-liquid extraction with linear saturated hydrocarbons such as pentane and n-hexane (Xu & Spink, 2007; Yao et al., 2014). While this approach is viable, recoveries of low-molecular weight OH-PAHs like 1-/2-NAP are relatively low. Additionally, we experienced evaporation of acetone at the described condition, resulting in test tube lids to pop out, which may lead to cross-contamination. Further, we studied kinetics of adducts formation (Figure 3) suggesting rapid product formation and their subsequent degradation. By observing kinetic curves, it can be concluded that the rate of adduct formation is different across compounds, likely resulting in heavier compounds to react faster. Kinetics data suggested us, that the approach will be hardly applicable towards routine analysis of a batch containing 40 samples.

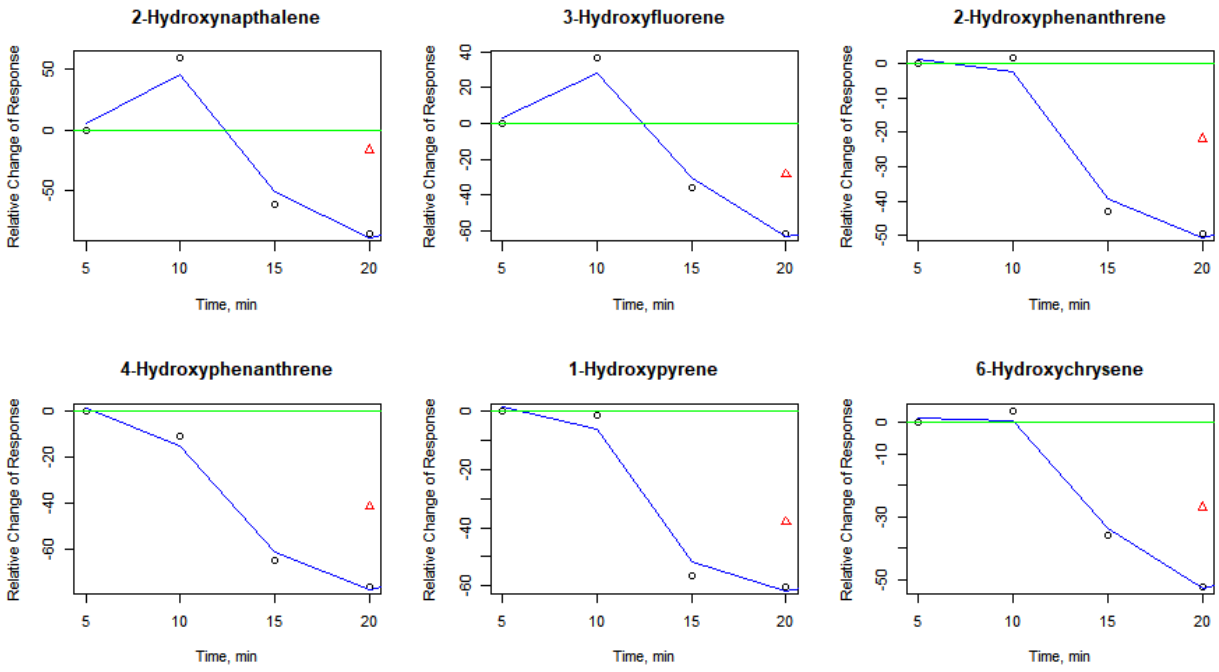



**Figure 3**. Monohydroxylated polycyclic aromatic hydrocarbons adducts formation with 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride (1,2-DMI-5-SC) at 60 ºC using n-hexane for liquid-liquid extraction. The data is expressed as response change (RC) to absolute response at time = 5 minutes. Red triangle represents RC of adducts spiked with additional 100 µL of 1,2-DMI-5-SC after 20 minutes of initial spiking with derivatizing reagent, carried for additional 20 minutes.

To overcome these limitations, we performed derivatization on the same SPE cartridge from which compounds were purified and extracted, expecting that derivatizing reagent may react with compounds that were not eluted from the SPE cartridge since it was previously reported that elution of OH-PAHs from SPE has low recoveries (Motorykin et al., 2015; C. J. Smith et al., 2002). Similarly to LLE approach, we performed kinetics on the SPE cartridge, specifically, the reaction was stopped by washing out salts from the cartridge with 2.5 mL of Milli-Q followed by 2 mL wash with 1:4 Milli-Q:methanol solution (Figure 4). We can observe that the formation OH-PAHs except 1-/2-NAP increases over time. For further method development and validation, we set reaction time to 30 minutes.

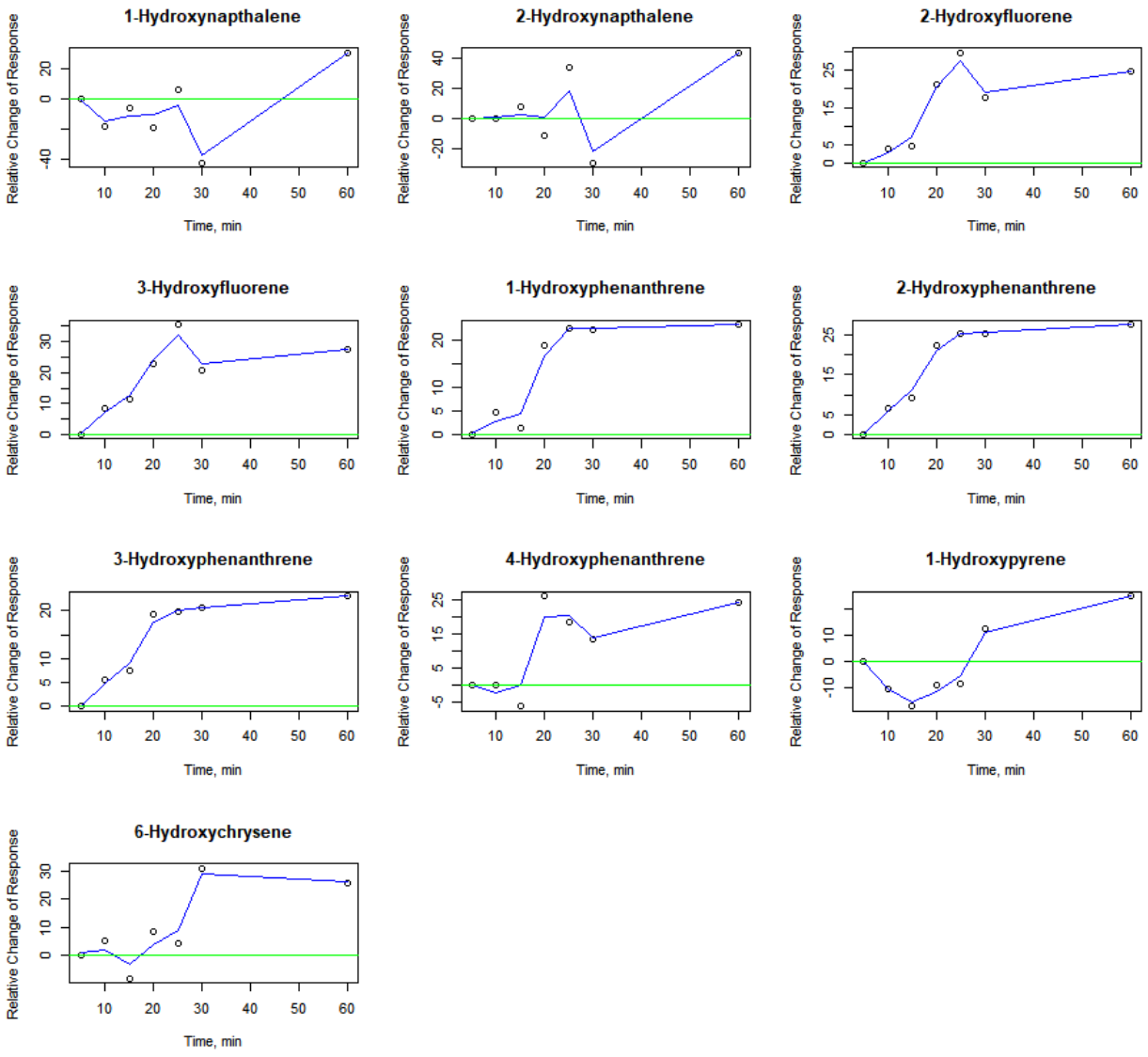



**Figure 4**. Kinetics of monohydroxylated polycyclic aromatic hydrocarbons adducts formation with 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride at room temperature (approximately 22 ºC). The data is expressed as response change (RC) to absolute response at time = 5 minutes.

Following selection of the derivatizing reagent and reaction settings, chromatographic separation conditions were optimized to achieve separation of target ions from co-eluted peaks. A typical chromatogram of the lowest level of quantification is presented on Figure 5. Total program runtime is 35 minutes. Due to relatively high LLOQ for 6-CHR which does not allow us to quantify any of samples in

the validation set, this compound was excluded from further validation. Chromatogram for OH-PAHs in their non-derivatized form at LLOQ of the method with derivatization are shown on Figure 6. Total program runtime is 20.2 minutes. Separation of 2-FLU and 3-FLU was not achieved under applied conditions. It can be seen, that certain peaks cannot be quantified at the same level without derivatization using SIM mode. While absolute response from the instrument is presented, it should not be used to compare sensitivity of the methods since these values were obtained from different instruments which have different "age" of electron multiplier, electron multiplier voltage, and instrument cleanliness.

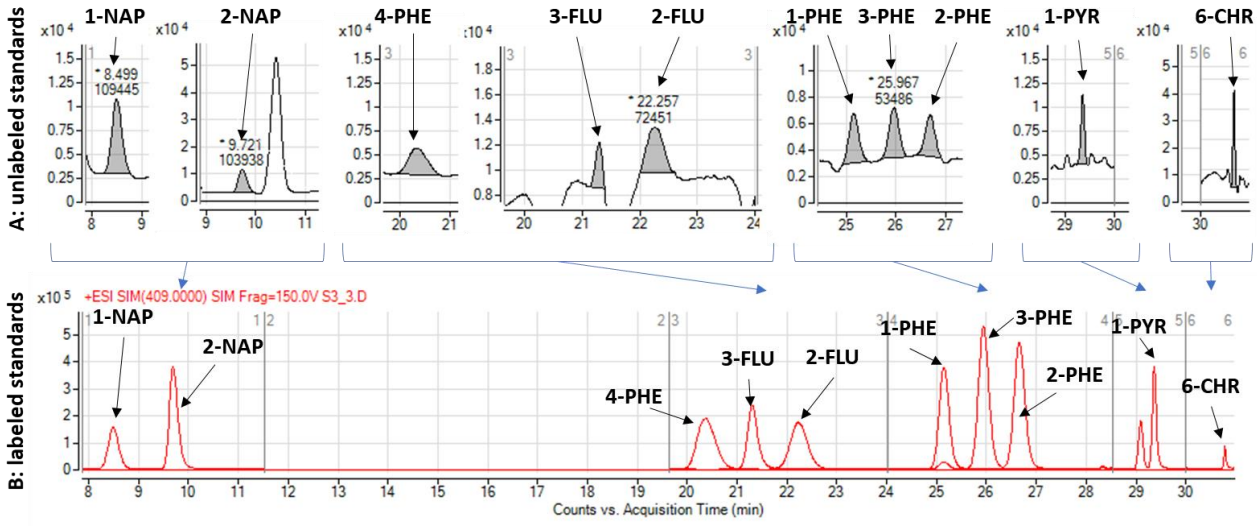



**Figure 5**. A: typical chromatograms of the lowest level of quantification (LLOQ) in ten times diluted urine obtained via derivatization of corresponding monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) metabolites with 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride (1,2-DMI-5-SC). Showed OH-PAHs LLOQ levels: 1-NAP – 1ng/ml, 2-NAP – 0.5 ng/ml, 4-PHE – 0.1 ng/ml, 3-FLU – 0.1 ng/ml, 2-FLU – 0.1 ng/ml, 1-PHE – 0.025 ng/ml, 3-PHE – 0.025 ng/ml, 2-PHE – 0.05 ng/ml, 1-PYR – 1 ng/ml, 6-HCR – 0.5 ng/ml. B: overall chromatogram of labeled internal standards of OH-PAHs-1,2-DMI-5-SC. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene.

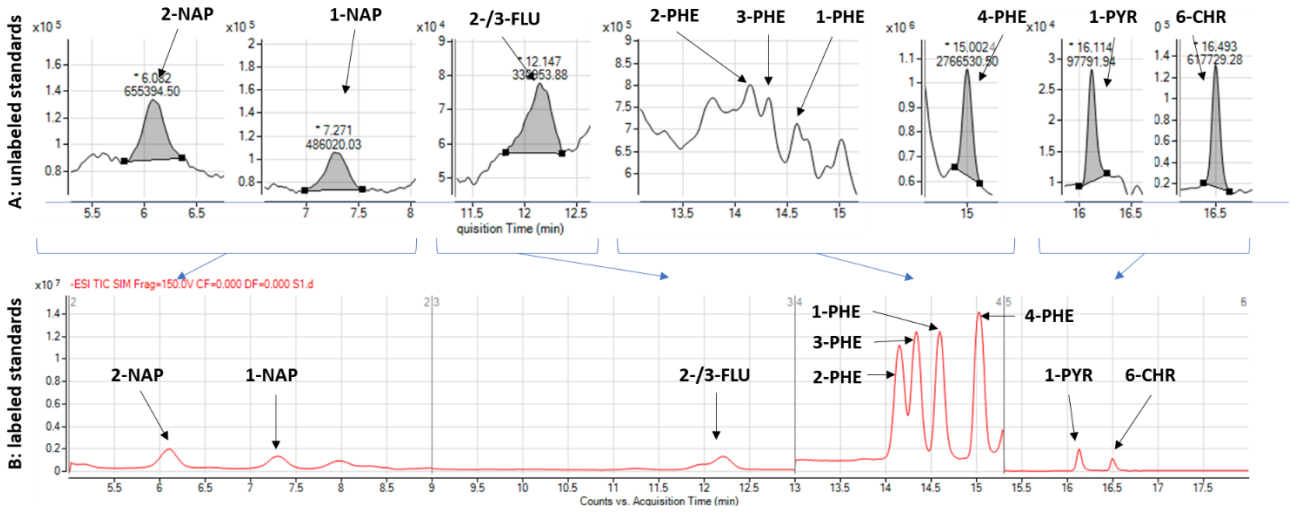



**Figure 6**. A: chromatograms achieved via injecting of the lowest level of quantification (LLOQ) obtained from the method with derivatization for corresponding monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) metabolites. Showed OH-PAHs levels: 1-NAP – 1ng/ml, 2-NAP – 0.5 ng/ml, 4-PHE – 0.1 ng/ml, 2-/3-FLU – 0.2 ng/ml, 1-PHE – 0.025 ng/ml, 3-PHE – 0.025 ng/ml, 2-PHE – 0.05 ng/ml, 1-PYR – 1 ng/ml, 6-HCR – 0.5 ng/ml. B: overall chromatogram of labeled internal standards of OH-PAHs. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene.

Overall, we developed a sensitive quantitative analytical method capable of measuring urinary levels of monohydroxylated polycyclic aromatic hydrocarbons metabolites using LC MS. To our knowledge, that is the first reported method capable of quantification of biologically relevant concentrations by that type of instrument. Single quadrupole instruments are typically 1/3 to ½ the cost of triple quadrupoles making them more accessible to LMIC, where most of the exposure to HAP occurs. In addition to usage of cost-effective instrument, our method uses the only SPE cartridge through the whole sample preparation including initial sample clean-up, derivatization of OH-PAHs, and a final clean-up operation. Commonly, methods with derivatization require a combination of two separate extraction appraoches (Motorykin et al., 2015; Xu & Spink, 2007).

**Selection of limit of quantification**

LLOQ was selected by taking into the consideration the following parameters: peak shape in blank compared to calibration level, relative retention time between labeled and unlabeled standard, and relative recovery not to exceed 80-120% range. LLOQs are summarized in Table 7.

**Method validation**

RR, inter- and intra-day precision, lowest limit of quantification and curve that was used to quantify relationship between relative response (ratio between unlabeled to labeled peak count) and concentration are shown in Table 7. Overall, the method showed acceptable RR and precisions in according to FDA guidance, specifically RR were in 100-109% range, inter-day precision in 2.3-5.7% range, and intra-day precision in 2.1-14% range for all compounds (*Guidance for industry, bioanalytical method validation*, 2018).

**Table 7.** Method performance at measuring monohydroxylated polycyclic aromatic hydrocarbon metabolites in urine in the derivatized form using 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride. R-squared calculated as an average r-squared of 5 runs. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, LLOQ – lowest limit of quantification.

| Analyte | R-squared | Curve | LLOQ | Relative recovery | | Precision | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Within-day | | Between-days | |
| | | | | QCL | QCH | QCL | QCH | QCL | QCH |
| **1-NAP** | 0.999 | Linear | 0.5 ng/ml | 108% | 108% | 5.7% | 3.6% | 5.7% | 13% |
| **2-NAP** | 0.999 | Quadratic | 0.25 ng/ml | 100% | 106% | 3.2% | 3.5% | 3.3% | 14% |
| **2-FLU** | 0.999 | Linear | 50 pg/ml | 109% | 105% | 2.9% | 3.1% | 2.6% | 5.9% |
| **3-FLU** | 0.999 | Linear | 50 pg/ml | 107% | 106% | 2.7% | 3.0% | 2.4% | 6.6% |
| **1-PHE** | 0.999 | Linear | 12.5 pg/ml | 107% | 103% | 2.7% | 4.4% | 2.4% | 7.8% |
| **2-PHE** | 0.999 | Linear | 12.5 pg/ml | 106% | 104% | 2.7% | 3.1% | 2.4% | 5.9% |
| **3-PHE** | 0.999 | Quadratic | 12.5 pg/ml | 104% | 102% | 2.8% | 3.6% | 2.5% | 6.4% |
| **4-PHE** | 0.999 | Linear | 50 pg/ml | 106% | 103% | 2.4% | 2.7% | 2.2% | 5.0% |
| **1-PYR** | 0.999 | Quadratic | 25 pg/ml | 102% | 102% | 2.3% | 2.9% | 2.1% | 6.2% |

Further we examine autosampler storage stability of derivatives. Relative change of concentration (Figure 7) and LIS (Figure 8) to its mean value at time = 0 are shown. From relative change of concentrations, we concluded that derivatives are stable over 9 days at a room temperature (approximately 22 ºC). At the same time, from LIS relative changes over time, we can observe that 1-NAP and 2-NAP response is not diminished, while the rest studied OH-PAHs experience degradation. Assuming that the loss of response may be as a result of instrument loss of sensitivity due to contamination, we performed correlation analysis for relative changes in LIS responses (Table 8). All relative loss/gain of LIS response were statistically positively correlated. However, we can see that while 1-NAP and 2-NAP are correlated, their correlation to other OH-PAHs is less than among other OH-PAHs themselves. Likely that suggests that 1-NAP and 2-NAP derivatives are more stable compared to other OH-PAHs derivatives. Overall, though we experienced loss in response, isotope-dilution approach compensates this loss equally, making changes to concentration insignificant (Figure 7). Our findings on degradation of derivatives suggest that use of surrogates instead of internal standards should be taken cautiously for analysis of OH-PAHs.

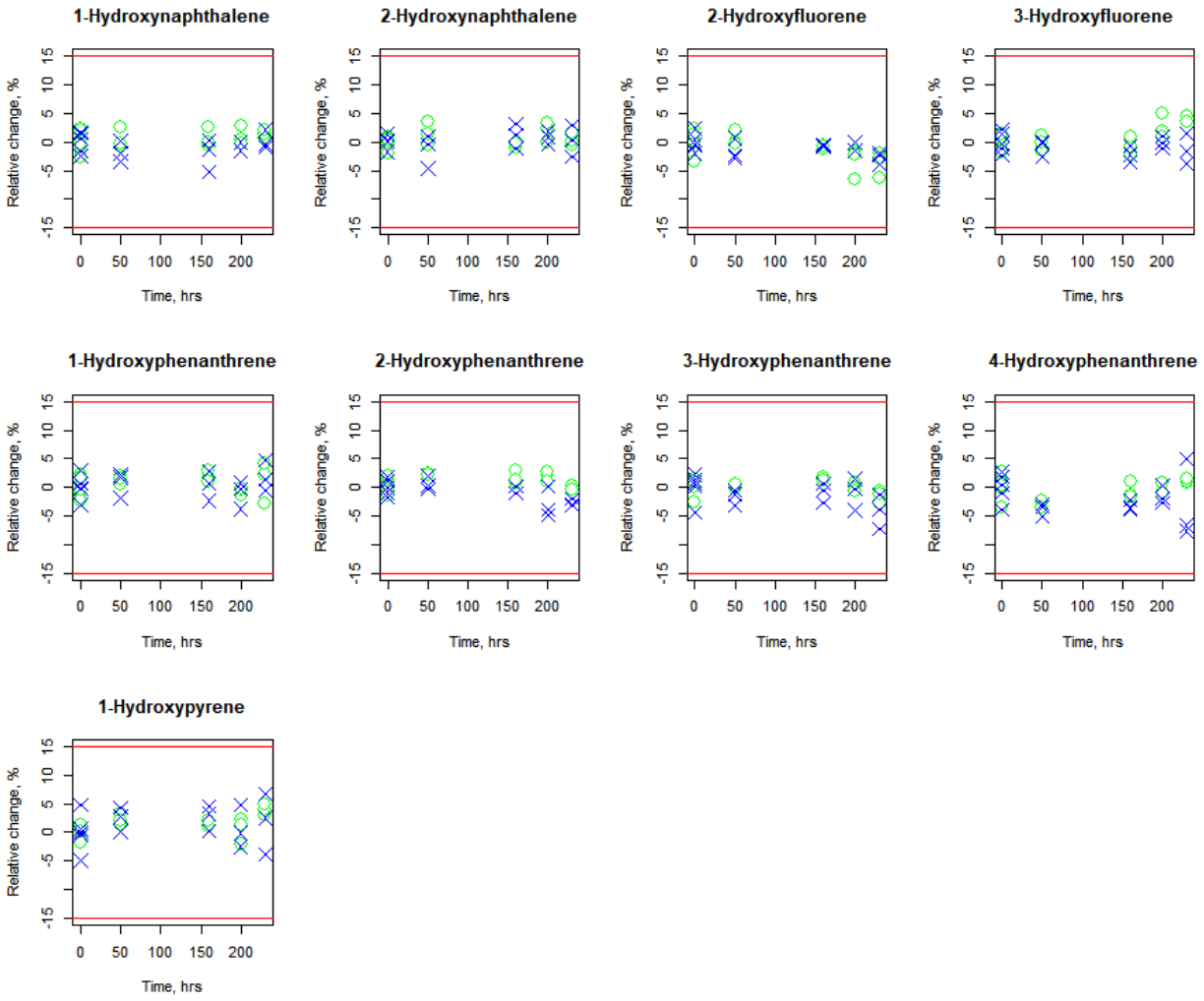



**Figure 7**. Relative change of two quality control level (high – green round, low – blue cross) to their mean concentration at time 0 (the first injection), expressed in percentage. Red line shows ±15% to relative change.

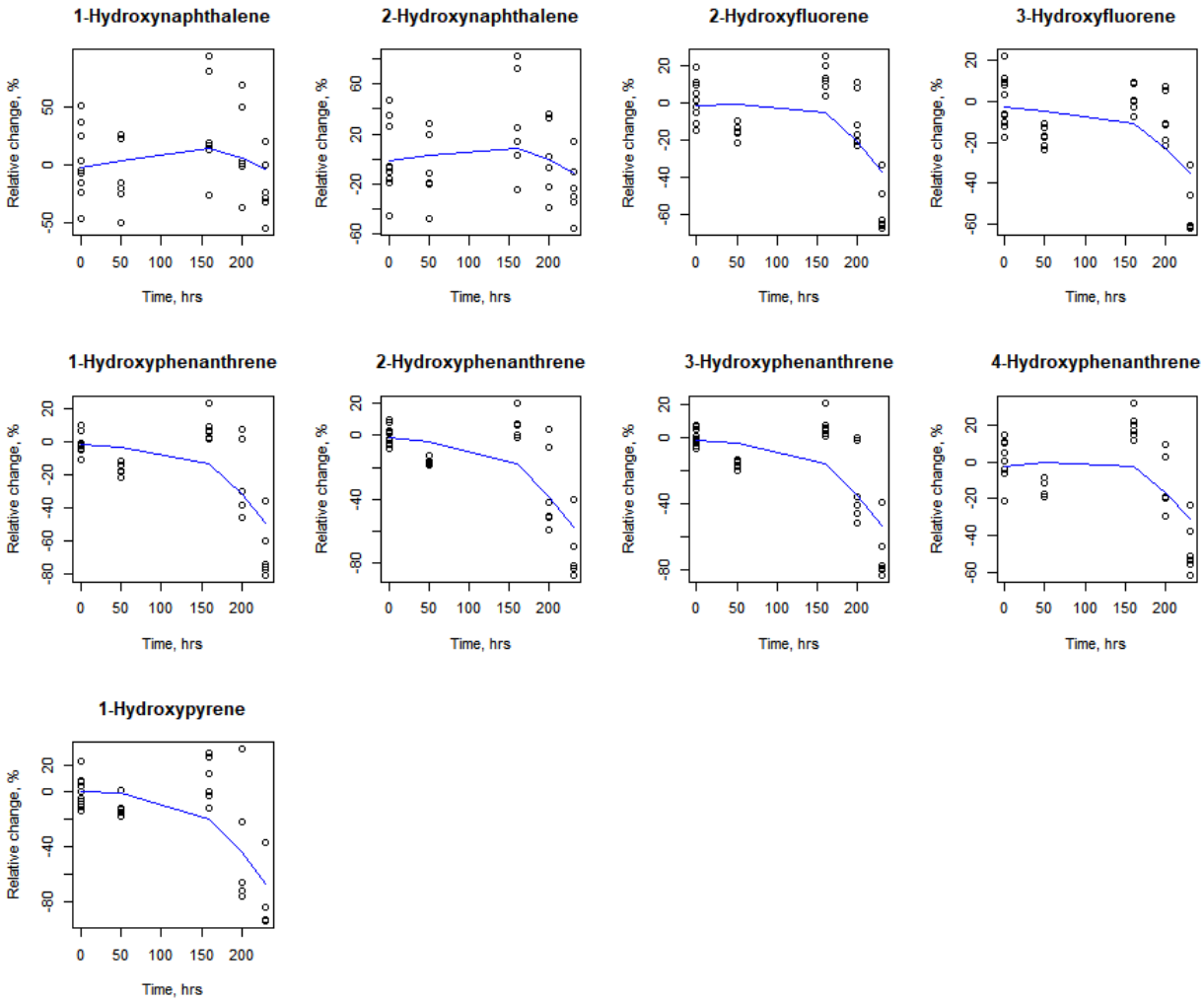



**Figure 8.** Relative change of labeled internal standard responses to its mean value at time 0, expressed in percentage. Blue line – smoothed fitting line.

**Table 8**. Spearman correlation coefficient of labeled internal standard (LIS) relative response change (a ratio between labeled IS at time i to average LIS response at time 0).

| | 1-NAP | 2-NAP | 2-FLU | 3-FLU | 1-PHE | 2-PHE | 3-PHE | 4-PHE | 1-PYR |
|---|---|---|---|---|---|---|---|---|---|
| **1-NAP** | 1 | 0.96 | 0.55 | 0.54 | 0.50 | 0.44 | 0.50 | 0.48 | 0.37 |
| **2-NAP** | | 1 | 0.54 | 0.51 | 0.49 | 0.43 | 0.51 | 0.49 | 0.31 |
| **2-FLU** | | | 1 | 0.94 | 0.96 | 0.91 | 0.94 | 0.92 | 0.84 |
| **3-FLU** | | | | 1 | 0.91 | 0.88 | 0.89 | 0.87 | 0.82 |
| **1-PHE** | | | | | 1 | 0.98 | 0.98 | 0.94 | 0.89 |
| **2-PHE** | | | | | | 1 | 0.97 | 0.93 | 0.92 |
| **3-PHE** | | | | | | | 1 | 0.94 | 0.87 |
| **4-PHE** | | | | | | | | 1 | 0.83 |
| **1-PYR** | | | | | | | | | 1 |

SPE recoveries of OH-PAHs with and without derivatization are shown on Figure 9. We can observe that for most compounds, derivatization performed on the same SPE cartridge increases recoveries, suggesting that elution in a free form may not be completed. Re-use of the SPE cartridge not only decreases cost of analysis but helps maintain stability of adducts as shown above.

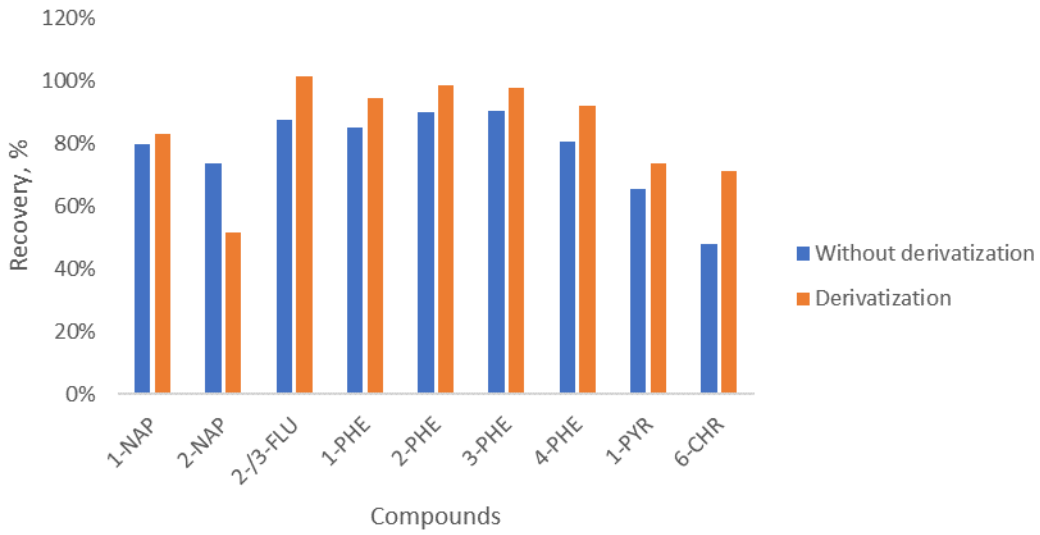



**Figure 9.** Recoveries of monohydroxylated polycyclic aromatic hydrocarbons on SPE with and without derivatization.

Note: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene

To ensure correct selection of the peaks, the method was transferred to LC MS/MS instrument with minor changes. Each adduct was scanned on product formation under collision-induced dissociation (CID). Registered fragments were identical to previously described (Xu & Spink, 2007) fragments obtained with 1,2-DMI-4-SC. However, an additional fragment was registered containing both a fragment of OH-PAH and a fragment of the derivatizing reagent. The formation of this product was described previously on estradiol (Keski-Rahkonen et al., 2015). Formation of products of CID are summarized in Figure 10, product resulting of rearrangement of the adduct that was observed only with 1,2-DMI-5-SC is shown after the arrow.

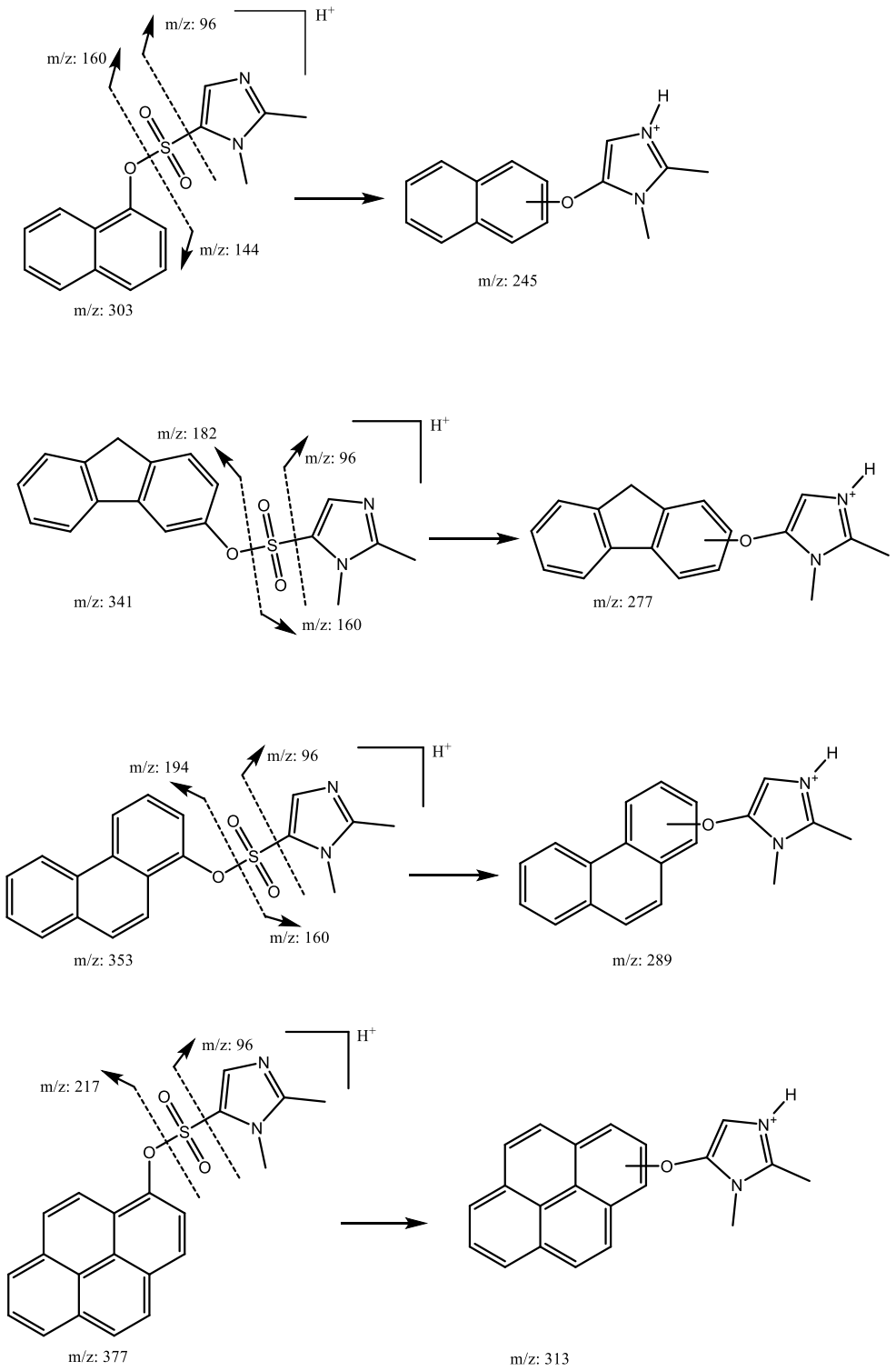


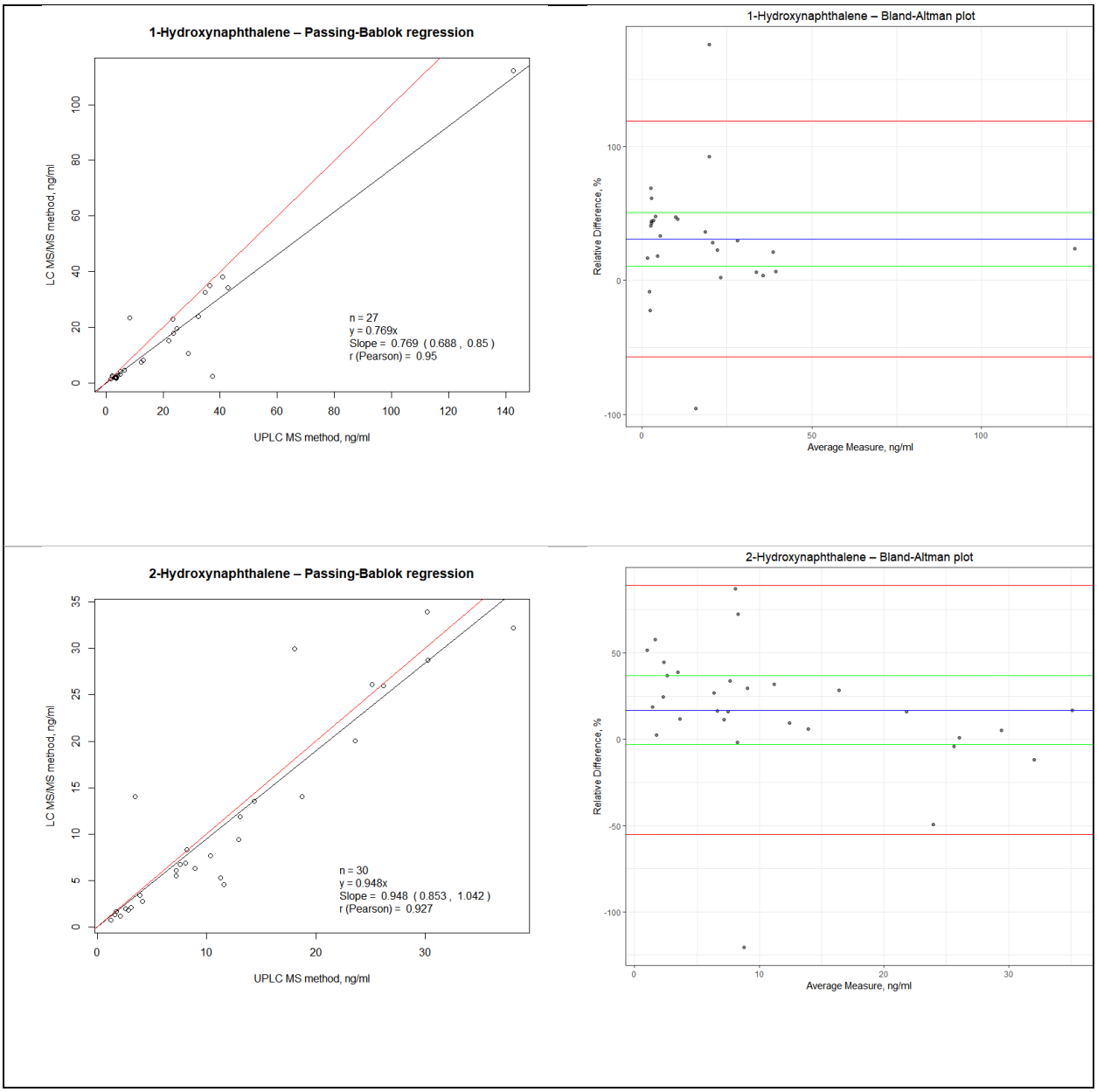

**Figure 10**. Adduct formation of 1-/2-hydroxynaphthalene (m/z = 303), 2-/3-hydroxyfluorene (m/z = 341), 1-/2-/3-/4-hydroxyphenanthrene (m/z = 353), 1-hydroxypyrene (m/z = 377). At a collision energy of 60-80 eV, we observed a fragment with m/z = 189 for 1-hydroxpyrene.

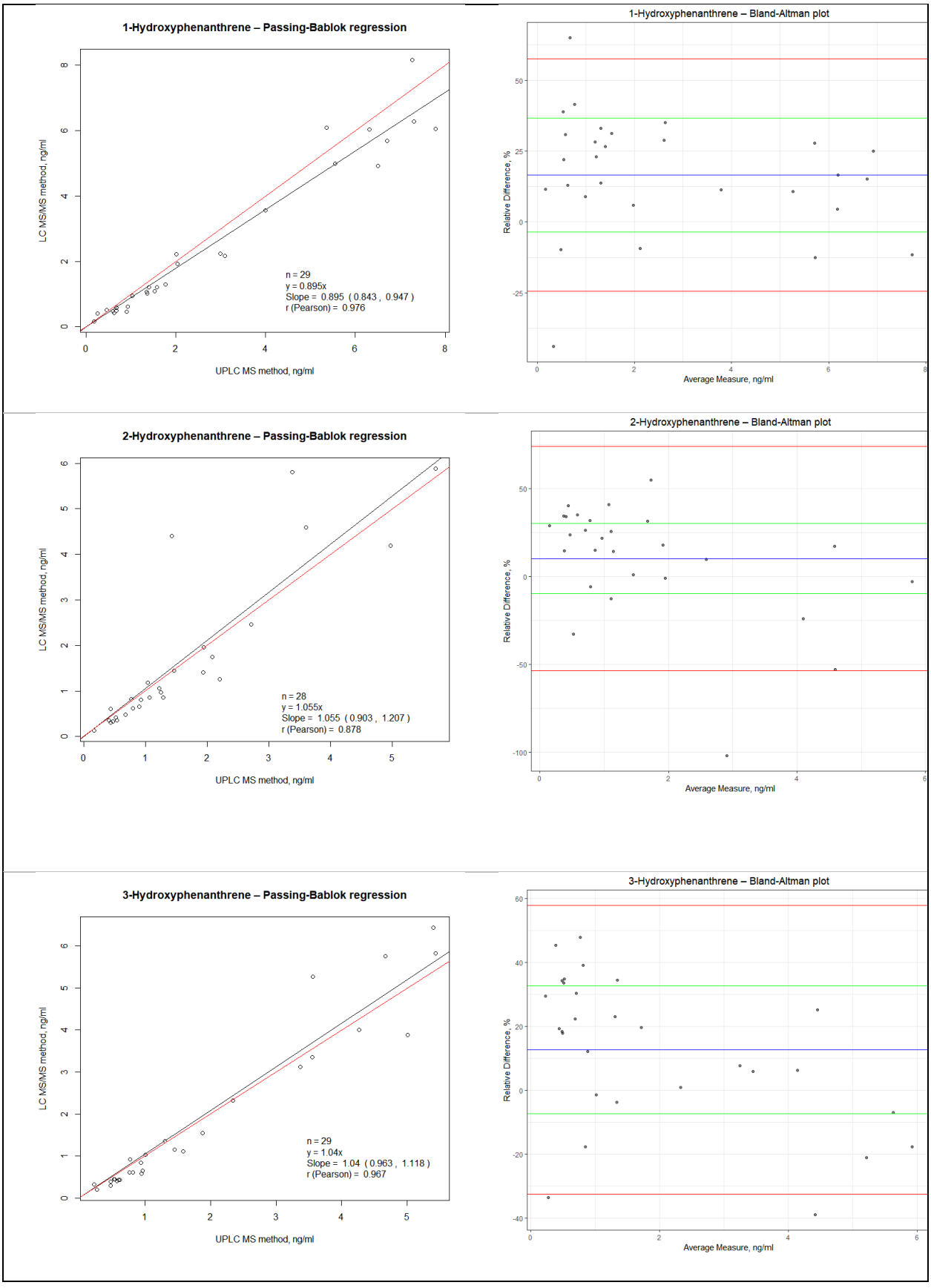
**Cross validation**

To complete cross-validation, we performed analysis for intraclass correlation coefficients (ICC) (Table 9), Passing-Bablok regression analysis (Passing & Bablok, 1983) (Figure 11), and Bland-Altman plots (Figure 11) (Giavarina, 2015; Keski-Rahkonen et al., 2015). ICC shows good to high method reliability. The results of Passing-Bablok and Bland-Altman analyses indicate that 2-NAP and 2-/3-PHE are not statistically significantly different from LC MS/MS method, while 1-NAP, 1-/4-PHE and 1-PYR shows proportional bias. Further we tested our method on NIST standard reference materials (SRM 3672 and SRM 3673) (Table 9). During analysis of 4-PHE a co-eluted peak with the same m/z appeared complecating quantification of the peak of interest. For this reason it was removed from the report; during analysis of CHAP samples similar case was not observed. Based on NIST values, established equations from Passing-Bablik regression analysis are necessary in order to provide correct values.

**Table 9**. Intraclass correlation coefficients between methods and quantification frequencies.

| **Criteria** | **1-NAP** | **2-NAP** | **Σ (2-/3-FLU)** | **1-PHE** | **2-PHE** | **3-PHE** | **4-PHE** | **1-PYR** |
|---|---|---|---|---|---|---|---|---|
| ICC | 0.92 | 0.93 | 0.58 | 0.97 | 0.86 | 0.96 | 0.91 | 0.86 |
| UPLC MS, >LOQ, % | 90 | 100 | 100 | 97 | 93 | 97 | 77 | 100 |
| LC MS/MS, >LOQ, % | 100 | 100 | 90 | 97 | 97 | 97 | 97 | 100 |


1-Hydroxynaphthalene – Passing-Bablok regression
LC MS/MS method, ng/ml
UPLC MS method, ng/ml
n = 27
y = 0.769x
Slope = 0.769 ( 0.688 , 0.85 )
r (Pearson) = 0.95
1-Hydroxynaphthalene – Bland-Altman plot
Relative Difference, %
Average Measure, ng/ml
2-Hydroxynaphthalene – Passing-Bablok regression
LC MS/MS method, ng/ml
UPLC MS method, ng/ml
n = 30
y = 0.948x
Slope = 0.948 ( 0.853 , 1.042 )
r (Pearson) = 0.927
2-Hydroxynaphthalene – Bland-Altman plot
Relative Difference, %
Average Measure, ng/ml


1-Hydroxyphenanthrene – Passing-Bablok regression
LC MS/MS method, ng/ml
UPLC MS method, ng/ml
n = 29
y = 0.895x
Slope = 0.895 ( 0.843 , 0.947 )
r (Pearson) = 0.976
1-Hydroxyphenanthrene – Bland-Altman plot
Relative Difference, %
Average Measure, ng/ml
2-Hydroxyphenanthrene – Passing-Bablok regression
LC MS/MS method, ng/ml
UPLC MS method, ng/ml
n = 28
y = 1.055x
Slope = 1.055 ( 0.903 , 1.207 )
r (Pearson) = 0.878
2-Hydroxyphenanthrene – Bland-Altman plot
Relative Difference, %
Average Measure, ng/ml
3-Hydroxyphenanthrene – Passing-Bablok regression
LC MS/MS method, ng/ml
UPLC MS method, ng/ml
n = 29
y = 1.04x
Slope = 1.04 ( 0.963 , 1.118 )
r (Pearson) = 0.967
3-Hydroxyphenanthrene – Bland-Altman plot
Relative Difference, %
Average Measure, ng/ml

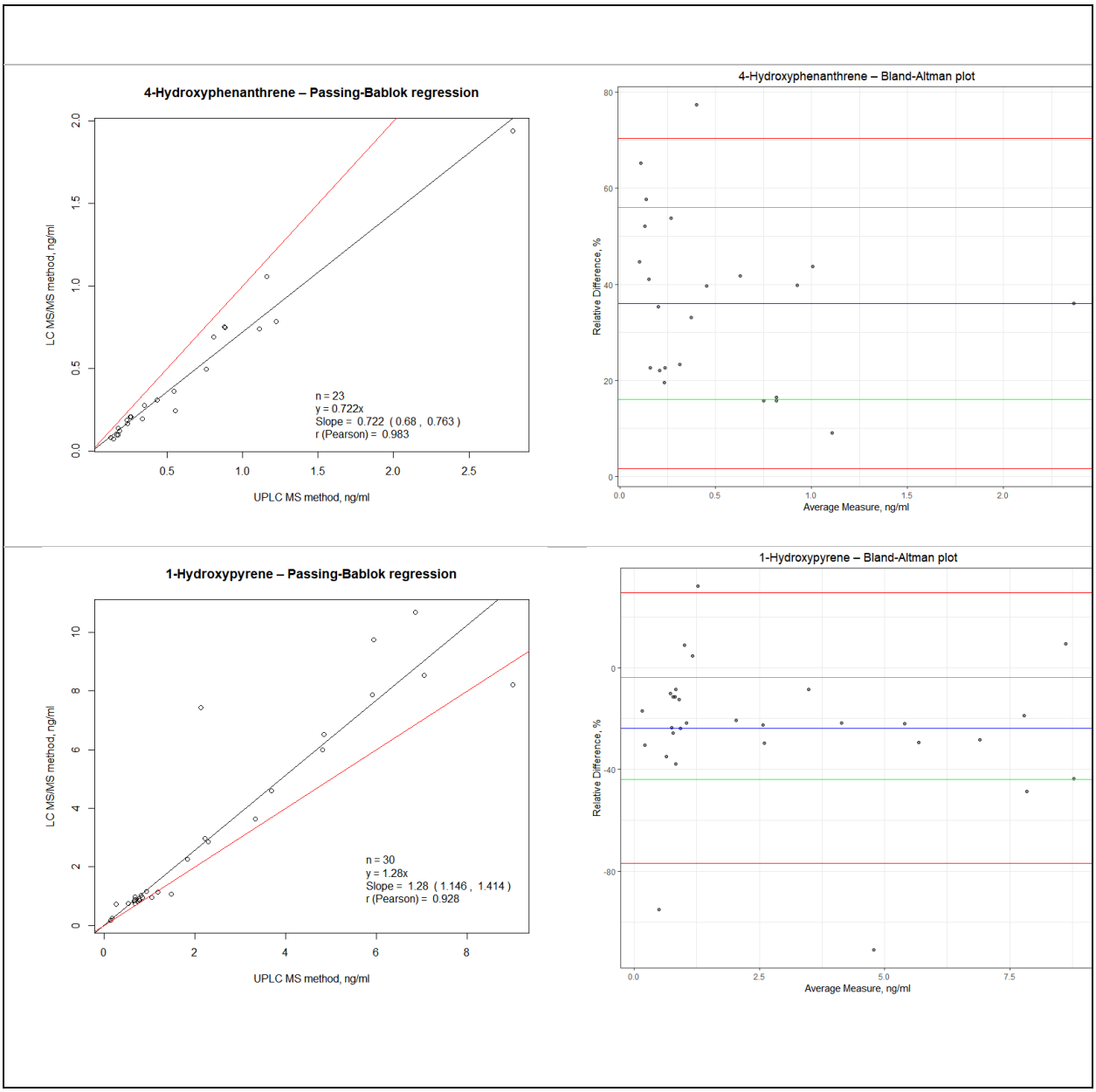



**Figure 11**. Passing-Bablok regression and Bland-Altman plots for method cross-validation. In Passing-Bablok plots, red line shows 1 to 1 relationship between methods, while black line shows regression line established in the experiment. In Bland-Altman plots, blue line represents mean relative bias, green lines shows ±20% to this bias, and red lines shows 95% agreement.

**Table 9.** Analysis of NIST standard reference material (SRM 3672 and 3673). 4-hydroxyphenanthrene had significant co-eluted peak and was removed from the report. Based Passing-Bablok regression analysis, samples concentrations were corrected via obtained equations. With asterisk marked levels that were below lowest limit of quantification, curve extrapolation was used to calculate their concentration.

| NIST Level | 1-Hydroxynaphthalene | | | 2-Hydroxynaphthalene | | |
|---|---|---|---|---|---|---|
| | Expected Range | Uncorrected | Corrected | Expected Range | Uncorrected | Corrected |
| 3673 | (165.6 - 284.4) | 249.0 | 191.5 | (1.06 - 1.584) | 1.549 | NA |
| 3672 | (27.04 - 40.56) | 47.37 | 36.43 | (6.856 - 10.284) | 9.458 | NA |
| NIST | 2-Hydroxyfluorene | | | 3-Hydroxyfluorene | | |
| | Expected Range | Uncorrected | Corrected | Expected Range | Uncorrected | Corrected |
| 3673 | (0.084 - 0.126) | 0.126 | NA | (0.031 - 0.046) | 0.065 | NA |
| 3672 | (0.683 - 1.02) | 0.777 | NA | (0.336 - 0.504) | 0.438 | NA |
| NIST | 1-Hydroxyphenanthrene | | | 2-Hydroxyphenanthrene | | |
| | Expected Range | Uncorrected | Corrected | Expected Range | Uncorrected | Corrected |
| 3673 | (0.038 - 0.097) | 0.073 | 0.066 | (0.019 - 0.029) | 0.021 | NA |
| 3672 | (0.106 - 0.159) | 0.192 | 0.172 | (0.683 - 1.02) | 0.070 | NA |
| NIST | 3-Hydroxyphenanthrene | | | 1-Hydroxypyrene | | |
| | Expected Range | Uncorrected | Corrected | Expected Range | Uncorrected | Corrected |
| 3673 | (0.022 - 0.033) | 0.022 | NA | (0.024 - 0.040) | 0.0202* | 0.026* |
| 3672 | (0.098 - 0.147) | 0.153 | NA | (0.136 - 0.204) | 0.155 | 0.199 |

## CONCLUSION

In the present study, we developed sensitive LC MS method for measurement of OH-PAHs in their derivative form with 1,2-DMI-5-SC. We showed novel approach for carrying out the derivatization reaction on the SPE cartridge, demonstrated its advantage against established approach with LLE and established kinetics required successful measurement of OH-PAHs in urine. We showed method agreement with established LC MS/MS instrument and provided equations for the correction of concentrations where bias was statistically significant.

**FUNDING**

This work was financially supported by NIH grants 5UM1HL134590-03, P50ES026071, EPA 83615301, P30ES019776 and the Laney Graduate School of Emory University.

## REFERENCES


1. Haritash, A.K. and C.P. Kaushik, *Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review.* Journal of Hazardous Materials, 2009. **169**(1): p. 1-15.
2. Kim, K.-H., et al., *A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects.* Environment International, 2013. **60**: p. 71-80.
3. Wang, Y., et al., *Polycyclic aromatic hydrocarbons (PAHs) in soils and vegetation near an e-waste recycling site in South China: Concentration, distribution, source, and risk assessment.* Science of The Total Environment, 2012. **439**: p. 187-193.
4. Yebra-Pimentel, I., et al., *A critical review about the health risk assessment of PAHs and their metabolites in foods.* 2015. **55**(10): p. 1383-1405.
5. Unwin, J., et al., *An assessment of occupational exposure to polycyclic aromatic hydrocarbons in the UK.* 2006. **50**(4): p. 395-403.
6. Abdel-Shafy, H.I. and M.S.M. Mansour, *A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation.* Egyptian Journal of Petroleum, 2016. **25**(1): p. 107-123.
7. Jary, H., et al., *Household Air Pollution and Acute Lower Respiratory Infections in Adults: A Systematic Review.* PLoS One, 2016. **11**(12): p. e0167656.
8. Dherani, M., et al., *Indoor air pollution from unprocessed solid fuel use and pneumonia risk in children aged under five years: a systematic review and meta-analysis.* Bull World Health Organ, 2008. **86**(5): p. 390-398C.
9. Assad, N.A., et al., *Chronic Obstructive Pulmonary Disease Secondary to Household Air Pollution.* Semin Respir Crit Care Med, 2015. **36**(03): p. 408-421.
10. Po, J.Y., J.M. FitzGerald, and C. Carlsten, *Respiratory disease associated with solid biomass fuel exposure in rural women and children: systematic review and meta-analysis.* Thorax, 2011. **66**(3): p. 232-9.
11. Smith, K.R., et al., *Millions dead: how do we know and what does it mean? Methods used in the comparative risk assessment of household air pollution.* 2014. **35**: p. 185-206.
12. Raspanti, G.A., et al., *Household air pollution and lung cancer risk among never-smokers in Nepal.* Environ Res, 2016. **147**: p. 141-5.
13. Josyula, S., et al., *Household air pollution and cancers other than lung: a meta-analysis.* Environ Health, 2015. **14**(1): p. 24.
14. Fatmi, Z. and D. Coggon, *Coronary heart disease and household air pollution from use of solid fuel: a systematic review.* Br Med Bull, 2016. **118**(1): p. 91-109.
15. Kim, K.H., S.A. Jahan, and E. Kabir, *A review of diseases associated with household air pollution due to the use of biomass fuels.* J Hazard Mater, 2011. **192**(2): p. 425-31.
16. Steenland, K., et al., *Modeling the potential health benefits of lower household air pollution after a hypothetical liquified petroleum gas (LPG) cookstove intervention.* Environment International, 2018. **111**: p. 71-79.
17. Epstein, M.B., et al., *Household fuels, low birth weight, and neonatal death in India: The separate impacts of biomass, kerosene, and coal.* International Journal of Hygiene and Environmental Health, 2013. **216**(5): p. 523-532.
18. Vyas, D., M.J.J.o.E.E. Vaghani, and Studies, *Health Impact Characterization of Traditional Biomass Cook Stove Usage on Indoor Air Quality in Rural Region–A State of Review.* 2018. **3**(1).
19. Urbancova, K., et al., *Evaluation of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns.* Science of The Total Environment, 2017. **577**: p. 212-219.
20. Bortey-Sam, N., et al., *Excretion of polycyclic aromatic hydrocarbon metabolites (OH-PAHs) in cattle urine in Ghana.* Environmental Pollution, 2016. **218**: p. 331-337.
21. Hansen, Å.M., et al., *Urinary 1-hydroxypyrene (1-HP) in environmental and occupational studies—A review.* International Journal of Hygiene and Environmental Health, 2008. **211**(5): p. 471-503.

22. Yang, B.-C., et al., *Quantification of monohydroxylated polycyclic aromatic hydrocarbons in human urine samples using solid-phase microextraction coupled with glass-capillary nanoelectrospray ionization mass spectrometry.* Analytica Chimica Acta, 2017. **973**: p. 68-74.
23. Lawal, A.T., *Polycyclic aromatic hydrocarbons. A review.* Cogent Environmental Science, 2017. **3**(1): p. 1339841.
24. Lankova, D., et al., *A novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites in urine using ultra-high-performance liquid chromatography with tandem mass spectrometry.* 2016. **408**(10): p. 2515-2525.
25. Panuwet, P., et al., *Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring.* Critical Reviews in Analytical Chemistry, 2016. **46**(2): p. 93-105.
26. Li, Z., et al., *Measurement of Urinary Monohydroxy Polycyclic Aromatic Hydrocarbons Using Automated Liquid−Liquid Extraction and Gas Chromatography/Isotope Dilution High-Resolution Mass Spectrometry.* Analytical Chemistry, 2006. **78**(16): p. 5744-5751.
27. Onyemauwa, F., et al., *Using liquid chromatography–tandem mass spectrometry to quantify monohydroxylated metabolites of polycyclic aromatic hydrocarbons in urine.* Journal of Chromatography B, 2009. **877**(11): p. 1117-1125.
28. Chetiyanukornkul, T., et al., *Simultaneous determination of urinary hydroxylated metabolites of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene as multiple biomarkers of exposure to polycyclic aromatic hydrocarbons.* 2006. **386**(3): p. 712-718.
29. Fan, R., et al., *Fast and Simultaneous Determination of Urinary 8-Hydroxy-2′-deoxyguanosine and Ten Monohydroxylated Polycyclic Aromatic Hydrocarbons by Liquid Chromatography/Tandem Mass Spectrometry.* Chemical Research in Toxicology, 2012. **25**(2): p. 491-499.
30. Jongeneelen, F.J., et al., *1-Hydroxypyrene in Urine as a Biological Indicator of Exposure to Polycyclic Aromatic Hydrocarbons in Severeal Work Environments.* The Annals of Occupational Hygiene, 1988. **32**(1): p. 35-43.
31. Ramsauer, B., et al., *A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of phenolic polycyclic aromatic hydrocarbons (OH-PAH) in urine of non-smokers and smokers.* 2011. **399**(2): p. 877-889.
32. Smith, C.J., et al., *Determination of selected monohydroxy metabolites of 2-, 3- and 4-ring polycyclic aromatic hydrocarbons in urine by solid-phase microextraction and isotope dilution gas chromatography–mass spectrometry*. Journal of Chromatography B, 2002. **778**(1): p. 157-164.
33. Zhao, G., et al., *Simultaneous determination of 11 monohydroxylated PAHs in human urine by stir bar sorptive extraction and liquid chromatography/tandem mass spectrometry.* Talanta, 2013. **116**: p. 822-826.
34. Itoh, N., H. Tao, and T. Ibusuki, *Optimization of aqueous acetylation for determination of hydroxy polycyclic aromatic hydrocarbons in water by stir bar sorptive extraction and thermal desorption–gas chromatography–mass spectrometry.* Analytica Chimica Acta, 2005. **535**(1): p. 243-250.
35. Motorykin, O., et al., *Determination of parent and hydroxy PAHs in personal PM2.5 and urine samples collected during Native American fish smoking activities.* Science of The Total Environment, 2015. **505**: p. 694-703.
36. Grova, N., et al., *Determination of PAHs and OH-PAHs in Rat Brain by Gas Chromatography Tandem (Triple Quadrupole) Mass Spectrometry.* Chemical Research in Toxicology, 2011. **24**(10): p. 1653-1667.
37. Bruce, N., et al., *WHO indoor air quality guidelines on household fuel combustion: Strategy implications of new evidence on interventions and exposure–risk functions.* Atmospheric Environment, 2015. **106**: p. 451-457.
38. Landrigan, P.J., *Air pollution and health.* Lancet Public Health, 2017. **2**(1): p. e4-e5.

39. Cohen, A.J., et al., *Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015.* Lancet, 2017. **389**(10082): p. 1907-1918.
40. Xu, L. and D.C. Spink, *1,2-Dimethylimidazole-4-sulfonyl chloride, a novel derivatization reagent for the analysis of phenolic compounds by liquid chromatography electrospray tandem mass spectrometry: Application to 1-hydroxypyrene in human urine.* Journal of Chromatography B, 2007. **855**(2): p. 159-165.
41. Keski-Rahkonen, P., et al., *Measurement of Estradiol in Human Serum by LC-MS/MS Using a Novel Estrogen-Specific Derivatization Reagent.* Analytical Chemistry, 2015. **87**(14): p. 7180-7186.
42. Wang, Q., C. Mesaros, and I.A. Blair, *Ultra-high sensitivity analysis of estrogens for special populations in serum and plasma by liquid chromatography–mass spectrometry: Assay considerations and suggested practices.* The Journal of Steroid Biochemistry and Molecular Biology, 2016. **162**: p. 70-79.
43. *Guidance for industry, bioanalytical method validation*, U.D.o.H.H. Services, Editor. 2018.
44. Chesher, D., *Evaluating Assay Precision.* The Clinical Biochemist Reviews, 2008. **29**(Suppl 1): p. S23-S26.
45. Li, Y., et al., *The use of chemical derivatization to enhance liquid chromatography/tandem mass spectrometric determination of 1-hydroxypyrene, a biomarker for polycyclic aromatic hydrocarbons in human urine.* Rapid Communications in Mass Spectrometry, 2005. **19**(22): p. 3331-3338.
46. Yao, L., et al., *Development of a sensitive method for the quantification of urinary 3-hydroxybenzo[a]pyrene by solid phase extraction, dansyl chloride derivatization and liquid chromatography-tandem mass spectrometry detection.* Analytical Methods, 2014. **6**(16): p. 6488-6493.
47. Passing, H. and W. Bablok, *A New Biometrical Procedure for Testing the Equality of Measurements from Two Different Analytical Methods. Application of linear regression procedures for method comparison studies in Clinical Chemistry, Part I*, in *Clinical Chemistry and Laboratory Medicine*. 1983. p. 709.
48. Giavarina, D., *Understanding bland altman analysis.* Biochemia medica: Biochemia medica, 2015. **25**(2): p. 141-151.

# CHAPTER 4

**Simultaneous quantification of metabolites of polycyclic aromatic hydrocarbon (OH-PAH), malondialdehyde (MDA), and 8-Oxo-2'-deoxyguanosine (8-Oxo-dG) in urine by high-performance liquid chromatography tandem quadrupole mass spectrometry**

Kostya Kartavenka[1], Parinya Panuwet[2], Volha Yakimavets[2], Priya Esilda D'Souza[2], Jeremy Sarnat[1], Dana Boyd Barr[1,2] and P. Barry Ryan[1,2]

---

1 Department of Environmental Health, Rollins School of Public Health, Emory University

2 Laboratory of Exposure Assessment and Development of Environmental Research (LEADER), Rollins School of Public Health, Emory University

## ABSTRACT

A single quantitative analytical method was developed for simultaneous measurement of urinary biomarker of exposure, polycyclic aromatic hydrocarbons monohydroxylated metabolites (OH-PAHs), and biomarkers of oxidative damage, 8-Oxo-2'-deoxyguanosine (8-Oxo-dG) and malondialdehyde (MDA). The method was validated using a set of two level quality control materials replicates, showing relative recoveries of 98 – 117%, inter-day precision of 2.6 – 18.8%, and intra-day precision of 3.2 – 17.2%. Autosampler storage stability and matrix effects were assessed. As part of the method validation, field samples analysis was performed using urine from pregnant agricultural works in Northern Thailand collected in 2012. A total of 88 samples were analyzed. While regression analysis shows statistically significant correlation between oxidative stress levels and exposure to OH-PAHs, further tertile analysis revealed only 8-Oxo-dG levels to be statistically different between low- and high-exposed groups. Additionally, statistically significant correlation was reported between 8-Oxo-dG and MDA.

## KEYWORDS

OH-PAHs, MDA, 8-Oxo-dG, HPLC MS/MS, derivatization

## INTRODUCTION

Biomass burning, which commonly includes wood, branches, dung, coal, and agricultural residues, is widely used around the world infood preparation, lighting, and heating. Despite growing general recognition of adverse health effect associated with biomass burning at household facilities, (Bruce et al., 2015; Nagel et al., 2016; Steenland et al., 2018) approximately one-third of the world still relies on this fuel (*Energy Access Outlook: From Poverty to Prosperity*, 2017; Vyas et al., 2018). Inefficient burning of biomass releases a significant amount of pollutants inside houses (commonly known as household air pollution (HAP)) (K. R. Smith et al., 2014) and even outside (Bruce et al., 2015). HAP has been reported to be characterized as a single most important environmental health risk factor worldwide (Cheng et al., 2016; K. R. Smith et al., 2014). HAP was reported to be associated with various respiratory (Assad et al., 2015; Dherani et al., 2008; Jary et al., 2016; Josyula et al., 2015; Po et al., 2011; Raspanti et al., 2016; K. R. Smith et al., 2014) and non-respiratory responses including cataracts (K. R. Smith et al., 2014), cardiovascular disease (Fatmi & Coggon, 2016; K. H. Kim et al., 2011), low birth weight and infant mortality (Epstein et al., 2013; Vyas et al., 2018).

One established technique to measure exposure to HAP is via measuring polycyclic aromatic hydrocarbons (PAHs), resulting from incomplete pyrolysis of biomass, and their metabolites, mono-(OH-PAHs) and poly-hydroxylated compounds, in human biological samples (Abdel-Shafy & Mansour, 2016) [Chapter 3]. At a high exposure levels, e.g., occupational exposure, a single biomarker can be used; however, at a more modest levels, a better understanding of exposure may be assessed via measuring multiple biomarkers (Rylance et al., 2013). The selection of additional candidate biomarkers can be derived from the understanding of metabolism of PAHs. Upon entering the organism, multiple enzymes are involved in the metabolism of PAHs. The detoxification starts with oxidation to epoxides following enzymatic glucuronidation or sulfonation, commonly described as a true detoxification. On the other hand, direct hydroxylation may take place to form OH-PAHs and poly-hydroxylated PAHs, which can covalently bind to proteins and nucleic acids, including DNA (Choi et al., 2010; Ramesh et al., 2004; Xue & Warshawsky, 2005).

In addition to direct binding to DNA, it was reported that the detoxification mechanism can also involve PAHs in a redox cycle with oxygen generating reactive oxygen species (ROS) (See Figure 1) (Delfino et al., 2011; Xue & Warshawsky, 2005). Due to lipophilic properties of PAHs (Lawal, 2017) and their potential in ROS generation, it would be expected to note elevated levels of biomarkers of oxidative stress in lipids such as malondialdehyde (MDA). Positive correlation between OH-PAHs and MDA has been reported in several studies (C-H Pan et al., 2008; Wu et al., 2017; Q. Yang et al., 2015).

An additional, common and abundant biomarker of oxidative damage to DNA is 8-Oxo-2'-deoxyguanosine as a result of the oxidative damage via hydroxyl radical with the nucleobase of the DNA strand (8-Oxo-dG) (Valavanidis et al., 2009). In a meta-analysis drawing upon data from 52 studies, it was concluded that exposure to combustion particles is consistently associated with elevated levels of both lipid peroxidation products (including MDA) and oxidative damaged to DNA products (including 8-Oxo-dG) in urine, plasma, EBC, and human blood cells, confirming both lipid peroxidation and oxidative DNA damage (Møller & Loft, 2010).

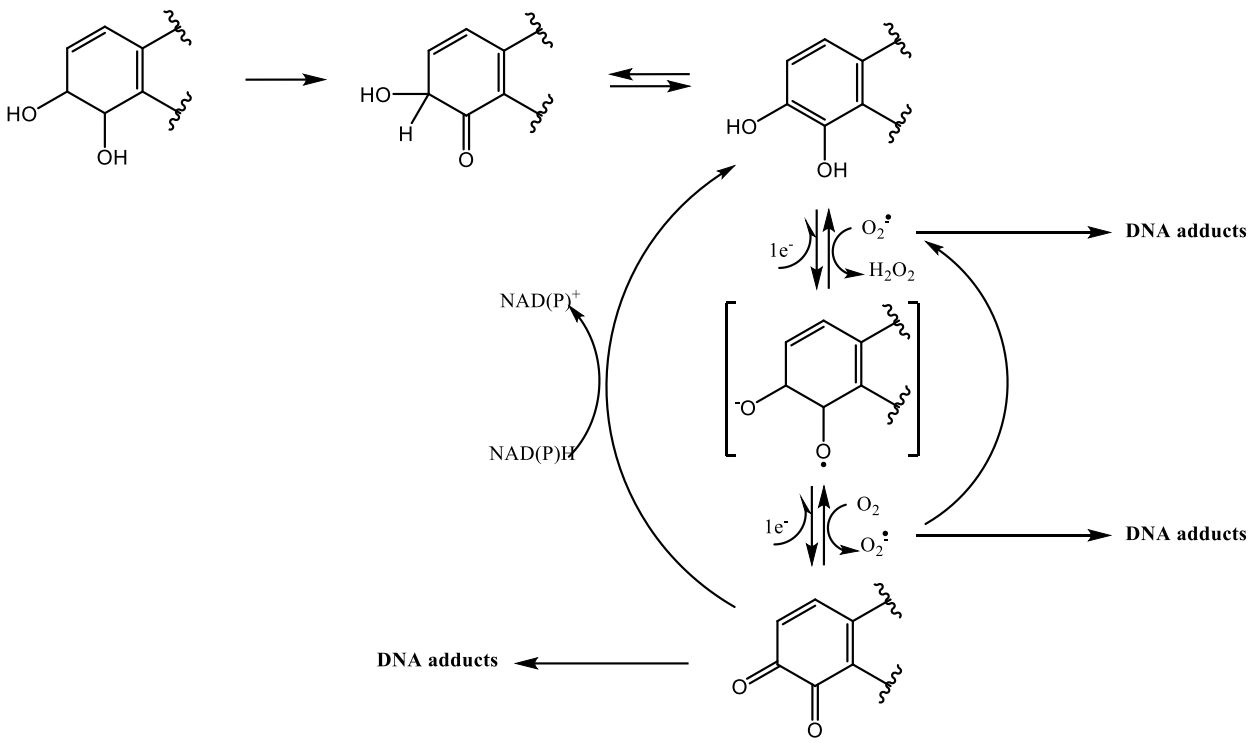



**Figure 1.** Formation of reactive oxygen species and DNA adducts as a results of metabolism of polycyclic aromatic hydrocarbons (Xue & Warshawsky, 2005).

A common way for measuring these three groups of compounds is via executing three individual methods therefore significantly increasing cost of analysis (Rylance et al., 2013; Wu et al., 2017). The first and, to our knowledge, the only successfully published approach to analyze both 8-Oxo-dG and OH-PAHs was performed by Fan, *et al*. (Fan, Wang, Ramage, et al., 2012), which found its further application in exposure studies on association of air pollution and oxidative damage (J. Li, Fan, et al., 2015; J. Li, Lu, et al., 2015; Lu et al., 2016); however, analysis of MDA was performed with a separate analytical method. Overall, compounds from each group can be analyzed via liquid chromatographic and gas chromatographic methods; the quantification can be performed via fluorescence, diode array, single quadrupole mass selective or tandem-quadrupole mass selective detectors. Among listed quantification techniques, mass spectrometric methods give the best results for specificity in analysis of biological samples. The importance of understanding of the relationship between both biomarkers of exposure (OH-PAHs) and biomarkers of effect (MDA, 8-Oxo-dG) and the absence of a single analytical method to

accomplish this, motived us in developing of such approach. We developed a liquid chromatography tandem mass spectrometry (LC MS/MS) method for simultaneous quantification of MDA, 8-Oxo-dG, and OH-PAHs in urine in their derivative forms, using dansylhydrazide (DH), dansylchloride (DNSCl), and 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride (1,2-DMI-5-SC) as derivatizing agents, respectively. To control for potential loss during sample preparation and or ionization suppression/enhancement (Panuwet et al., 2016), isotope-dilution quantification was used.

## MATERIAL AND METHODS

### Chemical and reagents

We obtained acetonitrile (HPLC grade), methanol (HPLC grade), sodium acetate from Fisher Chemical (Waltham, MA, USA), acetone (HPLC grade) and hydrochloric acid, 30% (for ultratrace analysis) from Sigma-Aldrich (Darmstadt, Germany). β-Glucuronidase from *Helix pomatia*, 1,2-dimethyl-1H-imidazole-5-sulphonyl chloride (Aldrich$^{CPR}$), malondialdehyde tetrabutylammonium salt, 96% (neat), dansyl chloride, 99%, 8-Hydroxy-2′-deoxyguanosine, 98% were purchased from Sigma-Aldrich, dansylhydrazine, 97% (reagent grade) from TCI America (Portland, OR, USA), sodium hydroxide 0.1N standardized solution was obtained from Alfa Aesar (Haverhill, MA, USA), sodium bicarbonate, ACS was obtained from VWR (Radnor, PA, USA). All unlabeled OH-PAHs standards (1-naphtol (1-NAP) (98%), 2-naphtol (2-NAP) (98%), 2-hydroxyfluorene (2-FLU) (98%), 3-hydroxyfluorene (3-FLU) (98%), 1-hydroxyphenanthrene (1-PHE) (98%), 2-hydroxyphenanthrene (2-PHE) (98%), 3-hydroxyphenanthrene (3-PHE) (98%), 4-hydroxyphenanthrene (4-PHE) (98%), 1-hydroxypyrene (1-PYR) (98%), 6-hydroxychrysene (6-CHR) (98%)) and their labeled standards (1-naphtol (13C6, 99%), 2-naphtol (13C6, 99%), 2-hydroxyfluorene (13C6, 99%), 3-hydroxyfluorene (13C6, 99%), 1-hydroxyphenanthrene (13C4, 99%), 2-hydroxyphenanthrene (13C6, 99%), 3-hydroxyphenanthrene (131C6, 98%), 4-hydroxyphenanthrene (13C4, 99%), 1-hydroxypyrene (13C6, 99%), 6-hydroxychrysene(13C6, 98%)), 8-hydroxy-2'-deoxyguanosine (15N5, 98%) were obtained from Cambridge Isotope Laboratories

(Tewksbury, MA, USA) as a stock standard of 50 ug/mL in toluene. 1,1,3,3-Tetraethoxypropane-1,3,-$D_2$ ($D_2$-TEP) was purchased from Santa Cruz Biotechnology.

1,2-DMI-5-SC and DNSCl derivatizing solutions were prepared by diluting neat compound in acetone to yield concentrations of 0.5 μg/ml and 100 μg/ml respectively. DH solution was prepared in acetonitrile to yield concentration of 2.3 mg/ml. Water was purified using a Milli-Q® Ultrapure water purification system (EMD Millipore, Billerica, MA).

**Preparation of standard, quality control, and labeled internal standard solutions**

We prepared a high-concentration stock solution of OH-PAHs via mixing of known amount of each stock standard solutions in amber glass volumetric flask in methanol. A set of ten calibration solutions (CS) were prepared via serial dilution (Table 1). A labeled internal standard (LIS) stock solution was prepared in methanol via mixing all 10 labeled standards (Table 1). Two levels (Table 1) quality control (QC) solutions were prepared in amber glass volumetric flask in methanol via mixing of a known amount of each stock standard solutions.

A high concentration 8-Oxo-dG and its LIS stock solutions were prepared in Milli-Q by dissolving neat compound to yield concentrations 2.5 μg/mL each. A set of eleven calibration and two QC solutions were prepared in amber glass vial via serial dilution from stock solutions (Table 1).

High concentration MDA stock solutions (CS and QC) were prepared via weighing a known amount of neat standard and dissolving it in acetonitrile to yield concentration of 7961 nM. An eight-point calibration curve and two QC solutions were prepared daily from the corresponding stock solution in Milli-Q via serial dilution. Labeled internal standard (LIS) stock solution was prepared via hydrolysis of $D_2$-TEP (Pilz et al., 2000) to yield $D_2$-MDA. This LIS solution was diluted with acetonitrile to yield concentration of 10 μM stock solution. A daily aliquot of LIS stock solution was diluted with Milli-Q to yield concentration of 250 nM. This step was designed to limit potential hydrogen exchange of deuterated standard by keeping it in aprotic solvent.

All OH-PAHs solutions were stored at -20 ºC, 8-Oxo-dG and MDA solutions were stored at +4 ºC prior to use.

**Table 1**. Summary of levels for calibration, quality control, and labeled internal standard solutions used in the method. All concentrations are expressed in ng/ml except MDA, which concentration is expressed in nano-mole per litter (nM). Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, MDA – malondialdehyde, 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine.

| Purpose | Concentration in Storage Vial | Concentration in Sample (after spiking) | Compounds |
|---|---|---|---|
| **Calibration** | 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 4000 | 0.125, 0.25, 0.5, 1.25, 2.5, 5, 12.5, 25, 50, 100 | 1-NAP, 2-NAP |
| | 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 400 | 0.0125, 0.025, 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, 5, 10 | 2-/3-FLU, 1-/2-/3-/4-PHE, 1-PYR, 6-CHR |
| | 2.5, 10, 20, 40, 80, 100, 160, 200, 250, 400, 800 | 0.125, 0.5, 1, 2, 4, 5, 8, 10, 12.5, 20, 40 | 8-Oxo-dG |
| | 9, 27, 45,75, 100, 250, 400, 800 | NA | MDA |
| **Quality control, low** | 50 | 1.25 | 1-NAP |
| | 100 | 2.5 | 2-NAP |
| | 20 | 0.5 | 2-/3-FLU, 1-/2-/3-/4-PHE, 1-PYR, 6-CHR |
| | 250 | 12.5 | 8-Oxo-dG |
| | 50 | NA | MDA |
| **Quality control, high** | 250 | 6.25 | 1-NAP |
| | 500 | 12.5 | 2-NAP |
| | 100 | 2.5 | 2-/3-FLU, 1-/2-/3-/4-PHE, 1-PYR, 6-CHR |
| | 100 | 5 | 8-Oxo-dG |
| | 200 | NA | MDA |
| **Labeled internal standard** | 500 | 25 | 1-/2-NAP |
| | 100 | 2.5 | 2-/3-FLU, 1-/2-/3-/4-PHE, 1-PYR, 6-CHR |
| | 100 | 5 | 8-Oxo-dg |
| | 250 | NA | MDA |

**Preparation of blank, calibration, quality control, and unknown samples**

**OH-PAHs and 8-Oxo-dG**

Calibration and QC solutions were prepared by spiking 25 μL of corresponding OH-PAHs standard and 25 μL of OH-PAHs LIS into 15 mL centrifuge tube, evaporated to dryness using under 10 psi

nitrogen flow at 45 ºC. After the evaporation, test tubes were spiked with 50 µL of 8-Oxo-dG of respective level and 50 µL of LIS. To account for matrix effects (*Guidance for industry, bioanalytical method validation*, 2018; Panuwet et al., 2016) each test tube was spiked with 1 mL of twenty-fold diluted pooled urine (Table 1). Blank[4] samples were prepared in the same manner as calibration curve in 20-fold diluted urine. Clean-up, derivatization and purification steps for calibration, QC and blanks solutions were performed in the same manner as unknown samples described below (Figure 1).

**MDA**

MDA calibration and QC solutions were prepared by transferring 200 µL of corresponding solution into new set of test tubes andspiking with 50 µL of LIS. Derivatization and clean-up operations were performed in the same manner as unknown samples described below (Figure 1).

**Sample preparation for analysis as a derivative**

Derivatization of MDA is performed in according to method described previous [Chapter 2]. Briefly, each urine sample was diluted 10-fold, 200 µL was transferred to a new set of test tubes, spiked with 50 µL of LIS following by 50 µL of 1.5 M hydrochloric acid, and 25 µL of DH solution. Samples were capped and placed into the incubator for 2 hours at 45 ºC.

Derivatization of OH-PAHs is based on previously developed [Chapter 3] approach with minor changes to address additional compounds in the analysis. Prior to analysis, each urine sample was stored at -80 ºC. On the day of analysis, urine was brought to room temperature, 1 mL of urine was transferred to 15 mL centrifuge tube, spiked with 25 µL LIS and with 1 mL of β-glucuronidase for deconjugation at 37 ºC for 16 hours (Figure 1). Upon completion of the deconjugation, the content of each tube was loaded into Strata X 33u cartridge (Phenomenex (Torrance, CA, USA)), conditioned with 1.5 mL acetonitrile and 2.5 mL Milli-Q. Cartridges were washed with 2.5 mL Milli-Q. Water was removed from the cartridge by applying vacuum for 10 s. A nNew set of test tubes were spiked with 0.3 mL of DNSCl solution and installed under the cartridges. Each cartridge was spiked with 1.2 mL of 4.5 % v/v acetonitrile in

[4] FDA uses term “zero calibrator” instead of blank

bicarbonate/sodium hydroxide buffer solution (pH 10.2), retained solvent was removed by applying vacuum for 10 s. This test tube set was capped and placed into the incubator at 45 ºC to allow derivatization of 8-Oxo-dG for 60 minutes. After the 8-Oxo-dG fraction was placed into the incubator, each cartridge was washed with 1 mL 1:4 methanol:Milli-Q, and 2 mL 1:1 acetone:Milli-Q solutions. Then cartridges were dried under vacuum for 5 minutes. A new set of test tubes was installed and cartridges were eluted with 0.5 mL 9:1 acetone:methanol and 2.5 mL 100% acetone, followed by vacuum to assure full elution of the eluent. Eluent was removed in the evaporator at 55 ºC under 10 psi nitrogen flow. During evaporation of the eluent, cartridges were conditioned with 2.5 mL Milli-Q followed by 0.5 mL of bicarbonate-sodium hydroxide buffer solution (pH 10.2). When samples were evaporated, 0.2 mL of the same buffer was added to each sample, then spiked with 0.2 mL of 1,2-DMI-5-SC solution (0.1 mg/mL), vortexed and transferred to corresponding cartridge. Derivatization was carried out on the SPE cartridge at room temperature for 30 minutes.

After completion of MDA derivatization with DH, this set of samples was spiked with 1.7 mL phosphate buffer (pH = 8), vortexed and transferred into corresponding test tubes with 8-Oxo-dG adduct. Test tubes, containing both MDA and 8-Oxo-dG in their derivatized form, was shortly vortexed and transferred into the corresponding SPE cartridge. This step was done one-by-one. Upon completion of combining all derivatives in one SPE cartridge each cartridge was washed with 3.3 mL Milli-Q to remove salts following by 2.5 mL 4:1 methanol:Milli-Q solution. Each cartridge was dried with vacuum for 5 minutes. Derivatives were eluted with 5 mL acetonitrile. Acetonitrile was evaporated under nitrogen flow at 55 ºC. Samples were reconstituted in 0.1 mL 2:3 acetonitrile:Milli-Q solution.

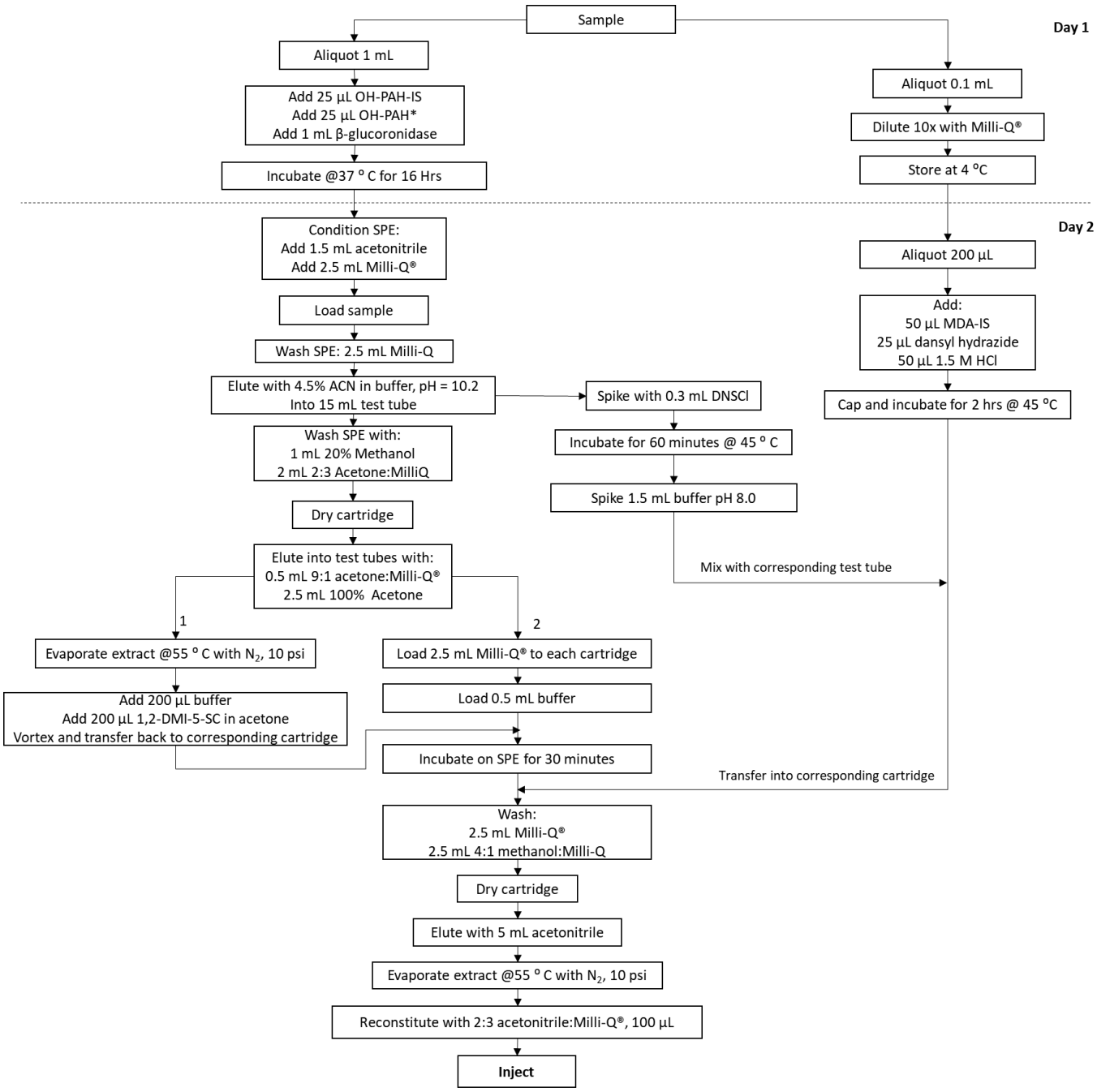



**Figure 1**. Sample preparation flow chart. Abbreviations: 1,2-DMI-5-SC – 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride, DNSCl – dansyl chloride, DH – dansyl hydrazide, LIS – labeled internal standard, * (asterisk) referred to calibration solutions preparation, where unlabeled standard is spiked into the matrix 20X diluted urine, SPE – stands for Strata-X 33u solid phase extraction cartridge, "buffer" stands for

sodium bicarbonate-sodium hydroxide buffer solution adjusted to pH 10.2, “buffer pH 8.0” stands for phosphate buffer adjusted to pH 8.0.

**Chromatographic analysis of OH-PAHs, 8-Oxo-dG, and MDA**

Sample analysis was performed on an Agilent Infinity 1260 high-performance liquid chromatograph (HPLC) with a G1367E autosampler connected to an Agilent G6460A tandem quadrupole mass spectrometer (Santa Clara, CA, USA). The separation was performed on Agilent Eclipse Plus Phenyl-Hexyl RRHD (1.8 μm 2.1x150 mm). The column temperature was set to 45 ºC. The binary mobile phase consists of A, 100% Milli-Q®, and B, 100% acetonitrile. Prior to injection of 8 μL, needle wash was performed in acetonitrile:Milli-Q 4:1 for 10 seconds, separation was achieved via a gradient elution program (Table 2). The total program runtime was 27.0 minutes.

**Table 2.** Graduate elution liquid chromatography program for simultaneous analysis of monohydroxylated polycyclic aromatic hydrocarbons, 8-Oxo-2'-deoxyguanosine, and malondialdehyde in urine samples. Where A and B stands for binary mobile phase composition expressed in percentage, A – Milli-Q®, B – 100% acetonitrile. No additives were used in either of solvents.

| Time, min | B composition | Flow rate, ml/min | Time, min | B composition | Flow rate, ml/min |
|---|---|---|---|---|---|
| 0.0 | 37.0 % | 0.3 | 19.0 | 100.0 % | 0.3 |
| 3.5 | 37.0 % | 0.3 | 20.0 | 100.0 % | 0.3 |
| 3.7 | 42.0 % | 0.3 | 20.3 | 100.0 % | 0.45 |
| 14.0 | 42.0 % | 0.3 | 22.5 | 100.0 % | 0.45 |
| 14.2 | 46.0 % | 0.3 | 22.7 | 37.0 % | 0.45 |
| 16.0 | 48.0 % | 0.3 | 23.5 | 37.0 % | 0.45 |
| 16.5 | 70.0 % | 0.3 | 24.0 | 37.0 % | 0.3 |
| 18.0 | 70.5 % | 0.3 | 27.0 | End of program | |

The target analytes including their LIS were ionized using positive electrospray ionization (ESI). The mass spectrometer was operated in multiple reaction monitoring (MRM) mode (Table 3). Across all time segments the following parameters were constant except stated: nozzle voltages was set to 2000 V, nitrogen was used as a dry and sheath gas, their flow rates were set to 5.7 L/min (except for 8-Oxo-dG

time fragment where it was set to 5.0  L/min) and 12.0 L/min, respectively, drying gas and sheath gas temperatures were set to 350 ºC and 360 ºC (except for 1-/2-NAP where it was set to 380 ºC), respectively, the nebulizer pressure was set to 30 psig (except for 8-Oxo-dG where it was set to 25 psig), the fragmentor voltage was set to 150 V (except 8-Oxo-dG where it was set to 135 V). Fragmentation occurs in collision induced dissociation mode (Table 3).

**Table 3**. Multiple reaction monitoring (MRM) mode parameters for quantification of monohydroxylated polycyclic aromatic hydrocarbons metabolites. Cell accelerated voltage was set to 5 V and fragmentor voltage across all transitions. Abbreviations: CE – collision energy, CA – cell accelerator voltage, NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine, MDA – malondialdehyde, Q – transition used for quantification, C – transition used for confirmation.

| Time segment, min | Analyte | MRM Transition | Purpose | CE, V | CA, V | CV, V | Delta EMV | Dwell time, ms |
|---|---|---|---|---|---|---|---|---|
| 4.2 – 4.85 | 8-Oxo-dG | 517 –> 401 | Q | 12 | 4 | 1800 | 300 | 170 |
| | | 517 –> 170 | C | 35 | | | | 170 |
| | 8-Oxo-dG, $^{15}$N5 | 522 –> 406 | Q | 12 | | | | 170 |
| 8.6 – 9.9 | 1-/2-NAP | 303 –> 239 | Q | 15 | 7 | 1900 | 700 | 150 |
| | | 303 –> 96 | C | 14 | | | | 150 |
| | 1-/2-NAP, $^{13}$C6 | 309 –> 245 | Q | 20 | | | | 200 |
| 14.3 – 16.9 | 2-/3-FLU | 341 –> 182 | Q | 27 | 7 | 2000 | 500 | 100 |
| | | 341 –> 96 | C | 22 | | | | 100 |
| | 2-/3-FLU, $^{13}$C6 | 347 –> 188 | Q | 20 | | | | 150 |
| | 4-PHE | 353 –> 289 | Q | 25 | 7 | 2000 | 500 | 100 |
| | | 353 –> 96 | C | 17 | | | | 100 |
| | 4-PHE, $^{13}$C4 | 357 –> 198 | Q | 20 | | | | 150 |
| | MDA | 302 –> 287 | Q | 25 | 7 | 2000 | 500 | 100 |
| | | 302 –> 223 | C | 25 | | | | 100 |
| | MDA, D2 | 302 –> 289 | Q | 25 | | | | 100 |
| 16.9 – 18.5 | 1-/2-/3-PHE | 353 –> 194 | Q | 25 | 7 | 2000 | 500 | 150 |
| | | 353 –> 96 | C | 20 | | | | 150 |
| | 1- PHE, $^{13}$C4 | 357 –> 293 | Q | 20 | | | | 150 |
| | 2-/3- PHE, $^{13}$C6 | 359 –> 200 | Q | 20 | | | | 150 |
| 18.5 – 19.5 | 1-PYR | 377 –> 218 | Q | 20 | 7 | 2100 | 700 | 150 |
| | | 377 –> 189 | C | 70 | | | | 150 |
| | 1-PYR, $^{13}$C6 | 383 –> 224 | Q | 20 | | | | 150 |
| 19.5 – 20.2 | 6-CHR | 403 –> 244 | Q | 20 | 7 | 2100 | 700 | 150 |
| | | 403 –> 215 | C | 60 | | | | 150 |
| | 6-CHR, $^{13}$C6 | 409 –> 250 | Q | 20 | | | | 150 |

**Method validation**

Method validation was performed only for UPLC MS as that is the purpose of this paper. The method precision was determined in according to protocol described by Chesher (Chesher, 2008) using two quality control levels (QCL and QCH, Table 1). Replicate samples (5 samples/day at each concentration

level) were prepared and analyzed daily during a 5-day period to determine inter-day and intra-day precision. The precisions were expressed as relative standard deviation (%RSD) values.

The method relative recoveries (RR) were determined, according to FDA guideline(*Guidance for industry, bioanalytical method validation*, 2018) using a set of replicates, quality control materials (QCL and QCH) prepared at concentrations listed in Table 1 (N = 25). The method RR were calculated by dividing the average, quantified values of the quality control materials with the respective, expected concentration values for both levels. The method RR were expressed as percentage values. Lowest calibration level was selected in according to FDA criteria for bioanalytical method validation(*Guidance for industry, bioanalytical method validation*, 2018). Limit of detection was selected as a lowest calibration level.

A study of autosampler storage stability was performed via a replicate injection of 29 samples including 27 individual urine samples after 46 hours stored in +8 ºC. Stability was assessed via comparison of loss/gain of mean responses for LIS. For compounds which had statistically significant mean difference, we performed further analysis on relative change in concentration to time = 0:

$$Relative\ change = \frac{Concentration_{Time\ i,replicate\ j}}{Concentration_{Time=0}} - 1$$

Further we evaluated if MRM transition leading to the non-specific ion (containing fragment of derivatizing agent only) can be used as an alternative to a more specific transition containing residue of both derivatizing agent and target analyte, where response from non-specific ion is statistically significantly greater than one from a specific transition. To evaluate this replaceability, we used Bland-Altman plots approach (Giavarina, 2015).

Matrix effects were evaluated by comparing mean responses (peak area) of labeled IS presented in ten-times diluted, across different analytical batches.

Urine samples were collected from pregnant agricultural works in Northern Thailand in 2012. The samples were collected multiple times during pregnancy for the purpose of establishing exposures to pesticides in this population and to ascertain the effects of such exposures on the neurodevelopment of the

offspring. All women in the investigation were agricultural workers who lived in the rural area of Fang in Chiang Mai province. The women were of mixed ethnicity, primarily ethnic Thai and ethnic Burmese. A total of 56 women participated in the study and supplied a minimum of five and a maximum of 13 urine samples. Since initial collection, samplers were stored at -80 C. For this investigation 88 samples were selected randomly from among those with sufficient remaining volume for analysis.

### Statistical analysis

All statistical analysis was conducted in RStudio (Version 1.0.14) using R (Version x64 3.5.1). The following packages were used to accomplish data transformation and analysis: data.table (Ver. 1.11.4), ICC (Ver. 2.3.0), shiny (Ver. 1.1.0), dplyr (Ver. 0.7.6), ggplot2 (Ver. 3.0.0), DescTools (Ver. 0.99.25). Shapiro-Wilks test was performed to determine if data is normally distributed, mean values were calculated for normally distributed data, geometric means was calculated otherwise. Mean values were reported with corresponding standard deviation. Since MDA should be present in all the samples, samples for which analysis was failed were removed from further analysis where MDA was involved. We performed correlation analysis, tertile analysis, and regression analysis

## RESULTS

### Chromatographic separation condition and characterization of MRM products

The optimum chromatographic conditions were achieved with Agilent Eclipse Plus Phenyl-Hexyl RRHD (1.8 μm 2.1x150 mm) column. Chromatogram of 20-fold diluted urine spiked with calibration point, containing standard at lowest level of quantification (LLOQ), is shown on Figure 2. In our previous study [UPLC OH-PAHs], we reported characterization of the fragmentation of OH-PAHs-1,2-DMI-5-SC derivatives under collision induced dissociation (CID) conditions. In the present study we use dansyl chloride to derivatize primary amino group of 8-Oxo-dG, the procedure of dansylation of amino group was previously reported by Kelman, *et al.*, for nucleotides, however fragmentation of the product under CID was not characterized (Kelman, Lilga, & Sharma, 1988). Proposed fragmentation pattern of 8-Oxo-dG-DNS adduct is shown on Figure 3.

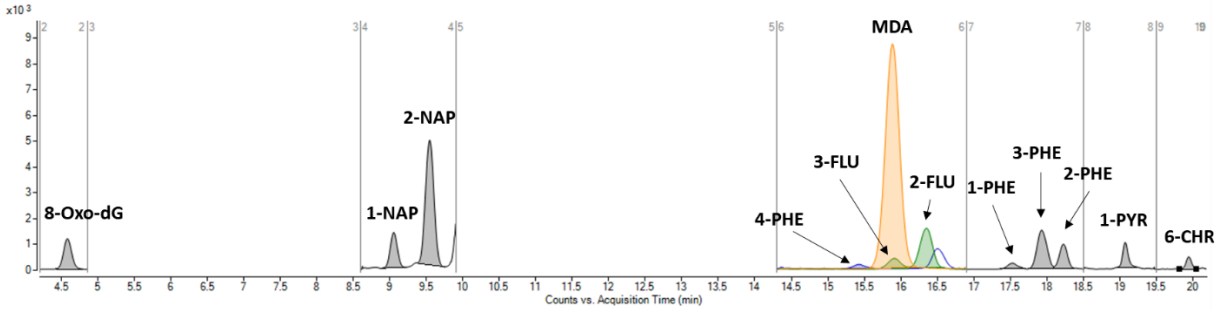



**Figure 2.** A: typical chromatograms of the lowest level of quantification (LLOQ) in twenty times diluted urine of monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs), 8-Oxo-dG, and malondialdehyde in their derivatized form using 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride, dansyl chloride, and dansylhydrazide respectively. Showed OH-PAHs LLOQ levels: 1-NAP – 0.125 ng/ml, 2-NAP – 0.125 ng/ml, 4-PHE – 0.0125 ng/ml, 3-FLU – 0.0125 ng/ml, 2-FLU – 0.0125 ng/ml, 1-PHE – 0.0125 ng/ml, 3-PHE – 0.0125 ng/ml, 2-PHE – 0.0125 ng/ml, 1-PYR – 0.0125 ng/ml, 6-HCR – 0.0125 ng/ml, 8-Oxo-dG – 0.5 ng/mL, MDA – 9 nM. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine, MDA – malondialdehyde.

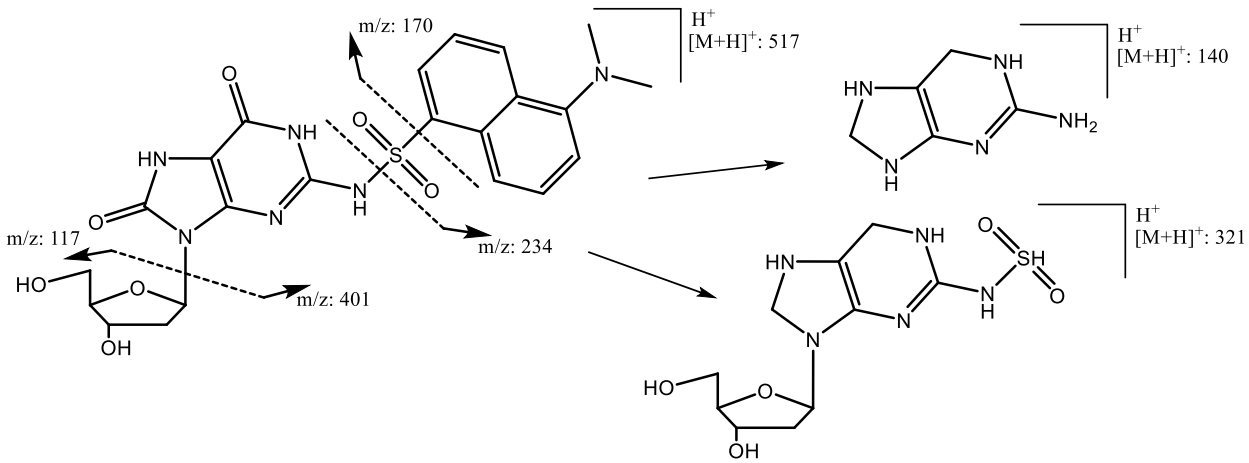



**Figure 3.** Characterization of the fragmentation of 8-Oxo-2'-deoxyguanosine–dansyl adduct under collision induced dissociation conditions, using nitrogen as a collision gas.

**Selection of limit of quantification**

LLOQ was selected by taking into the consideration the following parameters: peak shape in blanks compared to calibration level, relative retention time between labeled and unlabeled standard, and relative recovery not to exceed 80-120% range. In addition to reported calibration levels, 1/2 and 1/5 of the lowest level (Table 1) were analyzed in order to estimate LLOQ. LLOQs are summarized in Table 4. Overall, we anticipate that LLOQ for low-mass compounds maybe higher if a transition to a non-specific ion would be selected (discussed below).

**Method validation**

RR, inter- and intra-day precision, LLOQ and curve that was used to quantify relationship between relative response (ratio between unlabeled to labeled peak count) and concentration are shown in Table 4. Overall, the method showed acceptable RR and precisions, specifically RR were in 98-117% range for inter-day precision was in 2.6-18.8% range, intra-day precision was in 3.2-17.2% range.

**Table 4.** Method performance at measuring monohydroxylated polycyclic aromatic hydrocarbon metabolites in urine in the derivatized form using 1,2-dimethyl-1H-imidazole-5-sulfonyl chloride. R-squared calculated as an average r-squared of 5 runs. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine, MDA – malondialdehyde, LLOQ – lowest limit of quantification.

| | | | | Relative recovery | | Precision | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Within-day | | Between-days | |
| | R-squared | Curve | LLOQ | QCL | QCH | QCL | QCH | QCL | QCH |
| **8-Oxo-dG** | 0.9895 | Quadratic | 0.5 ng/ml | 117% | 112% | 2.6% | 2.7% | 3.5% | 3.2% |
| **MDA** | 0.9990 | Quadratic | 9.0 nM | 102% | 101% | 4.2% | 3.5% | 11.4% | 14.3% |
| **1-NAP** | 0.9970 | Quadratic | 125 pg/ml | 102% | 104% | 18.8% | 9.4% | 16.8% | 17.2% |
| **2-NAP** | 0.9929 | Quadratic | 125 pg/ml | 98% | 98% | 6.8% | 6.8% | 8.0% | 14.9% |
| **2-FLU** | 0.9954 | Quadratic | 12.5 pg/ml | 104% | 99% | 6.7% | 9.2% | 6.1% | 14.3% |
| **3-FLU** | 0.9939 | Quadratic | 12.5 pg/ml | 105% | 102% | 6.7% | 9.3% | 6.0% | 14.5% |
| **1-PHE** | 0.9929 | Quadratic | 12.5 pg/ml | 103% | 100% | 6.5% | 9.8% | 5.8% | 14.3% |
| **2-PHE** | 0.9934 | Quadratic | 12.5 pg/ml | 106% | 104% | 5.9% | 8.9% | 5.4% | 14.3% |
| **3-PHE** | 0.9937 | Quadratic | 12.5 pg/ml | 106% | 101% | 5.7% | 8.9% | 5.2% | 14.9% |
| **4-PHE** | 0.9931 | Quadratic | 12.5 pg/ml | 103% | 98% | 5.6% | 9.4% | 5.1% | 14.4% |
| **1-PYR** | 0.9916 | Quadratic | 12.5 pg/ml | 106% | 105% | 5.6% | 9.7% | 5.1% | 14.5% |
| **6-CHR** | 0.9904 | Quadratic | 12.5 pg/ml | 105% | 102% | 6.0% | 8.7% | 5.4% | 14.8% |

We evaluated possibility of using alternative MRM transitions leading to a non-specific product via Bland-Altman plots for those compounds that has a peak area for a transition leading to an ion containing both fragments, analyte and derivatizing agent. Bland-Altman plots and a summary of agreement are shown on Figure 4. Based on our analysis, MRM transition to a non-specific ion may be used for analysis of 1-NAP, 2-NAP, and 3-FLU, which will increase response from the instrument by 12.09, 8.87, and 2.28 times respectively, therefore likely increasing sensitivity.

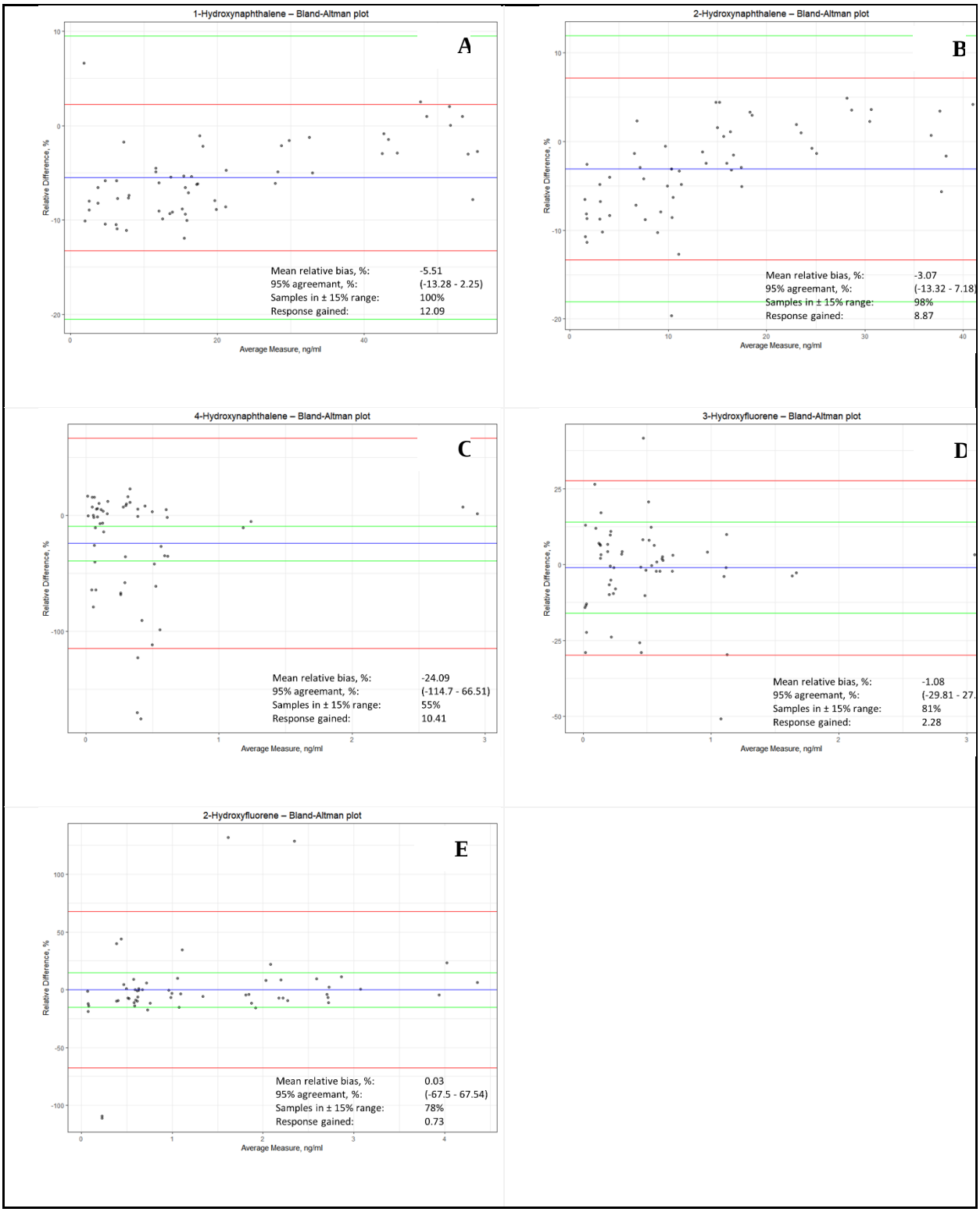



**Figure 4.** Bland-Altman analysis of the agreement between transition of monohydroxylated polycyclic aromatic hydrocarbons–1,2-dimethyl-1H-imidazole-5-sulfonyl chloride adduct leading to the formation of

a specific ion (containing fragment consist of both target analyte and derivatizing agent) and non-specific ion (containing fragment of derivatizing agent only). Plots are arranged to compounds elution order.

We evaluated matrix effects associated with urine matrix compared to twenty-fold diluted matrix by analysis of LIS responses (Figure 5). Overall, we observe statistically significant matrix effect, except for 2-NAP. However, by observing the plot data, it can be seen that LIS response had relatively higher variability compared to other compounds.

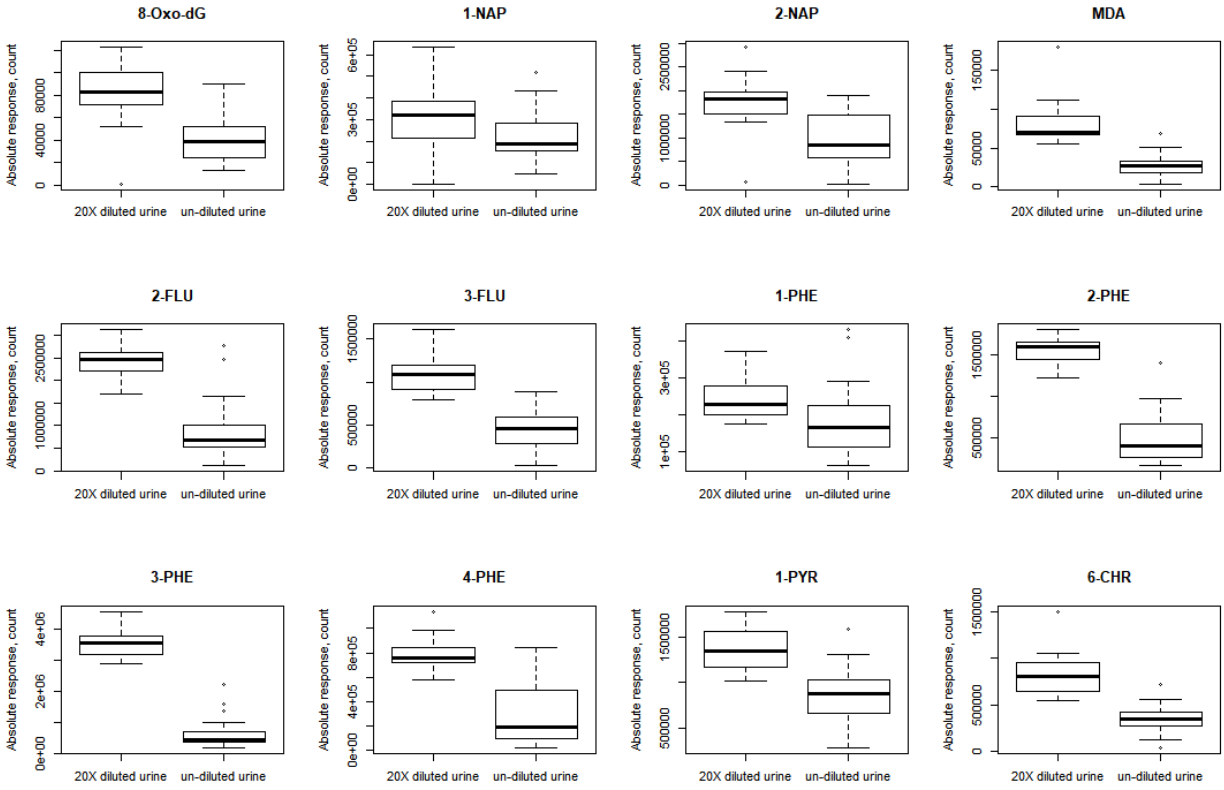



**Figure 5.** Comparison of isotopically labeled internal standard responses between 20-fold diluted urine used to prepare calibration solutions, and un-diluted urine (field samples). p-values for a two-sided t-test to compare means were all less than 0.01, except for 2-NAP where P-value = 0.072. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine, MDA – malondialdehyde.

The results of autosampler storage stability are shown on Figure 6, indicating that most of analytes were stable in their corresponding derivative forms for 46 hours without any significant loss of LIS response. 1-PYR, which had statistically significant difference in means, was tested on relative change of concentration compared initial value. We observed mean change of -0.25% (SD = 2.95) suggesting that no statistically significant difference relative response was observed over 46 hours, despite statistically significant changes in absolute response.

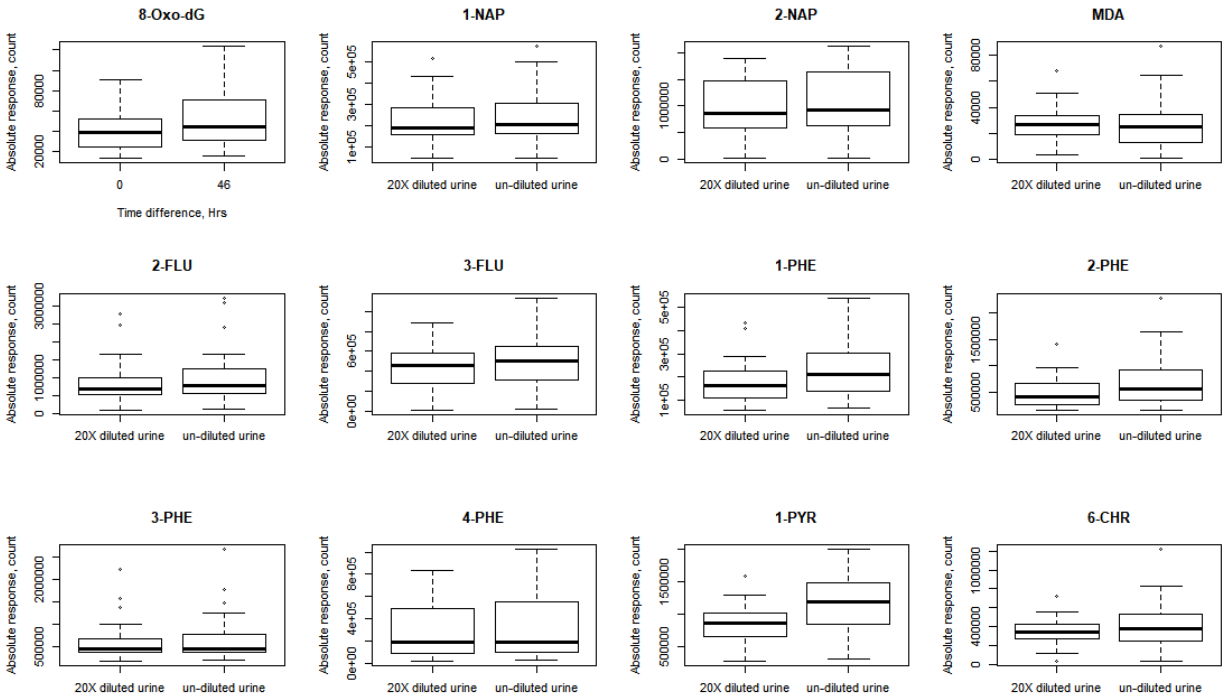



**Figure 6.** Comparison of isotopically labeled internal standard responses to evaluate autosampler storage stability. P-values calculated from two-sided t-test were all higher than 0.05, except 1-PYR (P-value = 0.005). Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine, MDA – malondialdehyde.

**Descriptive analysis**

The data on biomarkers levels obtained for studied population is shown in Table 5. We achieved more than 90% quantification frequency for the majority of compounds. Most of measured levels of 6-CHR were below LOQ; several peaks we observed in both quantification and confirmation transitions for 6-CHR, suggesting that other monohydroxylated metabolites were presented, however, due to absence of LIS we cannot confirm them.

**Table 5.** Descriptive analysis of biomarkers of oxidative stress (8-Oxo-dG, MDA) and biomarkers of exposure to polycyclic aromatic hydrocarbons (PAH) (1-/2-NAP, 2-/3-FLU, 1-/2-/3-/4-PHE, 1-PYR, 6-CHR) in urine. Data is shown in ng/ml except for MDA (nM) and was not corrected to creatinine levels. Contribution is shown as AM±SD for individual OH-PAH contributing to total concentration of measured PAH metabolites. Extrapolation of the calibration curve for 6-CHR increases detection frequency to 37.5%. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine, MDA – malondialdehyde, GM – geometric mean, GSD – geometric standard deviation, IQR - interquartile range, SD – arithmetic standard deviation, AM – arithmetic mean.

| | **8-Oxo-dG** | **MDA** | **1-NAP** | **2-NAP** | **2-FLU** | **3-FLU** |
|---|---|---|---|---|---|---|
| Shapiro-Wilks | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| GM | 4.17 | 1813.5 | 22.21 | 12.84 | 1.022 | 0.366 |
| GSD | 1.79 | 2.15 | 2.71 | 2.54 | 2.530 | 2.787 |
| Median | 3.79 | 1822.4 | 20.81 | 13.87 | 1.052 | 0.415 |
| IQR (25 - 75) | (2.75 - 6.67) | (1035.1 - 3052.1) | (12.11 - 47.25) | (7.02 - 24.3) | (0.647 - 2.140) | (0.197 - 0.713) |
| Contribution, % | NA | NA | 53±12 | 32±8 | 2.7±1.2 | 1.0±0.4 |
| Samples > LOQ, % | 100 | 91 | 100 | 100 | 100 | 100 |
| | **1-PHE** | **2-PHE** | **3-PHE** | **4-PHE** | **1-PYR** | **6-CHR** |
| Shapiro-Wilks | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| GM | 0.681 | 0.377 | 0.395 | 0.176 | 1.279 | NA |
| GSD | 2.16 | 2.498 | 2.576 | 3.132 | 2.710 | NA |
| Median | 0.749 | 0.384 | 0.368 | 0.242 | 1.303 | NA |
| IQR (25 - 75) | (0.436 - 1.200) | (0.187 - 0.720) | (0.235 - 0.803) | (0.088 - 0.384) | (0.661 - 2.518) | NA |
| Contribution, % | 2.1±1.3 | 1.1±0.5 | 1.1±0.6 | 0.54±0.34 | 3.9±2.4 | 0.02±0.07 |
| Samples > LOQ, % | 100 | 100 | 100 | 96.6 | 100 | 5.68 * |

### 3.5 Correlation analysis

Spearman correlation coefficients and p-values associated with them (Table 6) for association between OH-PAHs with their isomers and 8-Oxo-dG and MDA. We found statistically significant correlation

between biomarker of oxidative DNA damage (8-Oxo-dG) and biomarkers of exposure (OH-PAHs), less significant association was observed over biomarker of lipid peroxidation (MDA).

**Table 6.** Spearman correlation coefficients for biomarkers of exposure and effect; statistical significance showed via highlighted colors. Detection frequency of 6-CHR was not sufficient to evaluate most likelihood function of the distribution to assign values to non-detected samples, therefore analysis was performed by analysis only samples with detectable peaks of 6-CHR. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine, MDA – malondialdehyde.

| | 8-Oxo-dG | MDA | 1-NAP | 2-NAP | 2-FLU | 3-FLU | 1-PHE | 2-PHE | 3-PHE | 4-PHE | 1-PYR | 6-CHR* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8-Oxo-dG | 1 | 0.41 | 0.29 | 0.26 | 0.39 | 0.31 | 0.23 | 0.30 | 0.34 | 0.19 | 0.26 | 0.40 |
| MDA | | 1 | 0.28 | 0.15 | 0.23 | 0.19 | 0.15 | 0.22 | 0.23 | 0.17 | 0.13 | 0.34 |
| 1-NAP | | | 1 | 0.87 | 0.77 | 0.80 | 0.51 | 0.72 | 0.74 | 0.74 | 0.62 | 0.27 |
| 2-NAP | | | | 1 | 0.88 | 0.91 | 0.57 | 0.81 | 0.81 | 0.83 | 0.73 | 0.42 |
| 2-FLU | | | | | 1 | 0.93 | 0.75 | 0.93 | 0.95 | 0.86 | 0.87 | 0.49 |
| 3-FLU | | | | | | 1 | 0.65 | 0.85 | 0.88 | 0.82 | 0.83 | 0.48 |
| 1-PHE | | | | | | | 1 | 0.80 | 0.81 | 0.69 | 0.83 | 0.32 |
| 2-PHE | | | | | | | | 1 | 0.97 | 0.90 | 0.92 | 0.53 |
| 3-PHE | | | | | | | | | 1 | 0.87 | 0.93 | 0.53 |
| 4-PHE | | | | | | | | | | 1 | 0.82 | 0.49 |
| 1-PYR | | | | | | | | | | | 1 | 0.48 |
| 6-CHR | | | | | | | | | | | | 1 |

p<0.01
0.01<p<0.05
0.05<p<0.1

**Tertile and regression analysis**

Following analysis of correlation between biomarkers of effect and biomarker of exposure, we examined relation between biomarkers of effect and biomarkers of exposure. For this purpose, linear regression model was used following by tertile analysis to compare if there is a significant association between groups. No correction to p-value was used in the t-test pairwise comparison. OH-PAHs exposure was presented as a sum of concentration of individual metabolites transformed using natural logarithm (Figure 7A). Relation between biomarkers of oxidative stress was investigated in the same manner (Figure 7B). In addition to total OH-PAHs exposure, individual PAH metabolites were studied in the

same manner (Table 7). Regression analysis shows that most of OH-PAHs were associated with levels of both MDA and 8-Oxo-dG, however p-values indicate that association with 8-Oxo-dG is stronger. Tertile analysis shows statistically significant difference between high- and low-exposed groups for 8-Oxo-dG; pairwise comparison of MDA for high- and low- exposed groups had relatively lower statistically significance compared to 8-Oxo-dG.

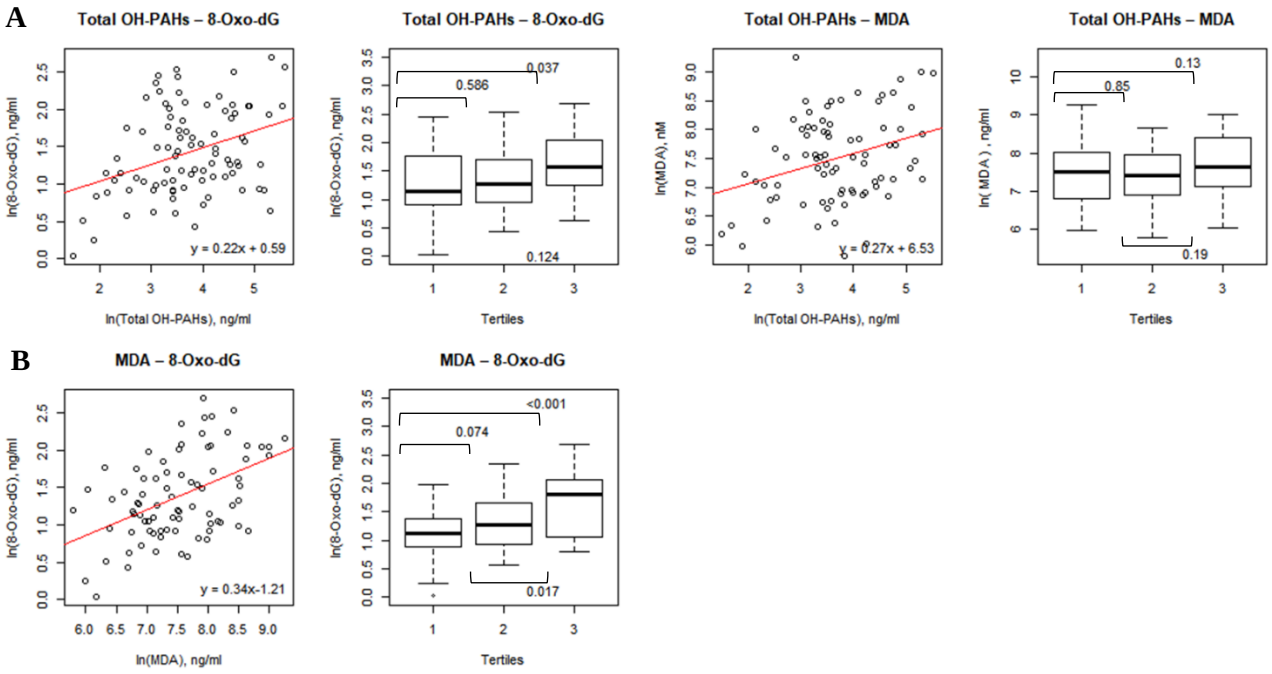



**Figure 7.** Simple regression linear and tertile analysis of association of **A)** monohydroxylated polycyclic aromatic hydrocarbon and biomarkers of oxidative stress (8-Oxo-dG, MDA), **B)** and 8-Oxo-dG and MDA. Abbreviations: 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine, MDA – malondialdehyde, asterisks – significance levels between low (first tertile) and high (third tertile) exposed groups are shown above boxplots.

**Table 7.** Summary of simple linear regression and tertile analysis (low compared to high) of individual polycyclic aromatic hydrocarbons metabolites compared to biomarkers of oxidative stress (MDA, 8-Oxo-dG). All intercepts were statistically significant, with p-value < 0.001. p-values associated with comparison of high- and low-exposed groups are provided in corresponding columns. 6-CHR results are calculated for only those samples, which concentration was non-zero. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, 8-Oxo-dG – 8-Oxo-2'-deoxyguanosine, MDA – malondialdehyde, Intcp – intercept.

| | 8-Oxo-dG | | | | MDA | | | |
|---|---|---|---|---|---|---|---|---|
| | **Intcp** | **β (95% CI)** | **p-value** | **High vs Low** | **Intcp** | **β (95% CI)** | **p-value** | **High vs Low** |
| 1-NAP | 0.822 | 0.195 (0.07 – 0.313) | 0.002 | 0.01 | 6.727 | 0.255 (0.090 – 0.419) | 0.003 | 0.032 |
| 2-NAP | 0.879 | 0.215 (0.089 – 0.340) | 0.001 | 0.085 | 6.993 | 0.203 (0.035 – 0.382) | 0.026 | 0.21 |
| 2-FLU | 1.421 | 0.285 (0.165 – 0.405) | <0.001 | 0.0037 | 7.51 | 0.245 (0.068 – 0.422) | 0.007 | 0.011 |
| 3-FLU | 1.658 | 0.229 (0.118 – 0.340) | <0.001 | 0.023 | 7.736 | 0.219 (0.059 – 0.380) | 0.008 | 0.135 |
| 1-PHE | 1.514 | 0.226 (0.071 – 0.380) | 0.005 | 0.025 | 7.59 | 0.2 (-0.021 – 0.421) | 0.075 | 0.48 |
| 2-PHE | 1.643 | 0.221 (0.093 – 0.349) | <0.001 | 0.026 | 7.728 | 0.223 (0.041 – 0.404) | 0.017 | 0.128 |
| 3-PHE | 1.651 | 0.24 (0.119 – 0.361) | <0.001 | 0.0026 | 7.733 | 0.236 (0.060 – 0.411) | 0.009 | 0.132 |
| 4-PHE | 1.552 | 0.072 (-0.036 – 0.18) | 0.19 | 0.081 | 7.706 | 0.114 (-0.034 – 0.262) | 0.13 | 0.082 |
| 1-PYR | 1.387 | 0.163 (0.043 – 0.283) | 0.008 | 0.019 | 7.478 | 0.125 (-0.044 – 0.295) | 0.145 | 0.489 |
| 6-CHR | 2.8 | 0.281 (0.073 – 0.489) | 0.01 | 0.057 | 8.822 | 0.252 (-0.016 – 0.519) | 0.064 | 0.088 |

## DISCUSSION

### Method performance

Overall, the method showed robustness in simultaneous analysis of 8-Oxo-dG, MDA, and OH-PAHs. Observed relatively high values of method's precision are likely due to the selection of small spiking volume of 25 µL in addition to potential difference in pipettes performances, since we do not experience

it in our previous study [Chapter 3], where 50 μL was used. For instance, median inter-day precision was 2.95% and median intra-day precision was 5.35% compared to 7.8% and 14.3% respectively in the present study. We also experienced loss of signal of labeled internal standard response for some MDA samples, which is likely due to error associated with low spiking volume of the derivatizing reagent.

Selection of MRM transitions for anlaysis of OH-PAHs is shown on Figure 4. Use of a transition to a non-specific ion, containing only fragment of derivatizing reagent, maybe beneficial when transition to a specific ion shows a low response. Based on our data, this transition can be used for 1-NAP, 2-NAP, and 3-FLU, where mean bias is close to 0, 95% agreemant range is narrow, and majority of samples are withing ±15 relative bias. At the same time, non-specific ion for 4-PHE (Figure 4C) should be used only as a confirmation due to high disperse of relative differences especially at low concentrations. For 2-FLU (Figure 4E) the signal gained from more specific transition is stronger than the one leading to a non-specific ion, making less specific transition impractical. Overall the trend of relative increase of response to specific ion is associated with number of benzene rings. This increase of response to a specific ion obtained under CID suggests incease of stability of positively charged intermediates.

In addition to DNSCl for derivatization of 8-Oxo-dG, we tried 1,2-dimethyl-1H-imidazole-4-sulfonyl chloride (1,2-DMI-4-SC), however we experienced multiple product formation, including mono-, di-, and tri-1,2-DMI-4-SC adducts and their ionization lead to multiple charges, therefore potentially decreasing sensitivity of the method. DNSCl revealed the only product, therefore we selected DNSCl as a derivatizing reagent for 8-Oxo-dG.

**Data analysis**

Concentrations of OH-PAHs obtained in the present work were compared to levels obtained in previous studies from rural population of Thailand (Naksen, Kawichai, Srinual, Salrasee, & Prapamontol, 2017), general urban population of China (Sun et al., 2017), and US National Health and Nutrition Examination Survey (*Fourth national report on human exposure to environmental chemicals*, 2018) (Table 8). We were not been able to quantify 6-CHR in a reasonable percent of population for further analysis, however the data, restricted to those sample where peak were detected (quatified either within

calibration range or via its extrapolation below LOQ), was analyzed showing similar correlation coefficients between monohydroxylated metabolites (Table 6). Analysis of the time segment where 6-CHR was eluted, revealed peaks of close proximity to 6-CHR and having fragmentation patterns similar to 6-CHR, suggesting that other monohydroxylated CHR may be presented in the samples potenatially at a higher abundance, therefore further confirmation via isotopically labeled standards is required. Overall, our data indicate that levels of OH-PAHs are much higher in Thailand population compared to US population, which is consistent with previously reported findings (Chetiyanukornkul et al., 2006; Chetiyanukornkul, Toriba, Kizu, & Hayakawa, 2004); compared to Chinese urban population several OH-PAHs were higher (1-/2-NAP) in our work, while other PAH metabolites (2-FLU, 1-/2-/3-/4-PHE, 1-PYR) were presented at a much lower concentration, including 1-PYR which is one of the most commonly used PAH metabolite.

**Table 8.** Levels of individual monohydroxylated polyclic aromatic hydrocarbon in selected previously reported studies. Abbreviations: NAP – hydroxynaphthalene, FLU – hydroxyfluorene, PHE – hydroxyphenanthrene, PYR – hydroxypyrene, CHR – hydroxychrysene, GM – geometric mean

| **1-NAP** | **2-NAP** | **2-FLU** | **3-FLU** | **1-PHE** | **2-PHE** | **3-PHE** | **4-PHE** | **1-PYR** | **Method** | **Ref.** |
|---|---|---|---|---|---|---|---|---|---|---|
| 22.21 | 12.84 | 1.02 | 0.366 | 0.681 | 0.377 | 0.395 | 0.176 | 1.279 | GM | Present |
| NA | NA | NA | NA | NA | NA | NA | NA | 0.82 | Median | (Naksen et al., 2017) |
| 1.41 | 4.14 | 0.24 | 0.0936 | 0.126 | 0.0608 | 0.062 | 0.0205 | 0.111 | GM | (*Fourth national report on human exposure to environmental chemicals*, 2018) |
| 6.82 | 14.35 | 3.29 | NA | 2.72 | 1.93 | 3.84 | 3.53 | 8.11 | GM | (Sun et al., 2017) |

Our data from analysis of 88 individual urine samples, indicate strong positive correlation between exposure to PAH and biomarker of oxidative stress to DNA, 8-Oxo-dG (Table 6). Similar findings were

confirmed via simple linear regression analysis with natural logarithm transformed data (Table 7). Still positive, but less stronger correlation was observed for MDA which may result from either contribution of other environmental factors (e.g. dietary, smoking status, smoking status, exercises, age, alcohol intake, health status (Block et al., 2002; Furukawa et al., 2017; Kelishadi, Mirghaffari, Poursafa, & Gidding, 2009; Reuter, Gupta, Chaturvedi, & Aggarwal, 2010; Schieber & Chandel, 2014)) or storage stability of MDA over 6 years with multiple freeze-cycles. Storage stability of either components in present analysis was never reported for a prolong period of time.

To compare our analysis on the association of biomakers of exposure and effect, several studies were identified. In the study of exposure of a village population to e-waste facilities located nearby (Q. Yang et al., 2015), the contribution of 1- and 2-NAP were similar to our work, accounting for 89% of total OH-PAHs compared to 85% reported in the present study. It was concluded, that exposure to PAHs from e-waste facilities was significantly positively correlated with levels of 8-Oxo-dG; on another hand, biomarker of oxidative stress, MDA, was statistically correlated to only 1-PYR. However, their reference, group presented by a rural area has statistically positive association of OH-PAHs with MDA, which is similar to our findings. The description of this reference area and their potential sources of PAHs were not specified. In the occupational exposure, slight increase in both 8-Oxo-dG and MDA was observed with increase of 1-PYR level, however the levels of both biomarkers were not statistically different between high- and low-exposedd groups (Jeng et al., 2011). Signficant positive correaltion of both 8-Oxo-dG and MDA was also associated with decrease of air quality (Wu et al., 2017).

Separately, studies were evaluating association between OH-PAHs and either 8-Oxo-dg or MDA. Overall, a positive correlation was reported for 8-Oxo-dG (Fan, Wang, Mao, et al., 2012; Kuang et al., 2013; Chih-Hong Pan, Chan, & Wu, 2008; Sun et al., 2017) and MDA (Bae et al., 2010; Bortey-Sam et al., 2017; Lin et al., 2016; Lu et al., 2016; Wu et al., 2017) under different exposure scenrious. On contrast, non-significant negative association of 8-Oxo-dG was reported (Fan et al., 2014) and non-significant positive (Fan, Wang, Mao, et al., 2012) with OH-PAHs.

The finding on correlation between individual OH-PAHs are in agreemant with previous studies (Fan, Wang, Mao, et al., 2012; Lu et al., 2016; Sun et al., 2017; Wu et al., 2017); however, as indicated in previous studies (Fan, Wang, Mao, et al., 2012; Lu et al., 2016), the use of a single PAH biomarker, specifically commonly used 1-PYR, is not sufficient since two-ring PAH metabolites are less corellated with 1-PYR. Additionally, we observed positive association between biomarkers of oxidative stress (Table 6, Figure 7B), which suggest that lipid peroxidation products affects DNA leading to its oxidative damage and subsequent release of 8-Oxo-dG (Marnett, 2000; Park & Floyd, 1992). At the same time, tertile analysis shows statistically significant means for 8-Oxo-dG but not MDA comparing low- and high-exposed groups to OH-PAHs. Together with high positive correlation between 8-Oxo-dG and MDA, this may indicate that other environmental co-factors may contribute to elevated levels of lipid peroxidation rather than OH-PAHs itself. Nevertherless, several studies (Lu et al., 2016; Wu et al., 2017) reported positive correlation between MDA and at least one of OH-PAHs suggesting, that MDA should be still used for profiling of air pollution associated oxidative stress.

## CONCLUSION

We developed highly sensitive liquid chromatography tandem mass spectrometry analytical method for simultaneous analysis of biomarkers of exposure (monohydroxylated PAH metabolites) and biomarkers of effect (8-Oxo-dG, MDA) in human. The method was validated on 88 individual urine samples. Our data indicate strong association between exposure to OH-PAHs and biomarker of oxidative DNA damage, 8-Oxo-dG. Levels of another biomarker of oxidative lipid peroxidation (MDA) also show positive association with OH-PAHs exposure, however tertile analysis was not statistically significant enough to demonstrate mean difference between high- and low-exposed groups. In the present work, we showed strong statistically significant association between biomarkers of effects, 8-Oxo-dg and MDA confirming hypothesis of DNA damage via intermediates of lipid peroxidation.

**FUNDING**

This work was financially supported by NIH grants 5UM1HL134590-03, P50ES026071, EPA 83615301, P30ES019776 and the Laney Graduate School of Emory University.

## REFERENCES


1. Bruce, N., et al., *WHO indoor air quality guidelines on household fuel combustion: Strategy implications of new evidence on interventions and exposure–risk functions.* Atmospheric Environment, 2015. **106**: p. 451-457.
2. Nagel, C.L., et al., *Study design of a cluster-randomized controlled trial to evaluate a large-scale distribution of cook stoves and water filters in Western Province, Rwanda.* Contemporary Clinical Trials Communications, 2016. **4**: p. 124-135.
3. Steenland, K., et al., *Modeling the potential health benefits of lower household air pollution after a hypothetical liquified petroleum gas (LPG) cookstove intervention.* Environment International, 2018. **111**: p. 71-79.
4. *Energy Access Outlook: From Poverty to Prosperity*. 2017, International Energy Agency: France. p. 144.
5. Vyas, D., M.J.J.o.E.E. Vaghani, and Studies, *Health Impact Characterization of Traditional Biomass Cook Stove Usage on Indoor Air Quality in Rural Region–A State of Review.* 2018. **3**(1).
6. Smith, K.R., et al., *Millions dead: how do we know and what does it mean? Methods used in the comparative risk assessment of household air pollution.* 2014. **35**: p. 185-206.
7. Cheng, S., et al., *Characterization of volatile organic compounds from different cooking emissions.* Atmospheric Environment, 2016. **145**: p. 299-307.
8. Jary, H., et al., *Household Air Pollution and Acute Lower Respiratory Infections in Adults: A Systematic Review.* PLoS One, 2016. **11**(12): p. e0167656.
9. Dherani, M., et al., *Indoor air pollution from unprocessed solid fuel use and pneumonia risk in children aged under five years: a systematic review and meta-analysis.* Bull World Health Organ, 2008. **86**(5): p. 390-398C.
10. Assad, N.A., et al., *Chronic Obstructive Pulmonary Disease Secondary to Household Air Pollution.* Semin Respir Crit Care Med, 2015. **36**(03): p. 408-421.
11. Po, J.Y., J.M. FitzGerald, and C. Carlsten, *Respiratory disease associated with solid biomass fuel exposure in rural women and children: systematic review and meta-analysis.* Thorax, 2011. **66**(3): p. 232-9.
12. Raspanti, G.A., et al., *Household air pollution and lung cancer risk among never-smokers in Nepal.* Environ Res, 2016. **147**: p. 141-5.
13. Josyula, S., et al., *Household air pollution and cancers other than lung: a meta-analysis.* Environ Health, 2015. **14**(1): p. 24.
14. Fatmi, Z. and D. Coggon, *Coronary heart disease and household air pollution from use of solid fuel: a systematic review.* Br Med Bull, 2016. **118**(1): p. 91-109.
15. Kim, K.H., S.A. Jahan, and E. Kabir, *A review of diseases associated with household air pollution due to the use of biomass fuels.* J Hazard Mater, 2011. **192**(2): p. 425-31.
16. Epstein, M.B., et al., *Household fuels, low birth weight, and neonatal death in India: The separate impacts of biomass, kerosene, and coal.* International Journal of Hygiene and Environmental Health, 2013. **216**(5): p. 523-532.
17. Abdel-Shafy, H.I. and M.S.M. Mansour, *A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation.* Egyptian Journal of Petroleum, 2016. **25**(1): p. 107-123.
18. Rylance, J., et al., *Household air pollution: a call for studies into biomarkers of exposure and predictors of respiratory disease.* 2013. **304**(9): p. L571-L578.
19. Choi, H., et al., *Polycyclic aromatic hydrocarbons.* 2010.
20. Ramesh, A., et al., *Bioavailability and Risk Assessment of Orally Ingested Polycyclic Aromatic Hydrocarbons.* 2004. **23**(5): p. 301-333.
21. Xue, W. and D. Warshawsky, *Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review.* Toxicology and Applied Pharmacology, 2005. **206**(1): p. 73-93.

22. Delfino, R.J., et al., *Air pollution and circulating biomarkers of oxidative stress.* 2011. **4**(1): p. 37-52.
23. Lawal, A.T., *Polycyclic aromatic hydrocarbons. A review.* Cogent Environmental Science, 2017. **3**(1): p. 1339841.
24. Pan, C.-H., et al., *Urinary 1-hydroxypyrene and malondialdehyde in male workers in Chinese restaurants.* 2008. **65**(11): p. 732-735.
25. Yang, Q., et al., *Polycyclic aromatic hydrocarbon (PAH) exposure and oxidative stress for a rural population from the North China Plain.* 2015. **22**(3): p. 1760-1769.
26. Wu, X., et al., *Determination of air pollution-related biomarkers of exposure in urine of travellers between Germany and China using liquid chromatographic and liquid chromatographic-mass spectrometric methods: a pilot study.* Biomarkers, 2017. **22**(6): p. 525-536.
27. Valavanidis, A., T. Vlachogianni, and C. Fiotakis, *8-hydroxy-2' -deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress and Carcinogenesis.* Journal of Environmental Science and Health, Part C, 2009. **27**(2): p. 120-139.
28. Møller, P. and S. Loft, *Oxidative Damage to DNA and Lipids as Biomarkers of Exposure to Air Pollution.* Environmental Health Perspectives, 2010. **118**(8): p. 1126-1136.
29. Fan, R., et al., *Fast and Simultaneous Determination of Urinary 8-Hydroxy-2'-deoxyguanosine and Ten Monohydroxylated Polycyclic Aromatic Hydrocarbons by Liquid Chromatography/Tandem Mass Spectrometry.* Chemical Research in Toxicology, 2012. **25**(2): p. 491-499.
30. Li, J., et al., *Co-exposure to polycyclic aromatic hydrocarbons, benzene and toluene and their dose–effects on oxidative stress damage in kindergarten-aged children in Guangzhou, China.* Science of The Total Environment, 2015. **524-525**: p. 74-80.
31. Li, J., et al., *Exposure to polycyclic aromatic hydrocarbons could cause their oxidative DNA damage: a case study for college students in Guangzhou, China.* 2015. **22**(3): p. 1770-1777.
32. Lu, S.-y., et al., *Associations between polycyclic aromatic hydrocarbon (PAH) exposure and oxidative stress in people living near e-waste recycling facilities in China.* Environment International, 2016. **94**: p. 161-169.
33. Panuwet, P., et al., *Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring.* Critical Reviews in Analytical Chemistry, 2016. **46**(2): p. 93-105.
34. Pilz, J., I. Meineke, and C.H. Gleiter, *Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative.* Journal of Chromatography B: Biomedical Sciences and Applications, 2000. **742**(2): p. 315-325.
35. *Guidance for industry, bioanalytical method validation*, U.D.o.H.H. Services, Editor. 2018.
36. Chesher, D., *Evaluating Assay Precision.* The Clinical Biochemist Reviews, 2008. **29**(Suppl 1): p. S23-S26.
37. Giavarina, D., *Understanding bland altman analysis.* Biochemia medica: Biochemia medica, 2015. **25**(2): p. 141-151.
38. Kelman, D.J., K.T. Lilga, and M. Sharma, *Synthesis and application of fluorescent labeled nucleotides to assay DNA damage.* Chemico-Biological Interactions, 1988. **66**(1): p. 85-100.
39. Naksen, W., et al., *First evidence of high urinary 1-hydroxypyrene level among rural school children during smoke haze episode in Chiang Mai Province, Thailand.* Atmospheric Pollution Research, 2017. **8**(3): p. 418-427.
40. Sun, H., et al., *Dose-response relationship between urinary polycyclic aromatic hydrocarbons metabolites and urinary 8-hydroxy-2'-deoxyguanosine in a Chinese general population.* Chemosphere, 2017. **174**: p. 506-514.
41. *Fourth national report on human exposure to environmental chemicals*. 2018, Centers for Disease Control Prevention: Atlanta, GA.

42. Chetiyanukornkul, T., et al., *Urinary 2-Hydroxyfluorene and 1-Hydroxypyrene Levels in Smokers and Nonsmokers in Japan and Thailand.* Polycyclic Aromatic Compounds, 2004. **24**(4-5): p. 467-474.
43. Chetiyanukornkul, T., et al., *Simultaneous determination of urinary hydroxylated metabolites of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene as multiple biomarkers of exposure to polycyclic aromatic hydrocarbons.* 2006. **386**(3): p. 712-718.
44. Block, G., et al., *Factors Associated with Oxidative Stress in Human Populations.* American Journal of Epidemiology, 2002. **156**(3): p. 274-285.
45. Kelishadi, R., et al., *Lifestyle and environmental factors associated with inflammation, oxidative stress and insulin resistance in children.* Atherosclerosis, 2009. **203**(1): p. 311-319.
46. Schieber, M. and Navdeep S. Chandel, *ROS Function in Redox Signaling and Oxidative Stress.* Current Biology, 2014. **24**(10): p. R453-R462.
47. Furukawa, S., et al., *Increased oxidative stress in obesity and its impact on metabolic syndrome.* 2017. **114**(12): p. 1752-1761.
48. Reuter, S., et al., *Oxidative stress, inflammation, and cancer: How are they linked?* Free Radical Biology and Medicine, 2010. **49**(11): p. 1603-1616.
49. Jeng, H.A., et al., *Polycyclic aromatic hydrocarbon-induced oxidative stress and lipid peroxidation in relation to immunological alteration.* 2011. **68**(9): p. 653-658.
50. Pan, C.-H., C.-C. Chan, and K.-Y. Wu, *Effects on Chinese Restaurant Workers of Exposure to Cooking Oil Fumes: A Cautionary Note on Urinary 8-Hydroxy-2'-Deoxyguanosine.* 2008. **17**(12): p. 3351-3357.
51. Fan, R., et al., *Preliminary study of children's exposure to PAHs and its association with 8-hydroxy-2'-deoxyguanosine in Guangzhou, China.* Environment International, 2012. **42**: p. 53-58.
52. Kuang, D., et al., *Dose-Response Relationships of Polycyclic Aromatic Hydrocarbons Exposure and Oxidative Damage to DNA and Lipid in Coke Oven Workers.* Environmental Science & Technology, 2013. **47**(13): p. 7446-7456.
53. Bae, S., et al., *Exposures to Particulate Matter and Polycyclic Aromatic Hydrocarbons and Oxidative Stress in Schoolchildren.* 2010. **118**(4): p. 579-583.
54. Bortey-Sam, N., et al., *Oxidative stress and respiratory symptoms due to human exposure to polycyclic aromatic hydrocarbons (PAHs) in Kumasi, Ghana.* Environmental Pollution, 2017. **228**: p. 311-320.
55. Lin, Y., et al., *Urinary Metabolites of Polycyclic Aromatic Hydrocarbons and the Association with Lipid Peroxidation: A Biomarker-Based Study between Los Angeles and Beijing.* Environmental Science & Technology, 2016. **50**(7): p. 3738-3745.
56. Fan, R., et al., *Biomass fuels and coke plants are important sources of human exposure to polycyclic aromatic hydrocarbons, benzene and toluene.* Environmental Research, 2014. **135**: p. 1-8.
57. Park, J.-W. and R.A. Floyd, *Lipid peroxidation products mediate the formation of 8-hydroxydeoxyguanosine in DNA.* Free Radical Biology and Medicine, 1992. **12**(4): p. 245-250.
58. Marnett, L.J., *Oxyradicals and DNA damage.* Carcinogenesis, 2000. **21**(3): p. 361-370.

# CONCLUSIONS

In the present work, we demonstrated that derivatization may improve and even enable analysis of several biomarkers of exposure and effect in urine samples at biological concentrations. Specifically, we developed a cost-effective method for analysis of MDA in urine and EBC samples using isotope dilution, additionally, we showed the possibility of such analysis in more sophisticated serum samples where advanced purification technique like solid phase extraction is required. Throughout the development of these analytical techniques, we showed that understanding and implementation of existing parts of the instrument as well as understanding chemistry can benefit analyst in developing optimized methods. To our knowledge, presented methods are the first reported methods for analysis of MDA in the listed biological samples using this instrument. Both methods were validated with respect to FDA guidance and showed their robustness on field samples, which guarantee methods applicability in epidemiological studies.

In the chapter 3, we described and advanced sample preparation and derivatization procedure which despite its relative complication enables analysis of urinary metabolites of PAHs exposure in the form of derivatives. The method was validated with respect to FDA guidance; it was cross-validated against established but more expensive LC MS/MS. We obtained equations necessary for conversion results from UPLC MSD to LC MS/MS results if data should be compared with LC MS/MS instrument. Kinetics associated with derivatives formation and degradation was studied, chemical structures were characterized via monitoring ions formed under collision induced dissociation. These products were compared to patterns described in the previously published papers on this derivatizing agent. Overall, the developed method can be applied in further epidemiological studies in the countries where primary household exposure to PAHs occurs without additional shipping to high-income countries where majority of established techniques, specifically LC MS/MS, are located. Presented method not only gives opportunity to characterized exposure locally, but also provide essential information for further methods development on similar compounds.

Adjusting physical-chemical properties of biomarkers of interest with various derivatizing agents enable us to combine them, consequently allowing their analysis in one single analytical method. This was achieved via extension of the method, described in chapter 3 combining with method described in chapter 2. Additionally, we incorporate analysis of a more specific biomarker of oxidative stress, 8-Oxo-dG, via utilizing third derivatizing reagent. Unfortunately, multi-fraction analysis introduces more matrix to the sample which suppressed several target analytes and therefore prevented analysis using more cost-effective instrument, like single-quadrupole mass spectrometer. The method was assessed on 88 field samples, the significance of combining biomarkers of exposure and effects was proven via statistical tests. Specifically, 8-Oxo-dG and MDA were positively correlated with levels of OH-PAHs. Additionally, positive correlation was obtained between MDA and 8-Oxo-dG suggesting that MDA can lead to further damage to DNA.

Overall, developed methods create a solid platform (theoretical and inspirational) for further analytical method improvements in both environments: with highly limited and relatively limited financial resources.

# FUTURE WORK

Our work shows that using multi-fraction separation followed by derivatization can enable simultaneous analysis of compounds with different retention properties. While we demonstrated it on a limited but important compound, this work can be expanded in the future to cover other urinary metabolites with similar properties and functioning groups. For examples, we anticipate that 4-hydroxynonenal (4-HNE) can be added, which was reported to be another biomarker of lipid peroxidation (Zhong & Yin, 2015), dose-dependent response can be studied for MDA and 4-HNE. While we include only monohydroxylated metabolites of PAHs, several other metabolites may be of interest, for example tetrols and amino-PAHs, which have a higher carcinogenic effect and were shown to be excreted in urine (Gong, Zhu, Kipen, Wang, Hu, Ohman-Strickland, Lu, Zhang, Wang, & Zhu, 2013; Riedel, Scherer, Engl, Hagedorn, & Tricker, 2006). This group of compounds can be likely quantified using 1,2-DMI-5-SC.

## REFERENCES


Abdel-Shafy, H. I., & Mansour, M. S. M. (2016). A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egyptian Journal of Petroleum, 25*(1), 107-123. doi:https://doi.org/10.1016/j.ejpe.2015.03.011

Ahmadzai, H., Huang, S., Hettiarachchi, R., Lin, J.-L., Thomas Paul, S., & Zhang, Q. (2013). Exhaled breath condensate: a comprehensive update. In *Clinical Chemistry and Laboratory Medicine* (Vol. 51, pp. 1343).

Armbruster, D. A., & Pry, T. (2008). Limit of Blank, Limit of Detection and Limit of Quantitation. *The Clinical Biochemist Reviews, 29*(Suppl 1), S49-S52.

Assad, N. A., Balmes, J., Mehta, S., Cheema, U., & Sood, A. (2015). Chronic Obstructive Pulmonary Disease Secondary to Household Air Pollution. *Semin Respir Crit Care Med, 36*(03), 408-421. doi:10.1055/s-0035-1554846

Ayala, A., Mu, #xf1, oz, M. F., Arg, #xfc, & elles, S. (2014a). Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxidative Medicine and Cellular Longevity, 2014*, 31. doi:10.1155/2014/360438

Ayala, A., Mu, #xf1, oz, M. F., Arg, #xfc, & elles, S. (2014b). Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal %J Oxidative Medicine and Cellular Longevity. *2014*, 31. doi:10.1155/2014/360438

Bae, S., Pan, X.-C., Kim, S.-Y., Park, K., Kim, Y.-H., Kim, H., & Hong, Y.-C. (2010). Exposures to Particulate Matter and Polycyclic Aromatic Hydrocarbons and Oxidative Stress in Schoolchildren. *118*(4), 579-583. doi:doi:10.1289/ehp.0901077

Baird, W. M., Hooven, L. A., & Mahadevan, B. (2005). Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. *45*(2-3), 106-114. doi:doi:10.1002/em.20095

Balazs, A., Gabriella, H., Orsolya, D., & Imre, B. (2014). Monitoring oxidative stress during chronic obstructive pulmonary disease exacerbations using malondialdehyde. *Respirology, 19*(1), 74-79. doi:doi:10.1111/resp.12155

Bartoli, M. L., Novelli, F., Costa, F., Malagrinò, L., Melosini, L., Bacci, E., . . . Paggiaro, P. L. (2011a). Malondialdehyde in Exhaled Breath Condensate as a Marker of Oxidative Stress in Different Pulmonary Diseases. *Mediators of Inflammation, 2011*, 7. doi:10.1155/2011/891752

Bartoli, M. L., Novelli, F., Costa, F., Malagrinò, L., Melosini, L., Bacci, E., . . . Paggiaro, P. L. (2011b). Malondialdehyde in Exhaled Breath Condensate as a Marker of Oxidative Stress in Different Pulmonary Diseases %J Mediators of Inflammation. *2011*, 7. doi:10.1155/2011/891752

Binding, N., Kläning, H., Karst, U., Pötter, W., Czeschinski, P. A., & Witting, U. J. F. J. o. A. C. (1998). Analytical reliability of carbonyl compound determination using 1,5-dansylhydrazine-derivatization. *362*(3), 270-273. doi:10.1007/s002160051072

Block, G., Dietrich, M., Norkus, E. P., Morrow, J. D., Hudes, M., Caan, B., & Packer, L. (2002). Factors Associated with Oxidative Stress in Human Populations. *American Journal of Epidemiology, 156*(3), 274-285. doi:10.1093/aje/kwf029

Boaz, M., Matas, Z., Biro, A., Katzir, Z. e., Green, M., Fainaru, M., & Smetana, S. (1999). Serum malondialdehyde and prevalent cardiovascular disease in hemodialysis. *Kidney International, 56*(3), 1078-1083. doi:https://doi.org/10.1046/j.1523-1755.1999.00613.x

Bortey-Sam, N., Ikenaka, Y., Akoto, O., Nakayama, S. M. M., Asante, K. A., Baidoo, E., . . . Ishizuka, M. (2017). Oxidative stress and respiratory symptoms due to human exposure to polycyclic aromatic hydrocarbons (PAHs) in Kumasi, Ghana. *Environmental Pollution, 228*, 311-320. doi:https://doi.org/10.1016/j.envpol.2017.05.036

Bortey-Sam, N., Ikenaka, Y., Akoto, O., Nakayama, S. M. M., Marfo, J., Saengtienchai, A., . . . Ishizuka, M. (2016). Excretion of polycyclic aromatic hydrocarbon metabolites (OH-PAHs) in cattle urine in Ghana. *Environmental Pollution, 218*, 331-337. doi:https://doi.org/10.1016/j.envpol.2016.07.008

Bruce, N., Pope, D., Rehfuess, E., Balakrishnan, K., Adair-Rohani, H., & Dora, C. (2015). WHO indoor air quality guidelines on household fuel combustion: Strategy implications of new evidence on interventions and exposure–risk functions. *Atmospheric Environment, 106*, 451-457. doi:10.1016/j.atmosenv.2014.08.064

Casimirri, E., Stendardo, M., Bonci, M., Andreoli, R., Bottazzi, B., Leone, R., . . . Boschetto, P. (2016). Biomarkers of oxidative-stress and inflammation in exhaled breath condensate from hospital cleaners. *Biomarkers, 21*(2), 115-122. doi:10.3109/1354750X.2015.1118541

Chen, J.-L., Huang, Y.-J., Pan, C.-H., Hu, C.-W., & Chao, M.-R. (2011). Determination of urinary malondialdehyde by isotope dilution LC-MS/MS with automated solid-phase extraction: A cautionary note on derivatization optimization. *Free Radical Biology and Medicine, 51*(9), 1823-1829. doi:https://doi.org/10.1016/j.freeradbiomed.2011.08.012

Cheng, S., Wang, G., Lang, J., Wen, W., Wang, X., & Yao, S. (2016). Characterization of volatile organic compounds from different cooking emissions. *Atmospheric Environment, 145*, 299-307. doi:https://doi.org/10.1016/j.atmosenv.2016.09.037

Chesher, D. (2008). Evaluating Assay Precision. *The Clinical Biochemist Reviews, 29*(Suppl 1), S23-S26.

Chetiyanukornkul, T., Toriba, A., Kameda, T., Tang, N., Hayakawa, K. J. A., & Chemistry, B. (2006). Simultaneous determination of urinary hydroxylated metabolites of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene as multiple biomarkers of exposure to polycyclic aromatic hydrocarbons. *386*(3), 712-718. doi:10.1007/s00216-006-0628-6

Chetiyanukornkul, T., Toriba, A., Kizu, R., & Hayakawa, K. (2004). Urinary 2-Hydroxyfluorene and 1-Hydroxypyrene Levels in Smokers and Nonsmokers in Japan and Thailand. *Polycyclic Aromatic Compounds, 24*(4-5), 467-474. doi:10.1080/10406630490471483

Choi, H., Harrison, R., Komulainen, H., & Saborit, J. M. D. (2010). Polycyclic aromatic hydrocarbons.

Cohen, A. J., Brauer, M., Burnett, R., Anderson, H. R., Frostad, J., Estep, K., . . . Forouzanfar, M. H. (2017). Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015. *Lancet, 389*(10082), 1907-1918. doi:10.1016/S0140-6736(17)30505-6

Corradi, M., Folesani, G., Andreoli, R., Manini, P., Bodini, A., Piacentini, G., . . . Baraldi, E. (2003). Aldehydes and Glutathione in Exhaled Breath Condensate of Children with Asthma Exacerbation. *American Journal of Respiratory and Critical Care Medicine, 167*(3), 395-399. doi:10.1164/rccm.200206-507OC

Corradi, M., Pignatti, P., Manini, P., Andreoli, R., Goldoni, M., Poppa, M., . . . Mutti, A. (2004). Comparison between exhaled and sputum oxidative stress biomarkers in chronic airway inflammation. *European Respiratory Journal, 24*(6), 1011-1017. doi:10.1183/09031936.04.00002404

Corradi, M., Rubinstein, I., Andreoli, R., Manini, P., Caglieri, A., Poli, D., . . . Mutti, A. (2003). Aldehydes in Exhaled Breath Condensate of Patients with Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine, 167*(10), 1380-1386. doi:10.1164/rccm.200210-1253OC

Cui, X., Gong, J., Han, H., He, L., Teng, Y., Tetley, T., . . . Zhang, J. (2018). Relationship between free and total malondialdehyde, a well-established marker of oxidative stress, in various types of human biospecimens. *Journal of Thoracic Disease, 10*(5).

Del Rio, D., Stewart, A. J., & Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism and Cardiovascular Diseases, 15*(4), 316-328. doi:https://doi.org/10.1016/j.numecd.2005.05.003

Delfino, R. J., Staimer, N., Vaziri, N. D. J. A. Q., Atmosphere, & Health. (2011). Air pollution and circulating biomarkers of oxidative stress. *4*(1), 37-52. doi:10.1007/s11869-010-0095-2

Dherani, M., Pope, D., Mascarenhas, M., Smith, K. R., Weber, M., & Bruce, N. (2008). Indoor air pollution from unprocessed solid fuel use and pneumonia risk in children aged under five years: a systematic review and meta-analysis. *Bull World Health Organ, 86*(5), 390-398C. doi:10.2471/BLT.07.044529

Ding, X., Wang, X.-M., Xie, Z.-Q., Xiang, C.-H., Mai, B.-X., Sun, L.-G., . . . Pöschl, U. (2007). Atmospheric polycyclic aromatic hydrocarbons observed over the North Pacific Ocean and the Arctic area: Spatial distribution and source identification. *Atmospheric Environment, 41*(10), 2061-2072. doi:10.1016/j.atmosenv.2006.11.002

*Energy Access Outlook: From Poverty to Prosperity*. (2017). Retrieved from France: https://www.iea.org/publications/freepublications/publication/WEO2017SpecialReport_EnergyAccessOutlook.pdf

Epstein, M. B., Bates, M. N., Arora, N. K., Balakrishnan, K., Jack, D. W., & Smith, K. R. (2013). Household fuels, low birth weight, and neonatal death in India: The separate impacts of biomass, kerosene, and coal. *International Journal of Hygiene and Environmental Health, 216*(5), 523-532. doi:https://doi.org/10.1016/j.ijheh.2012.12.006

Fan, R., Li, J., Chen, L., Xu, Z., He, D., Zhou, Y., . . . Li, J. (2014). Biomass fuels and coke plants are important sources of human exposure to polycyclic aromatic hydrocarbons, benzene and toluene. *Environmental Research, 135*, 1-8. doi:https://doi.org/10.1016/j.envres.2014.08.021

Fan, R., Wang, D., Mao, C., Ou, S., Lian, Z., Huang, S., . . . She, J. (2012). Preliminary study of children's exposure to PAHs and its association with 8-hydroxy-2′-deoxyguanosine in Guangzhou, China. *Environment International, 42*, 53-58. doi:https://doi.org/10.1016/j.envint.2011.03.021

Fan, R., Wang, D., Ramage, R., & She, J. (2012). Fast and Simultaneous Determination of Urinary 8-Hydroxy-2′-deoxyguanosine and Ten Monohydroxylated Polycyclic Aromatic Hydrocarbons by Liquid Chromatography/Tandem Mass Spectrometry. *Chemical Research in Toxicology, 25*(2), 491-499. doi:10.1021/tx200517h

Fandiño-Del-Rio, M., Goodman, D., Kephart, J. L., Miele, C. H., Williams, K. N., Moazzami, M., . . . Trials, H. A. P. t. T. I. J. (2017). Effects of a liquefied petroleum gas stove intervention on pollutant exposure and adult cardiopulmonary outcomes (CHAP): study protocol for a randomized controlled trial. *18*(1), 518. doi:10.1186/s13063-017-2179-x

Fatmi, Z., & Coggon, D. (2016). Coronary heart disease and household air pollution from use of solid fuel: a systematic review. *Br Med Bull, 118*(1), 91-109. doi:10.1093/bmb/ldw015

*Fourth national report on human exposure to environmental chemicals*. (2018). Retrieved from Atlanta, GA: https://www.cdc.gov/exposurereport/index.html

Frankel, E. N., & Neff, W. E. (1983). Formation of malonaldehyde from lipid oxidation products. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 754*(3), 264-270. doi:https://doi.org/10.1016/0005-2760(83)90141-8

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., . . . Shimomura, I. J. T. J. o. c. i. (2017). Increased oxidative stress in obesity and its impact on metabolic syndrome. *114*(12), 1752-1761.

Giavarina, D. (2015). Understanding bland altman analysis. *Biochemia medica: Biochemia medica, 25*(2), 141-151.

Giera, M., Kloos, D.-P., Raaphorst, A., Mayboroda, O. A., Deelder, A. M., Lingeman, H., & Niessen, W. M. A. (2011). Mild and selective labeling of malondialdehyde with 2-aminoacridone: assessment of urinary malondialdehyde levels. *Analyst, 136*(13), 2763-2769. doi:10.1039/C1AN15254H

Giera, M., Lingeman, H., & Niessen, W. M. A. (2012a). Recent Advancements in the LC- and GC-Based Analysis of Malondialdehyde (MDA): A Brief Overview. *Chromatographia, 75*(9), 433-440. doi:10.1007/s10337-012-2237-1

Giera, M., Lingeman, H., & Niessen, W. M. A. J. C. (2012b). Recent Advancements in the LC- and GC-Based Analysis of Malondialdehyde (MDA): A Brief Overview. *75*(9), 433-440. doi:10.1007/s10337-012-2237-1

Goldoni, M., Caglieri, A., Andreoli, R., Poli, D., Manini, P., Vettori, M. V., . . . Mutti, A. (2005). Influence of condensation temperature on selected exhaled breath parameters. *BMC Pulmonary Medicine, 5*(1), 10.

Gong, J., Zhu, T., Kipen, H., Wang, G., Hu, M., Ohman-Strickland, P., . . . Zhu, P. (2013). Malondialdehyde in exhaled breath condensate and urine as a biomarker of air pollution induced oxidative stress. *Journal of Exposure Science and Environmental Epidemiology, 23*(3), 322.

Gong, J., Zhu, T., Kipen, H., Wang, G., Hu, M., Ohman-Strickland, P., . . . Zhang, J. (2013a). Malondialdehyde in exhaled breath condensate and urine as a biomarker of air pollution induced oxidative stress. *Journal Of Exposure Science And Environmental Epidemiology, 23*, 322. doi:10.1038/jes.2012.127

https://www.nature.com/articles/jes2012127#supplementary-information

Gong, J., Zhu, T., Kipen, H., Wang, G., Hu, M., Ohman-Strickland, P., . . . Zhang, J. (2013b). Malondialdehyde in exhaled breath condensate and urine as a biomarker of air pollution induced oxidative stress. *Journal of exposure science & environmental epidemiology, 23*(3), 322-327. doi:10.1038/jes.2012.127

Grob, N., Aytekin, M., & Dweik, R. (2008). Biomarkers in exhaled breath condensate: a review of collection, processing and analysis. *Journal of breath research, 2*(3), 037004.

Grova, N., Salquèbre, G., Schroeder, H., & Appenzeller, B. M. R. (2011). Determination of PAHs and OH-PAHs in Rat Brain by Gas Chromatography Tandem (Triple Quadrupole) Mass Spectrometry. *Chemical Research in Toxicology, 24*(10), 1653-1667. doi:10.1021/tx2003596

*Guidance for industry, bioanalytical method validation*. (2018). Retrieved from https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064964.htm

Hansen, Å. M., Mathiesen, L., Pedersen, M., & Knudsen, L. E. (2008). Urinary 1-hydroxypyrene (1-HP) in environmental and occupational studies—A review. *International Journal of Hygiene and Environmental Health, 211*(5), 471-503. doi:https://doi.org/10.1016/j.ijheh.2007.09.012

Haritash, A. K., & Kaushik, C. P. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *Journal of Hazardous Materials, 169*(1), 1-15. doi:https://doi.org/10.1016/j.jhazmat.2009.03.137

Health, U. D. o., & Services, H. (2001). Guidance for industry, bioanalytical method validation. *http://www. fda. gov/cder/guidance/index. htm*.

Hecker, M., & Ullrich, V. (1989). On the mechanism of prostacyclin and thromboxane A2 biosynthesis. *264*(1), 141-150.

Hirayama, T., Yamada, N., Nohara, M., & Fukui, S. (1984). The high performance liquid chromatographic determination of total malondialdehyde in vegetable oil with dansyl hydrazine. *35*(3), 338-344. doi:doi:10.1002/jsfa.2740350314

Hogard, M. L., Lunte, C. E., & Lunte, S. M. (2017). Detection of reactive aldehyde biomarkers in biological samples using solid-phase extraction pre-concentration and liquid chromatography with fluorescence detection. *Analytical Methods, 9*(12), 1848-1854. doi:10.1039/C6AY03327J

Humans, I. W. G. o. t. E. o. C. R. t. (2010). Household use of solid fuels and high-temperature frying. *IARC monographs on the evaluation of carcinogenic risks to humans, 95*, 1-430.

Il'yasova, D., Scarbrough, P., & Spasojevic, I. (2012). Urinary biomarkers of oxidative status. *Clinica Chimica Acta, 413*(19), 1446-1453. doi:http://dx.doi.org/10.1016/j.cca.2012.06.012

Itoh, N., Tao, H., & Ibusuki, T. (2005). Optimization of aqueous acetylation for determination of hydroxy polycyclic aromatic hydrocarbons in water by stir bar sorptive extraction and thermal desorption–gas chromatography–mass spectrometry. *Analytica Chimica Acta, 535*(1), 243-250. doi:https://doi.org/10.1016/j.aca.2004.12.002

Ivica, J., Skoumalová, A., Topinková, E., & Wilhelm, J. J. C. (2011). HPLC Separation of Fluorescent Products of Lipid Peroxidation in Erythrocytes and Mitochondria. *73*(1), 67-73. doi:10.1007/s10337-010-1887-0

Jary, H., Simpson, H., Havens, D., Manda, G., Pope, D., Bruce, N., & Mortimer, K. (2016). Household Air Pollution and Acute Lower Respiratory Infections in Adults: A Systematic Review. *PLoS One, 11*(12), e0167656. doi:10.1371/journal.pone.0167656

Jeng, H. A., Pan, C.-H., Diawara, N., Chang-Chien, G.-P., Lin, W.-Y., Huang, C.-T., . . . Wu, M.-T. (2011). Polycyclic aromatic hydrocarbon-induced oxidative stress and lipid peroxidation in relation to immunological alteration. *68*(9), 653-658. doi:10.1136/oem.2010.055020 %J Occupational and Environmental Medicine

Jongeneelen, F. J., Anzion, R. B. M., Scheepers, P. T. J., Bos, R. P., Henderson, P. T., Nijenhuis, E. H., . . . Winkes, A. (1988). 1-Hydroxypyrene in Urine as a Biological Indicator of Exposure to Polycyclic Aromatic Hydrocarbons in Severeal Work Environments. *The Annals of Occupational Hygiene, 32*(1), 35-43. doi:10.1093/annhyg/32.1.35

Josyula, S., Lin, J., Xue, X., Rothman, N., Lan, Q., Rohan, T. E., & Hosgood, H. D., 3rd. (2015). Household air pollution and cancers other than lung: a meta-analysis. *Environ Health, 14*(1), 24. doi:10.1186/s12940-015-0001-3

Kabir, E., & Kim, K.-H. (2011). An investigation on hazardous and odorous pollutant emission during cooking activities. *Journal of Hazardous Materials, 188*(1), 443-454. doi:https://doi.org/10.1016/j.jhazmat.2011.01.113

Karatas, F., Karatepe, M., & Baysar, A. (2002). Determination of free malondialdehyde in human serum by high-performance liquid chromatography. *Analytical Biochemistry, 311*(1), 76-79. doi:https://doi.org/10.1016/S0003-2697(02)00387-1

Kelishadi, R., Mirghaffari, N., Poursafa, P., & Gidding, S. S. (2009). Lifestyle and environmental factors associated with inflammation, oxidative stress and insulin resistance in children. *Atherosclerosis, 203*(1), 311-319. doi:https://doi.org/10.1016/j.atherosclerosis.2008.06.022

Kelman, D. J., Lilga, K. T., & Sharma, M. (1988). Synthesis and application of fluorescent labeled nucleotides to assay DNA damage. *Chemico-Biological Interactions, 66*(1), 85-100. doi:https://doi.org/10.1016/0009-2797(88)90043-9

Keski-Rahkonen, P., Desai, R., Jimenez, M., Harwood, D. T., & Handelsman, D. J. (2015). Measurement of Estradiol in Human Serum by LC-MS/MS Using a Novel Estrogen-Specific Derivatization Reagent. *Analytical Chemistry, 87*(14), 7180-7186. doi:10.1021/acs.analchem.5b01042

Khoubnasabjafari, M., Ansarin, K., & Jouyban, A. (2015). Reliability of malondialdehyde as a biomarker of oxidative stress in psychological disorders. *BioImpacts : BI, 5*(3), 123-127. doi:10.15171/bi.2015.20

Khoubnasabjafari, M., Ansarin, K., & Jouyban, A. (2016). Critical Review of Malondialdehyde Analysis in Biological Samples. *Current Pharmaceutical Analysis, 12*(1), 4-17.

Kim, B., Jung, W., & Kho, Y. (2017). Quantification of Malondialdehyde in Human Urine by HPLC-DAD and Derivatization with 2,4-Dinitrophenylhydrazine. *Bulletin of the Korean Chemical Society, 38*(6), 642-645. doi:10.1002/bkcs.11143

Kim, K.-H., Jahan, S. A., Kabir, E., & Brown, R. J. C. (2013). A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. *Environment International, 60*, 71-80. doi:https://doi.org/10.1016/j.envint.2013.07.019

Kim, K. H., Jahan, S. A., & Kabir, E. (2011). A review of diseases associated with household air pollution due to the use of biomass fuels. *J Hazard Mater, 192*(2), 425-431. doi:10.1016/j.jhazmat.2011.05.087

Korchazhkina, O., Exley, C., & Andrew Spencer, S. (2003). Measurement by reversed-phase high-performance liquid chromatography of malondialdehyde in normal human urine following derivatisation with 2,4-dinitrophenylhydrazine. *Journal of Chromatography B, 794*(2), 353-362. doi:https://doi.org/10.1016/S1570-0232(03)00495-1

Kuang, D., Zhang, W., Deng, Q., Zhang, X., Huang, K., Guan, L., . . . Guo, H. (2013). Dose-Response Relationships of Polycyclic Aromatic Hydrocarbons Exposure and Oxidative Damage to DNA and Lipid in Coke Oven Workers. *Environmental Science & Technology, 47*(13), 7446-7456. doi:10.1021/es401639x

Landrigan, P. J. (2017). Air pollution and health. *Lancet Public Health, 2*(1), e4-e5. doi:10.1016/S2468-2667(16)30023-8

Lankova, D., Urbancova, K., Sram, R. J., Hajslova, J., Pulkrabova, J. J. A., & Chemistry, B. (2016). A novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites in urine using ultra-high-performance liquid chromatography with tandem mass spectrometry. *408*(10), 2515-2525. doi:10.1007/s00216-016-9350-1

Lawal, A. T. (2017). Polycyclic aromatic hydrocarbons. A review. *Cogent Environmental Science, 3*(1), 1339841. doi:10.1080/23311843.2017.1339841

Lee, J. S., Choi, Y. C., Shin, J. H., Lee, J. H., Lee, Y., Park, S. Y., . . . Yu, I. J. (2015). Health surveillance study of workers who manufacture multi-walled carbon nanotubes. *Nanotoxicology, 9*(6), 802-811. doi:10.3109/17435390.2014.978404

Li, J., Fan, R., Lu, S., Zhang, D., Zhou, Y., Lv, Y. J. E. S., & Research, P. (2015). Exposure to polycyclic aromatic hydrocarbons could cause their oxidative DNA damage: a case study for college students in Guangzhou, China. *22*(3), 1770-1777. doi:10.1007/s11356-014-2769-z

Li, J., Lu, S., Liu, G., Zhou, Y., Lv, Y., She, J., & Fan, R. (2015). Co-exposure to polycyclic aromatic hydrocarbons, benzene and toluene and their dose–effects on oxidative stress damage in kindergarten-aged children in Guangzhou, China. *Science of The Total Environment, 524-525*, 74-80. doi:https://doi.org/10.1016/j.scitotenv.2015.04.020

Li, P., Ding, G., Deng, Y., Punyapitak, D., Li, D., & Cao, Y. (2013). Determination of malondialdehyde in biological fluids by high-performance liquid chromatography using rhodamine B hydrazide as the derivatization reagent. *Free Radical Biology and Medicine, 65*, 224-231. doi:https://doi.org/10.1016/j.freeradbiomed.2013.06.032

Li, Y., Li, A. C., Shi, H., Zhou, S., Shou, W. Z., Jiang, X., . . . Lauterbach, J. H. (2005). The use of chemical derivatization to enhance liquid chromatography/tandem mass spectrometric determination of 1-hydroxypyrene, a biomarker for polycyclic aromatic hydrocarbons in human urine. *Rapid Communications in Mass Spectrometry, 19*(22), 3331-3338. doi:10.1002/rcm.2196

Li, Z., Romanoff, L. C., Trinidad, D. A., Hussain, N., Jones, R. S., Porter, E. N., . . . Sjödin, A. (2006). Measurement of Urinary Monohydroxy Polycyclic Aromatic Hydrocarbons Using Automated Liquid−Liquid Extraction and Gas Chromatography/Isotope Dilution High-Resolution Mass Spectrometry. *Analytical Chemistry, 78*(16), 5744-5751. doi:10.1021/ac0606094

Liang, Y., Yeligar, S. M., & Brown, L. A. S. (2012). Exhaled Breath Condensate: A Promising Source for Biomarkers of Lung Disease. *The Scientific World Journal, 2012*, 7. doi:10.1100/2012/217518

Lin, Y., Qiu, X., Yu, N., Yang, Q., Araujo, J. A., & Zhu, Y. (2016). Urinary Metabolites of Polycyclic Aromatic Hydrocarbons and the Association with Lipid Peroxidation: A Biomarker-Based Study between Los Angeles and Beijing. *Environmental Science & Technology, 50*(7), 3738-3745. doi:10.1021/acs.est.5b04629

Lord, H. L., Rosenfeld, J., Raha, S., & Hamadeh, M. J. (2008). Automated derivatization and analysis of malondialdehyde using column switching sample preparation HPLC with fluorescence detection. *31*(2), 387-401. doi:doi:10.1002/jssc.200700390

Lord, H. L., Rosenfeld, J., Volovich, V., Kumbhare, D., & Parkinson, B. (2009). Determination of malondialdehyde in human plasma by fully automated solid phase analytical derivatization. *Journal of Chromatography B, 877*(13), 1292-1298. doi:https://doi.org/10.1016/j.jchromb.2008.12.035

Lorente, L., Martín, M. M., Abreu-González, P., Ramos, L., Argueso, M., Solé-Violán, J., . . . Jiménez, A. (2015). Serum Malondialdehyde Levels in Patients with Malignant Middle Cerebral Artery Infarction Are Associated with Mortality. *PLOS ONE, 10*(5), e0125893. doi:10.1371/journal.pone.0125893

Lu, S.-y., Li, Y.-x., Zhang, J.-q., Zhang, T., Liu, G.-h., Huang, M.-z., . . . Qiu, R.-l. (2016). Associations between polycyclic aromatic hydrocarbon (PAH) exposure and oxidative stress in people living near e-waste recycling facilities in China. *Environment International, 94*, 161-169. doi:https://doi.org/10.1016/j.envint.2016.05.021

Marnett, L. J. (1999). Lipid peroxidation—DNA damage by malondialdehyde. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 424*(1), 83-95. doi:https://doi.org/10.1016/S0027-5107(99)00010-X

Marnett, L. J. (2000). Oxyradicals and DNA damage. *Carcinogenesis, 21*(3), 361-370. doi:10.1093/carcin/21.3.361

Mateos, R., & Bravo, L. (2007). Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins). *Journal of Separation Science, 30*(2), 175-191. doi:10.1002/jssc.200600314

Møller, P., & Loft, S. (2010). Oxidative Damage to DNA and Lipids as Biomarkers of Exposure to Air Pollution. *Environmental Health Perspectives, 118*(8), 1126-1136. doi:10.1289/ehp.0901725

Moselhy, H. F., Reid, R. G., Yousef, S., & Boyle, S. P. (2013). A specific, accurate, and sensitive measure of total plasma malondialdehyde by HPLC. *54*(3), 852-858. doi:10.1194/jlr.D032698

Motorykin, O., Schrlau, J., Jia, Y., Harper, B., Harris, S., Harding, A., . . . Massey Simonich, S. L. (2015). Determination of parent and hydroxy PAHs in personal PM2.5 and urine samples collected during Native American fish smoking activities. *Science of The Total Environment, 505*, 694-703. doi:https://doi.org/10.1016/j.scitotenv.2014.10.051

Nagel, C. L., Kirby, M. A., Zambrano, L. D., Rosa, G., Barstow, C. K., Thomas, E. A., & Clasen, T. F. (2016). Study design of a cluster-randomized controlled trial to evaluate a large-scale distribution of cook stoves and water filters in Western Province, Rwanda. *Contemporary Clinical Trials Communications, 4*, 124-135. doi:https://doi.org/10.1016/j.conctc.2016.07.003

Naksen, W., Kawichai, S., Srinual, N., Salrasee, W., & Prapamontol, T. (2017). First evidence of high urinary 1-hydroxypyrene level among rural school children during smoke haze episode in Chiang Mai Province, Thailand. *Atmospheric Pollution Research, 8*(3), 418-427. doi:https://doi.org/10.1016/j.apr.2016.11.002

Niedernhofer, L. J., Daniels, J. S., Rouzer, C. A., Greene, R. E., & Marnett, L. J. (2003). Malondialdehyde, a Product of Lipid Peroxidation, Is Mutagenic in Human Cells. *278*(33), 31426-31433. doi:10.1074/jbc.M212549200

Oh, J.-A., & Shin, H.-S. (2017). Simple and sensitive determination of malondialdehyde in human urine and saliva using UHPLC–MS/MS after derivatization with 3,4-diaminobenzophenone. *Journal of Separation Science, 40*(20), 3958-3968. doi:10.1002/jssc.201700490

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry, 95*(2), 351-358. doi:https://doi.org/10.1016/0003-2697(79)90738-3

Onyemauwa, F., Rappaport, S. M., Sobus, J. R., Gajdošová, D., Wu, R. a., & Waidyanatha, S. (2009). Using liquid chromatography–tandem mass spectrometry to quantify monohydroxylated metabolites of polycyclic aromatic hydrocarbons in urine. *Journal of Chromatography B, 877*(11), 1117-1125. doi:https://doi.org/10.1016/j.jchromb.2009.02.067

Pan, C.-H., Chan, C.-C., Huang, Y.-L., & Wu, K.-Y. (2008). Urinary 1-hydroxypyrene and malondialdehyde in male workers in Chinese restaurants. *65*(11), 732-735. doi:10.1136/oem.2007.036970 %J Occupational and Environmental Medicine

Pan, C.-H., Chan, C.-C., & Wu, K.-Y. (2008). Effects on Chinese Restaurant Workers of Exposure to Cooking Oil Fumes: A Cautionary Note on Urinary 8-Hydroxy-2′-Deoxyguanosine. *17*(12), 3351-3357. doi:10.1158/1055-9965.EPI-08-0075 %J Cancer Epidemiology Biomarkers & Prevention

Panuwet, P., Hunter, R. E., D'Souza, P. E., Chen, X., Radford, S. A., Cohen, J. R., . . . Barr, D. B. (2016). Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring. *Critical Reviews in Analytical Chemistry, 46*(2), 93-105. doi:10.1080/10408347.2014.980775

Park, J.-W., & Floyd, R. A. (1992). Lipid peroxidation products mediate the formation of 8-hydroxydeoxyguanosine in DNA. *Free Radical Biology and Medicine, 12*(4), 245-250. doi:https://doi.org/10.1016/0891-5849(92)90111-S

Passing, H., & Bablok, W. (1983). A New Biometrical Procedure for Testing the Equality of Measurements from Two Different Analytical Methods. Application of linear regression procedures for method comparison studies in Clinical Chemistry, Part I. In *Clinical Chemistry and Laboratory Medicine* (Vol. 21, pp. 709).

Pelclova, D., Zdimal, V., Kacer, P., Fenclova, Z., Vlckova, S., Syslova, K., . . . Barosova, H. (2016). Oxidative stress markers are elevated in exhaled breath condensate of workers exposed to nanoparticles during iron oxide pigment production. *Journal of breath research, 10*(1), 016004.

Pelclova, D., Zdimal, V., Kacer, P., Zikova, N., Komarc, M., Fenclova, Z., . . . Bello, D. (2017). Markers of lipid oxidative damage in the exhaled breath condensate of nano TiO2 production workers. *Nanotoxicology, 11*(1), 52-63. doi:10.1080/17435390.2016.1262921

Pelletier, G., Rigden, M., Kauri, L. M., Shutt, R., Mahmud, M., Cakmak, S., . . . Dales, R. (2017). Associations between urinary biomarkers of oxidative stress and air pollutants observed in a randomized crossover exposure to steel mill emissions. *International Journal of Hygiene and Environmental Health, 220*(2, Part B), 387-394. doi:https://doi.org/10.1016/j.ijheh.2016.11.010

Pilz, J., Meineke, I., & Gleiter, C. H. (2000). Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. *Journal of Chromatography B: Biomedical Sciences and Applications, 742*(2), 315-325. doi:http://dx.doi.org/10.1016/S0378-4347(00)00174-2

Plastaras, J. P., Guengerich, F. P., Nebert, D. W., & Marnett, L. J. (2000). Xenobiotic-metabolizing Cytochromes P450 Convert Prostaglandin Endoperoxide to Hydroxyheptadecatrienoic Acid and the Mutagen, Malondialdehyde. *275*(16), 11784-11790. doi:10.1074/jbc.275.16.11784

Po, J. Y., FitzGerald, J. M., & Carlsten, C. (2011). Respiratory disease associated with solid biomass fuel exposure in rural women and children: systematic review and meta-analysis. *Thorax, 66*(3), 232-239. doi:10.1136/thx.2010.147884

Rahman, I., & Kelly, F. (2003). ReviewBiomarkers in Breath Condensate: A promising New Non-invasive Technique in Free Radical Research. *Free Radical Research, 37*(12), 1253-1266. doi:10.1080/10715760310001623331

Ramesh, A., Walker, S. A., Hood, D. B., Guillén, M. D., Schneider, K., & Weyand, E. H. (2004). Bioavailability and Risk Assessment of Orally Ingested Polycyclic Aromatic Hydrocarbons. *23*(5), 301-333. doi:10.1080/10915810490517063

Ramsauer, B., Sterz, K., Hagedorn, H.-W., Engl, J., Scherer, G., McEwan, M., . . . Chemistry, B. (2011). A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of phenolic polycyclic aromatic hydrocarbons (OH-PAH) in urine of non-smokers and smokers. *399*(2), 877-889. doi:10.1007/s00216-010-4355-7

Raspanti, G. A., Hashibe, M., Siwakoti, B., Wei, M., Thakur, B. K., Pun, C. B., . . . Sapkota, A. (2016). Household air pollution and lung cancer risk among never-smokers in Nepal. *Environ Res, 147*, 141-145. doi:10.1016/j.envres.2016.02.008

Rengarajan, T., Rajendran, P., Nandakumar, N., Lokeshkumar, B., Rajendran, P., & Nishigaki, I. (2015). Exposure to polycyclic aromatic hydrocarbons with special focus on cancer. *Asian Pacific Journal of Tropical Biomedicine, 5*(3), 182-189. doi:https://doi.org/10.1016/S2221-1691(15)30003-4

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology and Medicine, 49*(11), 1603-1616. doi:https://doi.org/10.1016/j.freeradbiomed.2010.09.006

Rezaei, Z., Jamshidzadeh, A., & Sanati, E. (2013). A rapid and sensitive method for the determination of malondialdehyde as its hydralazine derivative in human plasma using high performance liquid chromatography. *Analytical Methods, 5*(12), 2995-2999. doi:10.1039/C3AY26525K

Riedel, K., Scherer, G., Engl, J., Hagedorn, H.-W., & Tricker, A. R. (2006). Determination of Three Carcinogenic Aromatic Amines in Urine of Smokers and Nonsmokers. *Journal of Analytical Toxicology, 30*(3), 187-195. doi:10.1093/jat/30.3.187

Roberta, A., Paola, M., Massimo, C., Antonio, M., & A., N. W. M. (2003). Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, *17*(7), 637-645. doi:doi:10.1002/rcm.960

Romieu, I., Barraza-Villarreal, A., Escamilla-Nuñez, C., Almstrand, A.-C., Diaz-Sanchez, D., Sly, P. D., & Olin, A.-C. (2008). Exhaled breath malondialdehyde as a marker of effect of exposure to air pollution in children with asthma. *Journal of Allergy and Clinical Immunology*, *121*(4), 903-909.e906. doi:https://doi.org/10.1016/j.jaci.2007.12.004

Rylance, J., Gordon, S. B., Naeher, L. P., Patel, A., Balmes, J. R., Adetona, O., . . . William J. Martin, I. (2013). Household air pollution: a call for studies into biomarkers of exposure and predictors of respiratory disease. *304*(9), L571-L578. doi:10.1152/ajplung.00416.2012

Schieber, M., & Chandel, Navdeep S. (2014). ROS Function in Redox Signaling and Oxidative Stress. *Current Biology*, *24*(10), R453-R462. doi:https://doi.org/10.1016/j.cub.2014.03.034

Smith, C. J., Walcott, C. J., Huang, W., Maggio, V., Grainger, J., & Patterson, D. G. (2002). Determination of selected monohydroxy metabolites of 2-, 3- and 4-ring polycyclic aromatic hydrocarbons in urine by solid-phase microextraction and isotope dilution gas chromatography–mass spectrometry. *Journal of Chromatography B*, *778*(1), 157-164. doi:https://doi.org/10.1016/S0378-4347(01)00456-X

Smith, K. R., Bruce, N., Balakrishnan, K., Adair-Rohani, H., Balmes, J., Chafe, Z., . . . Pope, D. J. A. r. o. p. h. (2014). Millions dead: how do we know and what does it mean? Methods used in the comparative risk assessment of household air pollution. *35*, 185-206.

Smith, K. R., Mehta, S., & Maeusezahl-Feuz, M. (2004). Indoor air pollution from household use of solid fuels.

Sobsey, C. A., Han, J., Lin, K., Swardfager, W., Levitt, A., & Borchers, C. H. (2016). Development and evaluation of a liquid chromatography–mass spectrometry method for rapid, accurate quantitation of malondialdehyde in human plasma. *Journal of Chromatography B*, *1029-1030*, 205-212. doi:https://doi.org/10.1016/j.jchromb.2016.07.013

Stalikas, C. D., & Konidari, C. N. (2001). Analysis of Malondialdehyde in Biological Matrices by Capillary Gas Chromatography with Electron-Capture Detection and Mass Spectrometry. *Analytical Biochemistry*, *290*(1), 108-115. doi:https://doi.org/10.1006/abio.2000.4951

Steenland, K., Pillarisetti, A., Kirby, M., Peel, J., Clark, M., Checkley, W., . . . Clasen, T. (2018). Modeling the potential health benefits of lower household air pollution after a hypothetical liquified petroleum gas (LPG) cookstove intervention. *Environment International*, *111*, 71-79. doi:https://doi.org/10.1016/j.envint.2017.11.018

Steghens, J.-P., van Kappel, A. L., Denis, I., & Collombel, C. (2001). Diaminonaphtalene, a new highly specific regent for HPLC-UV measurement of total and free malondialdehyde in human plasma or serum. *Free Radical Biology and Medicine*, *31*(2), 242-249. doi:https://doi.org/10.1016/S0891-5849(01)00578-0

Sun, H., Hou, J., Zhou, Y., Yang, Y., Cheng, J., Xu, T., . . . Yuan, J. (2017). Dose-response relationship between urinary polycyclic aromatic hydrocarbons metabolites and urinary 8-hydroxy-2′-deoxyguanosine in a Chinese general population. *Chemosphere*, *174*, 506-514. doi:https://doi.org/10.1016/j.chemosphere.2017.01.104

Syslová, K., Kačer, P., Kuzma, M., Najmanová, V., Fenclová, Z., Vlčková, Š., . . . Pelclová, D. (2009). Rapid and easy method for monitoring oxidative stress markers in body fluids of patients with asbestos or silica-induced lung diseases. *Journal of Chromatography B*, *877*(24), 2477-2486. doi:https://doi.org/10.1016/j.jchromb.2009.06.008

Tsikas, D. (2017). Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. *Analytical Biochemistry*, *524*, 13-30. doi:https://doi.org/10.1016/j.ab.2016.10.021

Tsikas, D., Rothmann, S., Schneider, J. Y., Suchy, M.-T., Trettin, A., Modun, D., . . . Frölich, J. C. (2016). Development, validation and biomedical applications of stable-isotope dilution GC–MS

and GC–MS/MS techniques for circulating malondialdehyde (MDA) after pentafluorobenzyl bromide derivatization: MDA as a biomarker of oxidative stress and its relation to 15(S)-8-iso-prostaglandin F2α and nitric oxide (NO). *Journal of Chromatography B, 1019*, 95-111. doi:https://doi.org/10.1016/j.jchromb.2015.10.009

Uchida, K. (2000). Role of reactive aldehyde in cardiovascular diseases. *Free Radical Biology and Medicine, 28*(12), 1685-1696. doi:https://doi.org/10.1016/S0891-5849(00)00226-4

Unwin, J., Cocker, J., Scobbie, E., & Chambers, H. J. A. o. O. H. (2006). An assessment of occupational exposure to polycyclic aromatic hydrocarbons in the UK. *50*(4), 395-403.

Urbancova, K., Lankova, D., Rossner, P., Rossnerova, A., Svecova, V., Tomaniova, M., . . . Pulkrabova, J. (2017). Evaluation of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns. *Science of The Total Environment, 577*, 212-219. doi:https://doi.org/10.1016/j.scitotenv.2016.10.165

Valavanidis, A., Vlachogianni, T., & Fiotakis, C. (2009). 8-hydroxy-2′ -deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress and Carcinogenesis. *Journal of Environmental Science and Health, Part C, 27*(2), 120-139. doi:10.1080/10590500902885684

Voitkun, V., & Zhitkovich, A. (1999). Analysis of DNA–protein crosslinking activity of malondialdehyde in vitro. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 424*(1), 97-106. doi:https://doi.org/10.1016/S0027-5107(99)00011-1

Vyas, D., Vaghani, M. J. J. o. E. E., & Studies. (2018). Health Impact Characterization of Traditional Biomass Cook Stove Usage on Indoor Air Quality in Rural Region–A State of Review. *3*(1).

Wang, Q., Mesaros, C., & Blair, I. A. (2016). Ultra-high sensitivity analysis of estrogens for special populations in serum and plasma by liquid chromatography–mass spectrometry: Assay considerations and suggested practices. *The Journal of Steroid Biochemistry and Molecular Biology, 162*, 70-79. doi:https://doi.org/10.1016/j.jsbmb.2016.01.002

Wang, Y., Tian, Z., Zhu, H., Cheng, Z., Kang, M., Luo, C., . . . Zhang, G. (2012). Polycyclic aromatic hydrocarbons (PAHs) in soils and vegetation near an e-waste recycling site in South China: Concentration, distribution, source, and risk assessment. *Science of The Total Environment, 439*, 187-193. doi:https://doi.org/10.1016/j.scitotenv.2012.08.018

Wu, X., Lintelmann, J., Klingbeil, S., Li, J., Wang, H., Kuhn, E., . . . Zimmermann, R. (2017). Determination of air pollution-related biomarkers of exposure in urine of travellers between Germany and China using liquid chromatographic and liquid chromatographic-mass spectrometric methods: a pilot study. *Biomarkers, 22*(6), 525-536. doi:10.1080/1354750X.2017.1306753

Xu, L., & Spink, D. C. (2007). 1,2-Dimethylimidazole-4-sulfonyl chloride, a novel derivatization reagent for the analysis of phenolic compounds by liquid chromatography electrospray tandem mass spectrometry: Application to 1-hydroxypyrene in human urine. *Journal of Chromatography B, 855*(2), 159-165. doi:https://doi.org/10.1016/j.jchromb.2007.04.039

Xue, W., & Warshawsky, D. (2005). Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. *Toxicology and Applied Pharmacology, 206*(1), 73-93. doi:https://doi.org/10.1016/j.taap.2004.11.006

Yang, B.-C., Fang, S.-F., Wan, X.-J., Luo, Y., Zhou, J.-Y., Li, Y., . . . Huang, O.-P. (2017). Quantification of monohydroxylated polycyclic aromatic hydrocarbons in human urine samples using solid-phase microextraction coupled with glass-capillary nanoelectrospray ionization mass spectrometry. *Analytica Chimica Acta, 973*, 68-74. doi:https://doi.org/10.1016/j.aca.2017.04.009

Yang, Q., Qiu, X., Li, R., Ma, J., Li, K., Li, G. J. E. S., & Research, P. (2015). Polycyclic aromatic hydrocarbon (PAH) exposure and oxidative stress for a rural population from the North China Plain. *22*(3), 1760-1769. doi:10.1007/s11356-014-3284-y

Yao, L., Yang, J., Liu, B., Zheng, S., Wang, W., Zhu, X., & Qian, X. (2014). Development of a sensitive method for the quantification of urinary 3-hydroxybenzo[a]pyrene by solid phase extraction, dansyl chloride derivatization and liquid chromatography-tandem mass spectrometry detection. *Analytical Methods, 6*(16), 6488-6493. doi:10.1039/C4AY00867G

Yebra-Pimentel, I., Fernández-González, R., Martínez-Carballo, E., Simal-Gándara, J. J. C. r. i. f. s., & nutrition. (2015). A critical review about the health risk assessment of PAHs and their metabolites in foods. *55*(10), 1383-1405.

Yeo, H. C., Helbock, H. J., Chyu, D. W., & Ames, B. N. (1994). Assay of Malondialdehyde in Biological Fluids by Gas Chromatography-Mass Spectrometry. *Analytical Biochemistry*, *220*(2), 391-396. doi:https://doi.org/10.1006/abio.1994.1355

Zhao, G., Chen, Y., Wang, S., Yu, J., Wang, X., Xie, F., . . . Xie, J. (2013). Simultaneous determination of 11 monohydroxylated PAHs in human urine by stir bar sorptive extraction and liquid chromatography/tandem mass spectrometry. *Talanta*, *116*, 822-826. doi:https://doi.org/10.1016/j.talanta.2013.07.071

Zhong, H., & Yin, H. (2015). Role of lipid peroxidation derived 4-hydroxynonenal (4-HNE) in cancer: Focusing on mitochondria. *Redox Biology*, *4*, 193-199. doi:https://doi.org/10.1016/j.redox.2014.12.011

Zorawar, S., Karthigesu, I. P., Pramjit, S., & Rupinder, K. (2014). Use of malondialdehyde as a biomarker for assessing oxidative stress in different disease pathologies: a review. *Iranian Journal of Public Health, 43*(3 Suppl.), 7-16.