BIOMARKERS OF OLIGOMERIC 1,6-HEXAMETHYLENE DIISOCYANATE EXPOSURE IN THE AUTOMOTIVE REFINISHING INDUSTRY

Zachary G. Robbins

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Sciences and Engineering in the Gillings School of Global Public Health.

Chapel Hill
2019

Approved by:

Leena Nylander-French

Wanda Bodnar

Rebecca Fry

Avram Gold

Zhenfa Zhang

ABSTRACT

Zachary G. Robbins: Biomarkers of oligomeric 1,6-hexamethylene diisocyanate exposure in the automotive refinishing industry
(Under the direction of Leena Nylander-French)

Spray-painters in automotive refinishing industries are exposed to the oligomeric trimer of 1,6-hexamethylene diisocyanate (HDI) monomer, HDI isocyanurate, a sensitizing agent that poses a significant risk for acute and chronic health effects. Biomonitoring of HDI exposures has been mostly limited to quantification of 1,6-diaminohexane (HDA), the hydrolysis product of HDI monomer, in urine or plasma. Because biomarkers of HDI monomer exposure are not appropriate biomarkers of oligomeric HDI exposures, the magnitude and distinct characteristics of HDI monomer and oligomer exposures and their relationships to their biomarkers need to be determined in exposed workers. We hypothesized that trisaminohexyl isocyanurate (TAHI), a hydrolysis product of HDI isocyanurate, is quantifiable in urine or plasma and is associated with HDI isocyanurate exposures. Towards this goal, sample extraction and analytical methods were developed and optimized to measure TAHI levels in urine and plasma of 47 spray-painters whose HDI monomer and HDI isocyanurate inhalation and skin exposures and urine and plasma HDA levels were previously characterized. Urine and plasma samples were acid hydrolyzed, extracted with dichloromethane, and derivatized with acetic anhydride for analysis of TAHI by nano-UPLC-ESI-MS/MS.

TAHI biomarker levels were significantly associated with HDI isocyanurate inhalation exposure levels and the duration of spray-painting task in both linear regression and linear mixed model analyses. Painting in downdraft booths significantly reduced exposure to HDI isocyanurate and urine TAHI levels. TAHI biomarker levels were also significantly correlated with HDI monomer exposures. As expected, HDA levels in urine or plasma were observed to be unsuitable biomarkers of HDI isocyanurate exposure. Based on the observations in this study, painting in downdraft booths, wearing nitrile or neoprene gloves, and wearing full-face air purifying, PAPR, or supplied-air respirators offers the greatest combination of respiratory and skin protection to reduce hazardous HDI exposures and biomarkers of exposure in automotive spray-painters. In summary, the developed method for quantification of HDI isocyanurate biomarker, TAHI, in urine and plasma is a significant advancement for HDI exposure assessment and will advance future investigations to oligomeric isocyanate exposures and biomarkers as well as associated health effects.

ACKNOWLEDGMENTS

There are many individuals I would like to thank for making this work possible and guiding me along the way. I am incredibly grateful for my MS and PhD advisor, Leena Nylander-French, for putting up with me and my irregular, procrastinating work habits throughout this decade. Leena was always able to quickly return edited papers no matter how busy she was with other work. According to friends and peers that was faster than any other professor. She also allowed me to try as many variations on experiments as humanly possible, even when most of them were dead ends. I am also thankful for the many past and current members of the Nylander-French lab that mentored me and assisted on this project and other projects not mentioned in this dissertation. Sheila Flack, Linda Gaines, and Jennifer Thomasen were all close to finishing their PhDs when I first arrived, yet they were all helpful in teaching me the ins and outs of the research that was spearheaded by Kenneth Fent before I started graduate school. Sheila and Linda both assisted with my introduction to the biological sample preparation and GC-MS analysis. Jennifer got me started on LC-MS analysis, and was integral in managing the sample collection work for our Seattle trip in 2010. I would not have the hands-on experience with inhalation and skin exposure monitoring without her help. Speaking of Seattle, Carolyn Reeb-Whitaker and the SHARP team, and Domonick Zanarini and the graduate students at the University of Washington were vital in making the summer in Seattle a fun.and intellectually immersive experience.

Lastly, Jayne Boyer has been an incredibly helpful lab manager and work friend since she arrived. Before she got here it was a nightmare to order any products through the school. She has made life easier in the lab being far more organized than what I imagine is the typical experience of a student in a laboratory. Plus, she has been the only constant in the laboratory as many students have come and gone while I've been in graduate school.

I also need to thank the members of my PhD committee (Wanda Bodnar, Avram Gold, Rebecca Fry, and Zhenfa Zhang) for their guidance during this process. The laboratory methods developed for this dissertation research would not have been possible without the chemical synthesis expertise and manual labor from Avram and Zhenfa. Wanda throughout the years, beginning with the end of my master's research, taught me how to use almost every LC-MS system in the UNC Biomarker Mass Spectrometry Facility. Every time I was disappointed with my experiments, we moved to another machine to try again. I may have broken a few things early on, but with her help I've acquired a vast amount of experience on my treasured nanospray-UPLC that no one wants to use anymore. Leonard Collins, although not directly associated with my project, was also instrumental in assisting with all LC-MS systems in the facility. Even when he tried to look busy, he was almost always willing to take a quick break and help with any question or problem I encountered. Benjamin Moeller was a post-doctoral fellow for Dr. Swenberg when I first started running samples in the facility and taught me tips and tricks for using UPLC and nano-UPLC. It was a shame when he left because it was easy to coordinate which days we would reserve a machine to run samples. Pete Cable has been working in the facility for years and has been my go-to conversationalist for all things sports and hiking despite his affinity for baseball.

I'd like to thank my family, especially my parents, for accepting me as a professional college student for over a decade without too much questioning about my decisions or lack thereof. After a few questions during past holidays they got the point and ceased needling me about graduating and finding a job. That will likely change after this is submitted. Finally, I need to thank all the friends I've made while at UNC, specifically my closest friends from the department whom I met during my first years in graduate school. They could have cracked many more jokes at my expense over the years about graduating last, but we usually kept school talk to a minimum. They still tried to take many shots at me for going to Duke, but it's difficult to land those shots when I went to the school with the greatest program and coach in college basketball.

This research was supported by grants from the National Institute for Occupational Safety and Health (R01-OH007598, R01-OH009364, R01-OH010476, and T42-OH008673), the North Carolina Occupational Safety and Health Education and Research Center's Pilot Projects Program, and the National Institute of Environmental Health Sciences (P30-ES010126).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ACGIH	American Conference of Governmental Industrial Hygienists
ADME	absorption, distribution, metabolism, and excretion
AIC	Akaike's Information Criterion
α-level	significance level
AM	arithmetic mean
amu	atomic mass unit
APF	OSHA assigned protection factor for respirator type
BEI	ACGIH biological exposure index
BMGV	UK HSE biological monitoring guidance value
^{13}C NMR	carbon-13 nuclear magnetic resonance spectroscopy
EPA	Environmental Protection Agency
ESI	electrospray ionization
eV	electron volts (collision energy)
GC-MS	gas chromatography-mass spectrometry
GM	geometric mean
GSD	geometric standard deviation
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
H_2MDI	methylene bis(4-cyclohexyl isocyanate)
H_2SO_4	sulfuric acid
HCl	hydrochloric acid
HDA	1,6-diaminohexane
HDI	1,6-hexamethylene diisocyanate

HFBA	heptafluorobutyric anhydride
HPLC	high-performance liquid chromatography
IgE	immunoglobulin E
IgG	immunoglobulin G
INH	inhalation exposure
INH-APF	inhalation exposure adjusted with APF
IPDA	isophorone diamine
IPDI	isophorone diisocyanate
ISO	HDI isocyanurate
K_3EDTA	tripotassium ethylenediamine tetraacetic acid (blood anticoagulant)
KH_2PO_4	monobasic potassium phosphate
LC-MS	liquid chromatography-mass spectrometry
Li-Heparin	lithium heparin (blood anticoagulant)
LLE	liquid-liquid extraction
LOD	limit of detection
M	molar concentration
M	molar mass
MAPE	mean absolute percentage error
MDA	methylenedianiline
MDI	methylene diphenyl diisocyanate
MDL	method detection limit
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio

NaOH	sodium hydroxide
NAT	*N*-acetyltransferase
NCI	negative chemical ionization
NCO	isocyanate group
NIOSH	National Institute for Occupational Safety and Health
NMR	nuclear magnetic resonance spectroscopy
OEHHA	California Office of Environmental Health Hazard Assessment
OSHA	Occupational Safety and Health Administration
PAPR	full-face powered air purifying respirator
PBZ	personal breathing-zone concentration
PBZ-APF	personal breathing-zone concentration adjusted with APF
PFPA	pentafluoropropionic anhydride
PPE	personal protective equipment
PROC	SAS procedure
r	Pearson correlation coefficient
R^2	marginal R^2 statistic for general linear model
RCF	relative centrifugal force
REL	recommended exposure limit
SAS	SAS 9.4 software suite
SD	arithmetic standard deviation
SPE	solid-phase extraction
SRM	selected reaction monitoring
STEL	short-term exposure limit

SWYPE™	surface pad sampling technique for skin exposure
TAAHI	trisacetamidohexyl isocyanurate
TAAHpI	trisacetamidoheptyl isocyanurate
TAHI	trisaminohexyl isocyanurate
TAHpI	trisaminoheptyl isocyanurate
TDI	toluene diisocyanate
TRIG	total reactive isocyanate groups
TWA	time-weighted average
UK HSE	United Kingdom Health and Safety Executive
UPLC	ultra-performance liquid chromatography
UV	ultraviolet light
var	variance
WEA	Swedish Work Environment Authority

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

1.1. Overview of isocyanates

Isocyanate is the general terminology for a family of semi-volatile organic compounds that include one or more isocyanate functional groups (–N=C=O). Isocyanate functional groups are electrophilic and favorably react with compounds containing nucleophilic functional groups such as: hydroxyl (–OH); carboxyl (–COOH); amino ($–NH_2$); and sulfhydryl (–SH) groups (Randall and Lee, 2002, Bello *et al.*, 2004, Thomas, 2015, Covestro, 2017). The isocyanate reaction with a hydroxyl group is of considerable importance for manufacturing and construction worldwide. Polyols (an alcohol with two or more hydroxyl groups) react with isocyanates to form a urethane linkage, an essential component of the class of chemicals and products we know as polyurethane (**Figure 1.1**) (Randall and Lee, 2002, Bello *et al.*, 2004, Lockey *et al.*, 2015, Thomas, 2015). Isocyanates are used to produce a wide variety of polyurethane-based products (Randall and Lee, 2002, Bello *et al.*, 2004, Bayer MaterialScience, 2005, Dow Chemical Company, 2010, Thomas, 2015, Covestro, 2017, Covestro, 2018a) that include:

- flexible foams used in furniture, bedding, carpeting, and packaging, or rigid foams for building insulation and refrigeration
- epoxies, adhesives, sealants, and wood binders
- waterproof coatings and paints for automobiles, aircraft, watercraft, bridges, parking decks, roofing, floors, and heavy equipment vehicles

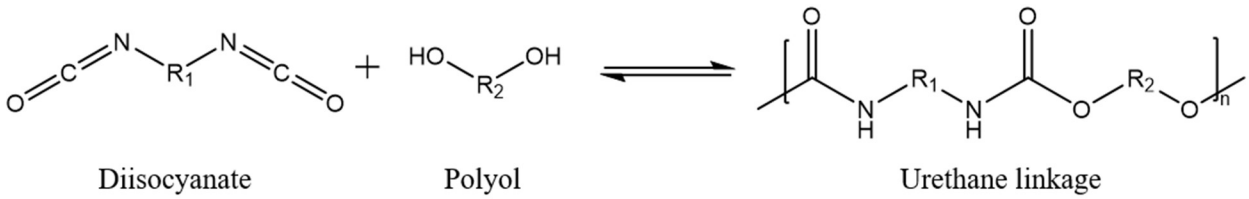

Figure 1.1. Representative chemical reaction of a diisocyanate and a polyol to form a urethane linkage in a polyurethane compound.

An estimated 4.4 million metric tons of isocyanates were produced globally in 2000, and the demand has risen in the last two decades (Randall and Lee, 2002). The most recognized class of isocyanates are diisocyanate monomers (containing two NCO groups) (Randall and Lee, 2002, Bello *et al.*, 2004, Bayer MaterialScience, 2005). Common diisocyanate monomers include: 2,4- and 2,6-toluene diisocyanate isomers (TDI), 2,4'- and 4,4'-methylenediphenyl diisocyanate isomers (MDI), 1,6-hexamethylene diisocyanate (HDI), and isophorone diisocyanate (IPDI) (**Figure 1.2**) (Randall and Lee, 2002, Bello *et al.*, 2004, NIOSH, 2004, Bayer MaterialScience, 2005, Lockey *et al.*, 2015). Isocyanates are further classified as either aromatic (NCO group directly attached to an aromatic ring) or saturated (aliphatic and cycloaliphatic) (Bello *et al.*, 2004, Thomas, 2015, Covestro, 2017). Saturated isocyanates are more commonly known as aliphatic isocyanates which are further classified as linear and cycloaliphatic (also known as alicyclic) structures (Thomas, 2015, Covestro, 2017). Additionally, isocyanates with NCO groups not directly attached to the aromatic ring are considered aliphatic or benzylic isocyanates (Thomas, 2015).

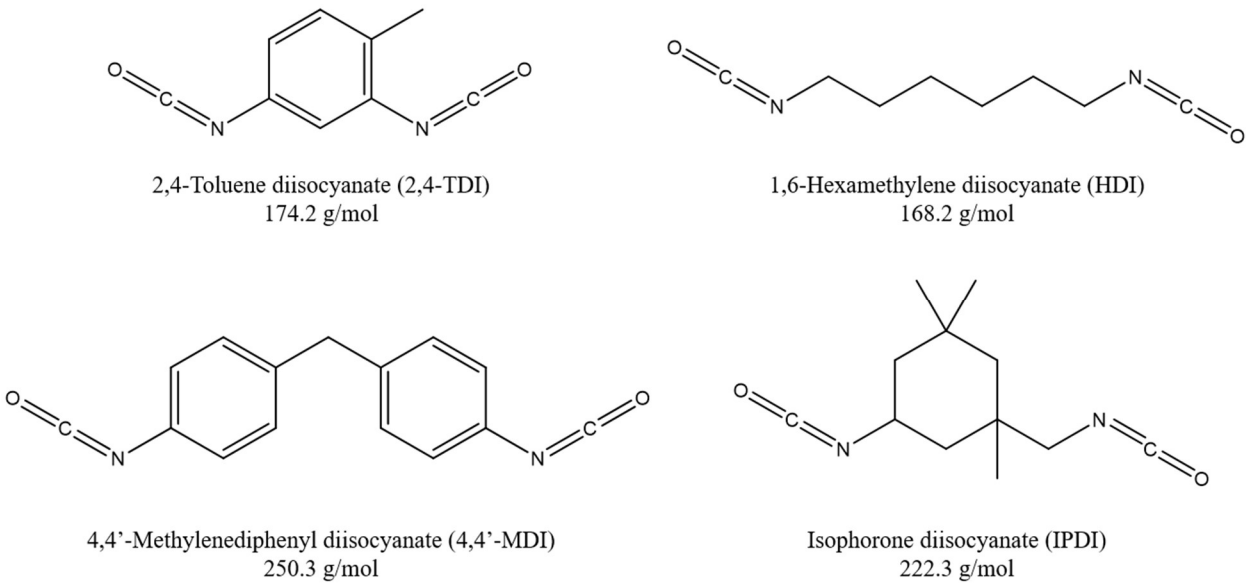

Figure 1.2. Structures and molar masses of 2,4-toluene diisocyanate (2,4-TDI), 1,6-hexamethylene diisocyanate (HDI), 4,4'-methylenediphenyl diisocyanate (4,4'-MDI), and isophorone diisocyanate (IPDI).

Aromatic isocyanates MDI and TDI and aliphatic isocyanates HDI and IPDI accounted for approximately >95% and <5% of the global market in 2000, respectively (Randall and Lee, 2002). Aromatic isocyanates are more reactive than aliphatic isocyanates and are susceptible to degradation by UV radiation (Randall and Lee, 2002, Bello *et al.*, 2004). Cured aromatic isocyanates will rapidly yellow from UV exposure and may structurally degrade over time. Aromatic isocyanates are primarily found in applications with little to no UV exposure or are pigmented to disguise color change (Bayer MaterialScience, 2005). By contrast, aliphatic isocyanates are highly resistant to UV radiation and are preferred for exterior coatings/paints due to their long-term resistance to weather corrosion and UV radiation (Randall and Lee, 2002, Bello *et al.*, 2004, Thomas, 2015, Covestro, 2018b). The lower reactivity of aliphatic isocyanates is also desirable for slow-curing applications (Randall and Lee, 2002, Bello *et al.*, 2004).

Although isocyanates are classified by the naming conventions of the diisocyanate monomers, the actual use of diisocyanate monomers is limited in most industrial applications due to their volatility, reactivity, and health concerns (Bello *et al.*, 2004, NIOSH, 2004). The majority of isocyanates used in manufacturing and construction industries are oligomers of the diisocyanate monomers (Bello *et al.*, 2004, NIOSH, 2004). In the literature, oligomers may also be denoted as polyisocyanates, pre-polymers, or polymeric isocyanates (Bello *et al.*, 2004, NIOSH, 2004). Oligomer and oligomeric will be used in this dissertation to classify these chemicals. Oligomeric isocyanate structures may consist of repeated chains of monomers, central aliphatic moieties that link monomers and side chains, or complex adducts of monomers and oligomers (Bello *et al.*, 2004, Bayer MaterialScience, 2005). **Figure 1.3** displays some common oligomeric isocyanates originating from the diisocyanate monomers of MDI, TDI, HDI, and IPDI (Bello *et al.*, 2004, Bayer MaterialScience, 2005).

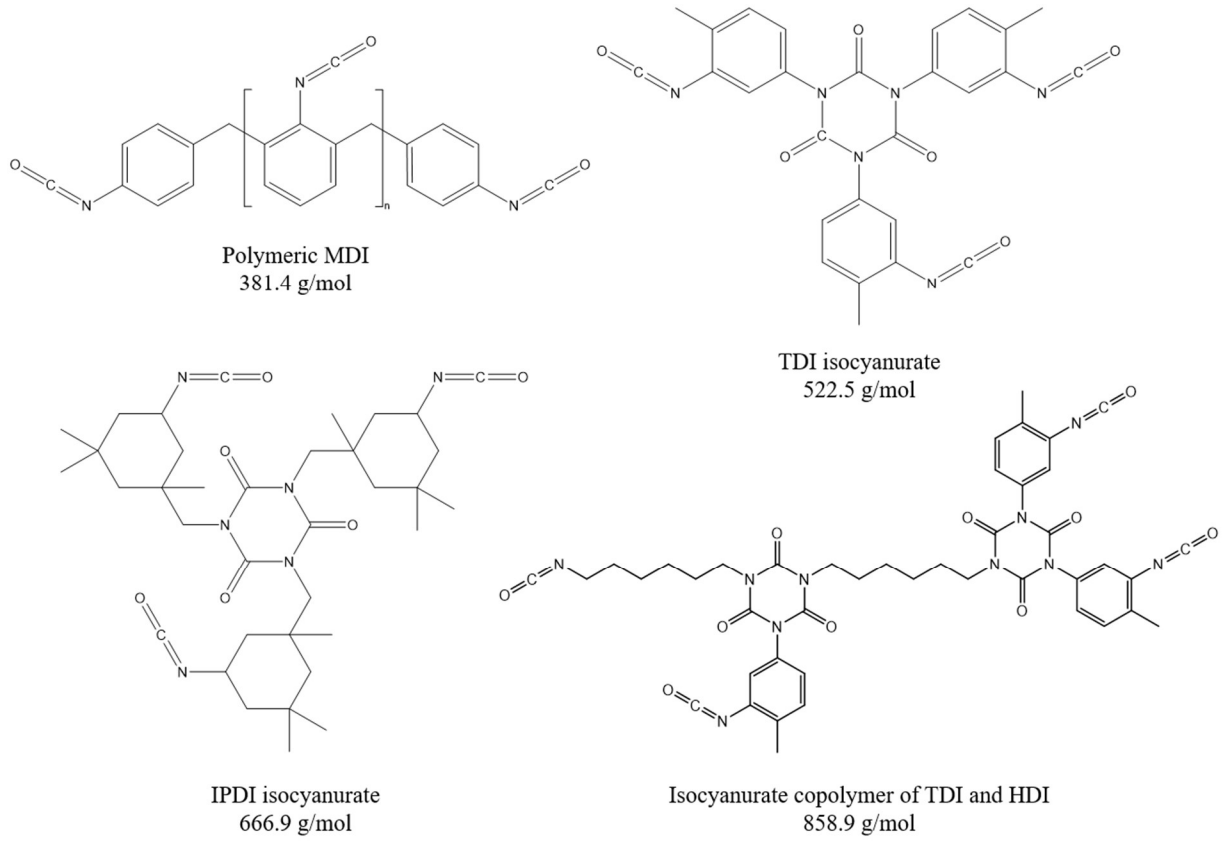

Figure 1.3. Structures and molar masses of polymeric MDI, TDI isocyanurate, IPDI isocyanurate, and isocyanurate copolymer of TDI and HDI.

1.2. Health effects associated with isocyanate exposures

Isocyanate exposures are associated with acute and chronic adverse health effects of the respiratory tract and of the skin (NIOSH, 1978, Bernstein, 1996, NIOSH, 1996, Liu and Wisnewski, 2003, Bello *et al.*, 2004, NIOSH, 2004, Bello *et al.*, 2007a, Lockey *et al.*, 2015, Covestro, 2017, California OEHHA, 2019). Acute health effects may include shortness of breath, rhinitis, pulmonary edema, asthma induction, irritation of the eyes, irritation of the respiratory tract, or irritant contact dermatitis (NIOSH, 1978, NIOSH, 1996, Bello *et al.*, 2004, Bello *et al.*, 2007a, Lockey *et al.*, 2015, Covestro, 2017, California OEHHA, 2019). Chronic health effects associated with sensitization from respiratory and skin exposures to isocyanates may include occupational asthma, allergic contact dermatitis, or hypersensitivity pneumonitis (NIOSH, 1978,

Malo *et al.*, 1983, Wilkinson *et al.*, 1991, Vandenplas *et al.*, 1992, Vandenplas *et al.*, 1993a, Vandenplas *et al.*, 1993b, Vandenplas *et al.*, 1993c, Chan-Yeung and Malo, 1995, Bernstein, 1996, NIOSH, 1996, Piirila *et al.*, 2000, Goossens *et al.*, 2002, Frick *et al.*, 2003a, Frick *et al.*, 2003b, Liu and Wisnewski, 2003, Bello *et al.*, 2004, Bello *et al.*, 2007a, Liippo and Lammintausta, 2008, Aalto-Korte *et al.*, 2010, Aalto-Korte *et al.*, 2012, Kiec-Swierczynska *et al.*, 2014, Lockey *et al.*, 2015, Covestro, 2017, California OEHHA, 2019).

Exposure to isocyanates is considered a leading cause of occupational asthma worldwide (Bernstein, 1996, Lockey *et al.*, 2015). It has been estimated that <1 – 30% of workers in production facilities and end-user applications develop isocyanate-induced asthma (Bernstein, 1996, Bello *et al.*, 2004, NIOSH, 2004, Bello *et al.*, 2007a, Lockey *et al.*, 2015). After sensitization, levels of isocyanates below occupational exposure limits can induce an asthmatic response (NIOSH, 1996, NIOSH, 2004, Bello *et al.*, 2007a). Inhalation exposure was previously considered the dominant pathway for sensitization and development of asthma, however, animal studies have shown that skin exposure is an important route of isocyanate sensitization (Karol *et al.*, 1981, Rattray *et al.*, 1994, Zissu *et al.*, 1998, Herrick *et al.*, 2002, Bello *et al.*, 2004, Bello *et al.*, 2007a). Skin exposure may also induce respiratory sensitization and/or a respiratory response without concomitant inhalation exposure (Rattray *et al.*, 1994, Petsonk *et al.*, 2000, Redlich and Karol, 2002, Bello *et al.*, 2004, Bello *et al.*, 2007a, Redlich, 2010, Wisnewski *et al.*, 2011, Henriks-Eckerman *et al.*, 2015). In addition to sensitization linked to the development of asthma, sensitization from skin exposure may also lead to the development of allergic contact dermatitis (Goossens *et al.*, 2002, Frick *et al.*, 2003a, Frick *et al.*, 2003b, Bello *et al.*, 2007a, Liippo and Lammintausta, 2008, Aalto-Korte *et al.*, 2010, Aalto-Korte *et al.*, 2012, Kiec-Swierczynska *et al.*, 2014).

1.3. Oligomeric isocyanate exposures

As mentioned in **Section 1.1**, oligomeric isocyanates are prevalent in formulations for end-user industrial applications of isocyanates. The higher volatility and reactivity of diisocyanate monomers is undesirable for coatings/paints, additionally, pressurized applications of liquid formulations combined with potential higher work environment temperatures may lead to substantial vapor hazards (Bello *et al.*, 2004, NIOSH, 2004). By contrast, the lower volatility and reactivity of oligomers compared to their diisocyanate monomer counterparts makes them easier to work with in occupational settings (Bello *et al.*, 2004, NIOSH, 2004). Notwithstanding the practical advantages of applying formulations with high concentrations of oligomeric isocyanates, these chemicals still contain two or more reactive isocyanate functional groups that present considerable exposure hazards (Bello *et al.*, 2004, Bayer MaterialScience, 2005). Exposures to oligomeric isocyanates can lead to acute and chronic adverse health effects historically associated with diisocyanate monomer exposures (Vandenplas *et al.*, 1993a, Bello *et al.*, 2004, Bello *et al.*, 2007a, Aalto-Korte *et al.*, 2010, California OEHHA, 2019). Inhalation was considered the primary route of exposure leading to respiratory sensitization and development of isocyanate-induced asthma. Less data are available for health hazards associated with oligomeric isocyanate exposures because their prevalence in industrial products is a recent development over the last few decades (Bello *et al.*, 2004). Thus, vapor exposures of semi-volatile and volatile diisocyanate monomers were considered the major source of respiratory adverse health effects while exposures to aerosols that contain high levels of semi-volatile and/or non-volatile oligomers were largely ignored. However, exposures to aerosols containing oligomeric isocyanates may also cause adverse respiratory health effects and furthermore present a more

significant skin exposure hazard than diisocyanate monomers (Bello *et al.*, 2004, Bello *et al.*, 2007a).

Aromatic isocyanate formulations have similar concentrations of oligomers and diisocyanate monomers in mixtures. TDI exposure assessment has been primarily limited to measuring 2,4- and 2,6-TDI monomers while levels of TDI oligomers and adducts are largely unreported in surveillance of occupational settings (Maitre *et al.*, 1993, Lind *et al.*, 1996, Lind *et al.*, 1997, Tinnerberg *et al.*, 1997, Kaaria *et al.*, 2001, Jarand *et al.*, 2002, Yeh *et al.*, 2002, Austin, 2007, De Palma *et al.*, 2012, Geens *et al.*, 2012, Gui *et al.*, 2014, Tinnerberg *et al.*, 2014, Brzeznicki and Bonczarowska, 2015, Swierczynska-Machura *et al.*, 2015). Polymeric MDI exposure concentrations have been recently reported in an occupational setting (Bello *et al.*, 2019). However, polymeric MDI exposures during MDI applications are not typically measured or are included with MDI monomers in the total NCO concentration (Skarping *et al.*, 1996, Crespo and Galan, 1999, Karoly *et al.*, 2004, Lesage *et al.*, 2007, Booth *et al.*, 2009, Liljelind *et al.*, 2010, Tinnerberg *et al.*, 2014, Jones *et al.*, 2017). Although formulations of aromatic isocyanates contain significant levels of oligomers, the lack of exposure monitoring for aromatic oligomers limits further investigation of associated health effects and biomarkers.

Low volatility and reactivity are highly desirable characteristics for exterior coating/paint applications; thus, formulations are comprised of high concentrations of oligomeric aliphatic isocyanates. Polyurethane-based coatings/paints are applied to vehicle, bridge, and building surfaces to prevent weather corrosion and degradation by UV radiation contain monomers and oligomers of HDI and/or IPDI (Randall and Lee, 2002, Bello *et al.*, 2004, Bayer MaterialScience, 2005, Dow Chemical Company, 2010, California OEHHA, 2019). The U.S. Bureau of Labor Statistics estimates that approximately 58,000 painters were employed in motor

vehicle manufacturing and refinishing industries (U.S. Bureau of Labor Statistics, 2019b), and approximately 382,000 painters were employed in construction and maintenance in 2016 (U.S. Bureau of Labor Statistics, 2019a). Despite the prevalence of polyurethane-based coating/paint applications across vehicle and construction industries, exposures to aliphatic isocyanates have mostly been assessed in the automotive refinishing industry (Janko *et al.*, 1992, Maitre *et al.*, 1996, Woskie *et al.*, 2004, Pronk *et al.*, 2006a, Pronk *et al.*, 2006b, Fent *et al.*, 2008, Fent *et al.*, 2009a, Fent *et al.*, 2009b, Reeb-Whitaker *et al.*, 2012, Reeb-Whitaker and Schoonover, 2016), and to a lesser extent in the aircraft refinishing industry (Carlton and England, 2000, Ceballos *et al.*, 2017, Bennett *et al.*, 2018). The established research of exposures in the automotive refinishing industry affords further investigation of oligomeric aliphatic isocyanate exposures when exposure monitoring of oligomers in aromatic isocyanate applications is lacking.

1.4. HDI exposures in the automotive refinishing industry

In 2016, the U.S. automotive refinishing industry employed an estimated 20,000 – 25,000 painters and >227,000 workers in >33,900 auto body shops, with a projected job growth of 7% for painters from 2016 to 2026 (U.S. Census Bureau, 2018, U.S. Bureau of Labor Statistics, 2019b). Workers in the automotive refinishing industry are potentially exposed to HDI monomer and its oligomers: the dimer HDI uretdione, and the trimers HDI biuret and HDI isocyanurate (**Figure 1.4**). Isocyanates in formulations for clearcoat paint applications are comprised of small amounts of HDI monomer (<1%), small amounts of HDI uretdione and HDI biuret (<1-10%), and much higher amounts of HDI isocyanurate (>80%) (Janko *et al.*, 1992, Bello *et al.*, 2004, Woskie *et al.*, 2004, Pronk *et al.*, 2006a, Pronk *et al.*, 2006b, Bello *et al.*, 2007a, Fent *et al.*, 2008, Fent *et al.*, 2009a, Fent *et al.*, 2009b, Reeb-Whitaker *et al.*, 2012). The highest exposures to HDI monomer and HDI oligomers occur during a paint task when vapors and aerosols are

generated by a spray gun (Fent *et al.*, 2009a, Fent *et al.*, 2009b). Paint tasks normally occur in partially or fully enclosed ventilated booths (*e.g.*, crossdraft, semi-downdraft, or downdraft) (Fent *et al.*, 2009a, Fent *et al.*, 2009b). However, >200,000 technicians and office workers may also be exposed to HDI vapors and aerosols (Woskie *et al.*, 2004, Boutin *et al.*, 2006, Pronk *et al.*, 2006a, Pronk *et al.*, 2006b).

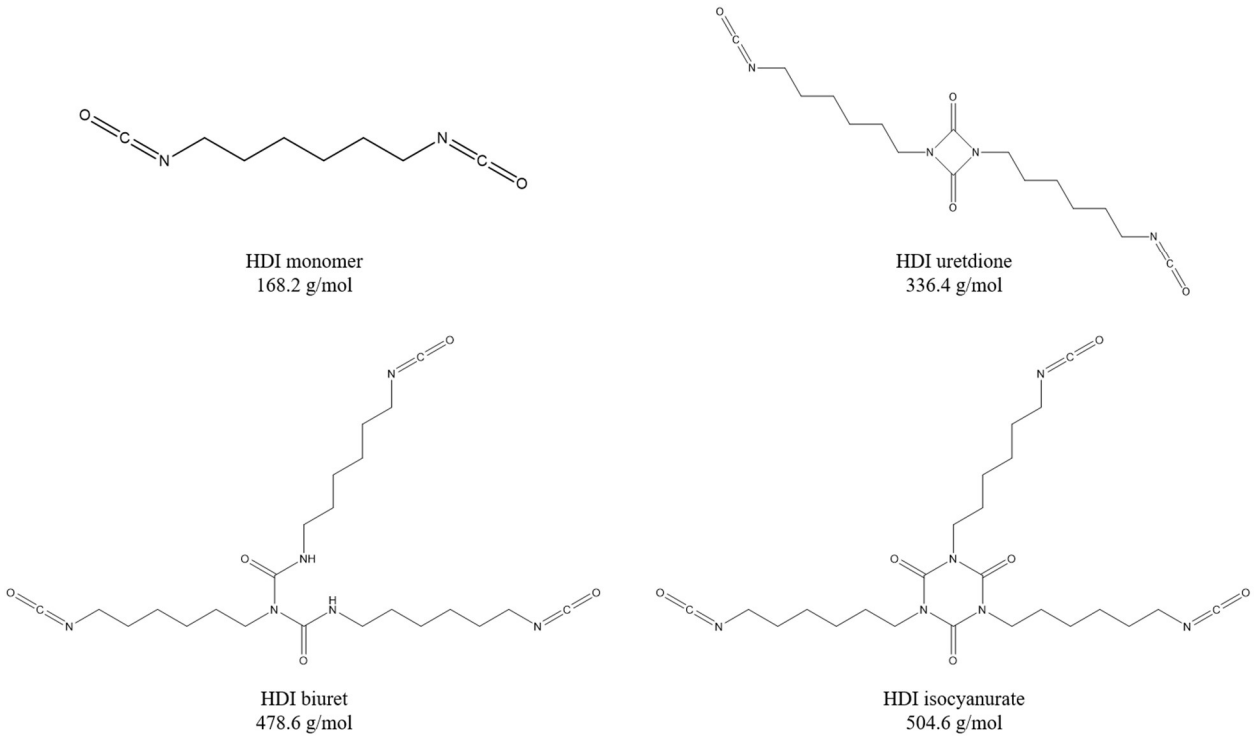

Figure 1.4. Structures and molar masses of HDI monomer and its oligomers HDI uretdione, HDI biuret, and HDI isocyanurate.

1.4.1. Inhalation exposures to HDI

Inhalation exposures to HDI monomer and oligomers are monitored by area sampling or personal breathing-zone (PBZ) sampling (Janko *et al.*, 1992, Rudzinski *et al.*, 1995, Sparer *et al.*, 2004, Woskie *et al.*, 2004, Pronk *et al.*, 2006a, Pronk *et al.*, 2006b, Henneken *et al.*, 2007, Fent *et al.*, 2008, Fent *et al.*, 2009a, Reeb-Whitaker *et al.*, 2012). However, measurements of PBZ concentrations are potentially confounded by respiratory protection. To account for respirator use, the Occupational Safety and Health Administration (OSHA) established assigned protection factors (APF) for respirator type [1 = no respirator; 10 = half-face negative-pressure air purifying respirator; 50 = full-face negative-pressure air purifying respirator; 1000 = powered-air purifying respirator (PAPR) or continuous flow supplied-air respirator] to adjust personal exposure concentrations for workers who use respirators (OSHA, 2009). Adjustment with APF may be confounded by improper fit, poor respirator maintenance, or facial hair that prevents a tight seal for half- and full-face negative-pressure air purifying respirators (Liu *et al.*, 2006, Fent *et al.*, 2008, OSHA, 2009, Floyd *et al.*, 2018). Liu *et al.* monitored 22 workers wearing half-face air purifying respirators during spray-painting and priming and calculated respirator protection factors of 17 and 388 for HDI monomer and HDI oligomers, respectively, which were both higher than the OSHA APF of 10 for this respirator type (Liu *et al.*, 2006). However, 20% of 142 spray-painters failed the first respirator fit factor test due to loose fitting respirators, wrong sizes, and facial hair, and 8% failed the second fit test after respirator use training (Liu *et al.*, 2006). Floyd *et al.* demonstrated a significant reduction in the respirator fit factor in 19 subjects with beard lengths equal to or greater than 0.25 in (Floyd *et al.*, 2018). Additionally, Bello *et al.* measured HDI monomer and HDI oligomers in 30% and 80% of face skin samples, respectively, collected by SWYPE™ sampling surfaces occluded by half-face air purifying respirators for 20

workers (Bello *et al.*, 2008). Furthermore, Reeb-Whitaker *et al.* determined that in order to reduce HDI monomer and HDI oligomer exposures below occupational exposure limits, automotive spray-painters would need to wear a respirator with a minimum APF value of 25 (Reeb-Whitaker *et al.*, 2012). These studies indicate that respiratory protection, most notably half-face air purifying respirators, may not sufficiently reduce inhalation exposures to HDI in the automotive refinishing industry.

1.4.2. Skin exposures to HDI

Isocyanate aerosols generated by spray-painting present significant skin exposure hazards in the automotive refinishing industry (Pronk *et al.*, 2006b, Bello *et al.*, 2007a, Liu *et al.*, 2007, Bello *et al.*, 2008, Fent *et al.*, 2008, Fent *et al.*, 2009b, Fletcher, 2015). Workers may wear protective clothing (*e.g.*, coveralls, hat) and/or gloves to minimize exposures during painting, however, their use is less prevalent than respirators amongst automotive spray-painters (Bello *et al.*, 2007a, Fent *et al.*, 2008, Fent *et al.*, 2009b, Ceballos *et al.*, 2011, Ceballos *et al.*, 2014a). Additionally, contact exposure with unreacted HDI species on surfaces may occur after painting or during tasks unrelated to painting (*e.g.*, sanding, buffing, taping and untaping, mechanical work, detailing, compounding) (Liu *et al.*, 2000, Pronk *et al.*, 2006b, Bello *et al.*, 2007b, Liu *et al.*, 2007, Bello *et al.*, 2008). In a recent study, skin exposure from surface contact appeared to be minimal for 18 automotive spray-painters in 5 auto body shops (De Vries *et al.*, 2012). However, more research is warranted to assess whether unreacted isocyanates present a significant exposure hazard when PPE is not worn during tasks unrelated to painting.

1.4.3. Challenges for monitoring skin exposures

Exposure assessment in the automotive refinishing industry has focused on inhalation exposures, therefore, skin exposure assessment and associated adverse health effects are not well

understood. Skin exposures have been primarily monitored with removal techniques that collected unreacted HDI species on the skin after a paint task has ended. Qualitative and quantitative SWYPE™ sampling have been used to collect unreacted HDI species on skin surfaces post-exposure (Liu *et al.*, 2000, Bello *et al.*, 2007a, Liu *et al.*, 2007, Bello *et al.*, 2008). Tape-stripping is an alternative removal technique that was developed to monitor skin exposure and penetration after painting (Fent *et al.*, 2006). Unlike SWYPE™ sampling, multiple tape-strips applied to the same skin site collects unreacted HDI species in the outermost layers of the stratum corneum, thereby providing an estimate of percutaneous absorption (Fent *et al.*, 2006). This method has been utilized in two independent exposure assessment studies of automotive spray-painters (Fent *et al.*, 2008, Fent *et al.*, 2009b, Fletcher, 2015). However, neither of these techniques measure skin exposure during a spray-painting task and thus may underestimate exposure due to evaporation, physical removal, chemical removal, or skin absorption of the isocyanates (Bello *et al.*, 2007a, Bello *et al.*, 2008, Thomasen *et al.*, 2011, Thomasen and Nylander-French, 2012).

HDI species on skin surfaces, particularly the more volatile HDI monomer, may evaporate quickly from the skin post-exposure. Additionally, isocyanates may be physically removed from the skin by surface contact, glove contact and removal, or coverall removal to expose skin for sampling. There may be chemical removal of isocyanates due to reactions with curing agents in the paint, or with water and proteins on or in the skin. Chemical removal will reduce quantitative measurements because SWYPE™ and tape-strip sampling rely on piperazine derivatization of unreacted isocyanates (Fent *et al.*, 2006, Bello *et al.*, 2007a, Bello *et al.*, 2008, Fent *et al.*, 2008, Fent *et al.*, 2009b). Lastly, significant levels of HDI monomer and HDI oligomers may be absorbed based on two studies of excised skin dosed with HDI. Bello *et al.*

dosed excised guinea pig skin with HDI-containing Desmodur® products diluted with ethyl acetate and observed that approximately 10% of HDI oligomers were absorbed in the skin after 5 min and approximately 20% were absorbed after 2 h with minimal evaporation of the unreacted oligomers (Bello *et al.*, 2006). Thomasen *et al.* performed a similar experiment dosing excised human skin with slow- and fast-drying clearcoat containing HDI monomer and HDI oligomers (Thomasen and Nylander-French, 2012). Approximately 20% of HDI monomer and 15 – 25% of HDI isocyanurate were absorbed in the skin after a 10-min exposure. Similar absorption levels were observed in the 30- and 60-min exposures, however, the recovery of isocyanates in the occlusion material dropped from 70 – 80% in the 10- and 30-min exposures to 55 – 60% in the 60-min exposure. Absorption and reactions with water or proteins in the skin may have led to low recovery of dosed HDI monomer and HDI isocyanurate after 60 min (Thomasen and Nylander-French, 2012). These permeation studies suggest that a longer task would lead to more absorption of HDI monomer and HDI isocyanurate. However, the conditions of both studies did not allow for investigation of the porosity of living human skin in an occupational environment where heat stress, physical stress, PPE use and contact, and perspiration may enhance skin porosity and absorption of isocyanates (Bello *et al.*, 2006, Thomasen and Nylander-French, 2012).

Interception techniques have been developed to capture skin isocyanate exposure during painting. Thomsen *et al.* developed an impregnated felt patch to be worn around the forearms of a painter (Thomasen *et al.*, 2011). This method measured much higher skin exposures to HDI monomer and HDI isocyanurate than tape-strip sampling for 25 automotive spray-painters in Washington State (Fletcher, 2015). Two other promising methods have been developed to capture breakthrough exposure through coveralls or gloves. Blake *et al.* impregnated arm-length

cotton sleeves to measure monomeric and polymeric MDI and methylene bis(4-cyclohexyl isocyanate) (H_2MDI) exposures (Blake *et al.*, 2012), and Harari *et al.* impregnated cotton gloves to measure monomeric and polymeric MDI exposures (Harari *et al.*, 2016). The combination of these two methods may provide the best estimate of skin exposure during a paint task; however, neither method has been utilized to measure HDI exposures in occupational settings. Impregnated felt patches have been field tested and producing patches and sample extraction is both less laborious and requires less disposables and solvents than similar procedures for producing impregnated cotton gloves or sleeves and extracting derivatized isocyanates from these materials (Thomasen *et al.*, 2011, Blake *et al.*, 2012, Harari *et al.*, 2016). These recently developed sampling techniques may soon supplant removal techniques for measuring isocyanate skin exposures in the workplace if they are proven reliable and reproducible during multiple exposure assessment studies.

1.4.4. HDI monomer and HDI oligomer inhalation exposures in the literature

Exposure assessment studies conducted in the automotive refinishing industry have reported HDI oligomer exposures as a sum of HDI uretdione, HDI biuret, and HDI isocyanurate concentrations (Janko *et al.*, 1992, Maitre *et al.*, 1996, Liu *et al.*, 2006, Pronk *et al.*, 2006a, Pronk *et al.*, 2006b, Bello *et al.*, 2008). Alternatively, the combined concentrations of HDI monomer and HDI oligomers have been reported as a total NCO concentration, also known as total reactive isocyanate groups (TRIG) in the literature (Sparer *et al.*, 2004, Woskie *et al.*, 2004, Creely *et al.*, 2006, Liu *et al.*, 2006, Pronk *et al.*, 2006a, Pronk *et al.*, 2006b, Bello *et al.*, 2008, Fent *et al.*, 2008, De Vries *et al.*, 2012, Reeb-Whitaker *et al.*, 2012, Jones *et al.*, 2017). The analysis of combined HDI oligomer concentration or total NCO concentration offers simplicity and uniformity of measurement across multiple studies in compliance with current occupational

exposure limits (see **Section 1.6**). However, these measurements treat HDI species homogeneously and may be inadequate for investigating the contributions of individual HDI oligomer exposures to biomarker levels and development of adverse health outcomes (Bello *et al.*, 2004, Bello *et al.*, 2007a, Fent *et al.*, 2008). Combined measurements would be appropriate if the physical and chemical properties of each isocyanate were the same. However, the reactivity of the NCO groups may vary due to stereochemical configuration and size of the isocyanate, and electronegativity of attached moieties (Bello *et al.*, 2004, Thomas, 2015). Additionally, the properties of the moieties may affect hydrophilicity and lipophilicity of the chemical altering pulmonary and skin absorption rates and toxicokinetics between HDI species (Bello *et al.*, 2004, Bello *et al.*, 2007a). Recent exposure assessment studies have reported concentrations of individual HDI oligomer exposures in the automotive refinishing industry to aid investigation of adverse health effects and biomarkers of exposures that may not be strongly associated with combined measurements of HDI species (Pronk *et al.*, 2006a, Pronk *et al.*, 2006b, Fent *et al.*, 2008, Fent *et al.*, 2009a, Fent *et al.*, 2009b, Reeb-Whitaker *et al.*, 2012, Reeb-Whitaker and Schoonover, 2016).

1.5. Concerns for exposures to HDI isocyanurate

Although HDI monomer is more reactive and volatile than HDI isocyanurate (Pauluhn, 2015), animal and human studies have shown HDI isocyanurate exposures lead to acute and chronic adverse health effects without concomitant exposure to HDI monomer (Vandenplas *et al.*, 1993a, Zissu *et al.*, 1998, Bello *et al.*, 2004, Bello *et al.*, 2007a, Aalto-Korte *et al.*, 2010, California OEHHA, 2019). Murine studies have shown that HDI isocyanurate is an acute respiratory irritant (Ferguson *et al.*, 1987, Pauluhn, 2000, Pauluhn and Mohr, 2001, Pauluhn, 2004, Ma-Hock *et al.*, 2007). Decreased lung function has also been observed in workers after

short-term and long-term exposures to HDI oligomers (Alexandersson *et al.*, 1987, Dahlqvist *et al.*, 1995, Randolph *et al.*, 1997, Glindmeyer *et al.*, 2004, Pourabedian *et al.*, 2010, California OEHHA, 2019). In a controlled inhalation study, 4 volunteers had asthmatic reactions after HDI oligomer exposure (with unspecified concentrations of HDI biuret and HDI isocyanurate) but not after HDI monomer exposure (Vandenplas *et al.*, 1993a). In the same study, HDI oligomer-specific immunoglobulin E (IgE) and immunoglobulin G (IgG) in human serum were significantly correlated with HDI oligomer exposures (Vandenplas *et al.*, 1993a). Additionally, HDI isocyanurate-specific IgE and IgG in human serum have been identified in occupationally exposed populations, however, these markers were not validated as biomarkers of HDI isocyanurate exposures (Campo *et al.*, 2007, Pronk *et al.*, 2007). HDI isocyanurate also exhibits skin sensitizing capacity without inhalation exposure and without concomitant exposure to HDI monomer or HDI biuret in guinea pigs and humans (Zissu *et al.*, 1998, Pauluhn *et al.*, 2002, Aalto-Korte *et al.*, 2010). Lastly, HDI oligomers are known causes of allergic contact dermatitis in humans (Aalto-Korte *et al.*, 2010). Because HDI isocyanurate is the predominant HDI exposure in the automotive refinishing industry, it is important to identify and investigate HDI isocyanurate inhalation and skin exposures during spray-painting.

1.6. Exposure limits to HDI monomer, HDI oligomers, and total NCO

Although adverse health outcomes are associated with both HDI monomer and HDI oligomer exposures, OSHA has not established permissible exposure limits (PEL) or short-term exposure limits (STEL) for HDI monomer or HDI oligomers (Bello *et al.*, 2004, OSHA, 2018, OSHA, 2019b, OSHA, 2019a). Other governmental agencies and recommendatory bodies in the U.S. and abroad have established exposures limits for HDI monomer, HDI oligomers, or total NCO content (**Table 1.1**). In the U.S., the National Institute for Occupational Safety and Health

(NIOSH) and the American Conference of Governmental Industrial Hygienists (ACGIH) established 10-h and 8-h recommended exposure limits (REL), respectively, for HDI monomer (Bello *et al.*, 2004, OSHA, 2018, ACGIH, 2019). NIOSH also established a 10-min ceiling STEL for HDI monomer (Bello *et al.*, 2004, OSHA, 2018). Neither NIOSH nor ACGIH have established exposure limits for HDI oligomers (Bello *et al.*, 2004, ACGIH, 2019, OSHA, 2019b, OSHA, 2019a). Oregon OSHA is the only governmental agency in the U.S. with established PEL and STEL for HDI oligomers (sum of HDI biuret and HDI isocyanurate) (Oregon OSHA, 2017). These limits were adopted in 1986 from the Bayer Corporation and were primarily based on one animal pulmonary irritation study to HDI biuret (Weyel *et al.*, 1982, Janko *et al.*, 1992, Bello *et al.*, 2004, Oregon OSHA, 2017).

Globally, the United Kingdom Health and Safety Executive (UK HSE) and the Swedish Work Environment Authority (WEA) have established short-term and full-shift exposure limits for total NCO content (Bello *et al.*, 2004, UK HSE, 2005a). The total NCO content may be converted to equivalent concentrations of HDI monomer, HDI biuret, or HDI isocyanurate, but the limits are based on total NCO content of all isocyanates measured (Bello *et al.*, 2004). In **Table 1.1**, the UK HSE and Swedish WEA exposure limits for total NCO content are shown as the equivalent concentrations of HDI isocyanurate. Using the conversion factor of 4.64 for HDI isocyanurate (Bello *et al.*, 2004), the total NCO content equivalent exposure limits are lower than the current exposure limits established by Oregon OSHA (Oregon OSHA, 2017). In 2017, the California Office of Environmental Health Hazard Assessment (OEHHA) within the California Environmental Protection Agency proposed reference exposure levels for HDI monomer and HDI oligomers with the goal of protecting human health in occupational and residential settings (California OEHHA, 2019). Although the proposed reference exposure levels are not explicitly

intended for occupational settings, they are derived from animal and human exposure studies that have been conducted since the establishment of Oregon OSHA exposure limits for HDI oligomers. The 1-h limit (0.3 μg/m^3) and 8-h limit (0.06 μg/m^3) for HDI monomer, and 1-h limit (4.5 μg/m^3) and 8-h limit (0.8 μg/m^3) for HDI oligomers are considerably lower than the current exposure limits established by the Oregon OSHA, UK HSE, and Swedish WEA (Bello *et al.*, 2004, Oregon OSHA, 2017, California OEHHA, 2019).

Table 1.1. Occupational and recommended exposure limits (μg/m^3) publicized by governmental agencies and recommendatory bodies for HDI monomer, HDI oligomers, and total NCO content.

	HDI monomer		**HDI oligomers**		**Total NCO**	
Agency	TWA	STEL	TWA	STEL	TWA	STEL
OSHA	**	**	**	**	**	**
NIOSH	35	140	**	**	17.5[a]	70[a]
ACGIH	34	**	**	**	17[a]	**
Oregon OSHA	**	**	500	1000	107.8[a]	215.5[a]
UK HSE	40[b]	140[b]	92.8[b]	324.8[b]	20	70
Swedish WEA	40[b]	88[b]	92.8[b]	204.2[b]	20	44
California OEHHA	0.06	0.3	0.8	4.5	0.2[c]	1.0[c]

TWA = full-shift time-weighted average concentration (μg/m^3); STEL = short-term time-weighted average concentration (μg/m^3); ** = Agency does not have occupational exposure limit for this chemical.

TWA values represent an 8-hour full-shift limit for ACGIH, Oregon OSHA, UK HSE, Swedish WEA, and California OEHHA, and a 10-hour full-shift limit for NIOSH (Bello *et al.*, 2004, UK HSE, 2005a, Oregon OSHA, 2017, OSHA, 2018, ACGIH, 2019, California OEHHA, 2019, OSHA, 2019a, OSHA, 2019b).

STEL values represent a 5-min short-term limit for Swedish WEA, a 10-min ceiling limit for NIOSH and UK HSE, a 15-min short-term limit for Oregon OSHA, and a 1-hour short-term limit for California OEHHA (Bello *et al.*, 2004, UK HSE, 2005a, Oregon OSHA, 2017, OSHA, 2018, ACGIH, 2019, California OEHHA, 2019, OSHA, 2019a, OSHA, 2019b).

[a]Total NCO equivalent concentration (μg NCO/m^3) of HDI monomer or HDI oligomer exposure limit, calculated by dividing the exposure limit by the conversion factor (HDI monomer = 2.00; HDI isocyanurate = 4.64) (Bello *et al.*, 2004).

[b]HDI monomer or HDI isocyanurate equivalent concentration (μg/m^3) of total NCO exposure limit, calculated by multiplying the exposure limit by the conversion factor (HDI monomer = 2.00; HDI isocyanurate = 4.64) (Bello *et al.*, 2004).

[c]Total NCO equivalent concentration (μg NCO/m^3) of HDI oligomer exposure limit, calculated by multiplying the exposure limit by the conversion factor (HDI isocyanurate = 4.64) (Bello *et al.*, 2004).

1.7. Biomonitoring metabolites of HDI exposures in urine and blood

Exposure assessment of HDI is complicated by time and effort associated with workplace surveillance and variability of sampling methods for monitoring multiple exposure routes. Additionally, inter-individual physical characteristics, varying PPE use, and workplace exposure controls can modify exposures. Thus, traditional sampling methods may not provide accurate exposure measurements and furthermore, are unable to predict past exposures. Biomonitoring complements exposure assessment by integrating multiple exposure routes into one measurement and may shed light on modification of exposures. Monitoring inhalation, ingestion, or skin exposure routes will not provide information on the absorption, distribution, metabolism, and excretion (ADME) of the chemical/s of interest. Levels of metabolites measured in urine or blood may provide an estimate of rapid ADME from recent exposure or may also indicate favored metabolic pathways by measurement of long-lived macromolecule conjugates.

1.7.1. Urine HDA levels in controlled laboratory exposure settings

Biomonitoring to estimate rapid systemic availability and blood circulation of HDI monomer exposures largely relies on measuring unbound 1,6-diaminohexane (HDA), the hydrolysis product of HDI monomer, in urine (Rosenberg and Savolainen, 1986, Brorson *et al.*, 1990a, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Williams *et al.*, 1999, Liu *et al.*, 2004, Creely *et al.*, 2006, Pronk *et al.*, 2006b, Flack *et al.*, 2010a, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Budnik *et al.*, 2011, Jones *et al.*, 2013, Hu *et al.*, 2017, Jones *et al.*, 2017). HDA has been measured in urine shortly after controlled exposure challenges or occupational exposures to HDI monomer and/or HDI oligomers. Rosenberg *et al.* were the first to quantify HDA in urine by exposing 5 volunteers to HDI monomer and HDI oligomers in an exposure chamber (Rosenberg and Savolainen, 1986). Brorson *et al.* validated urinary HDA as a biomarker of short-term HDI

monomer exposure by exposing 5 volunteers to HDI monomer in an exposure chamber and calculated a urinary HDA excretion half-life of 1.2 h (Brorson *et al.*, 1990a). Tinnerberg *et al.* calculated a longer urinary HDA excretion half-life of 2.5 h after exposing 3 volunteers to HDI monomer in an exposure chamber (Tinnerberg *et al.*, 1995). More recently, Liu *et al.* and Budnik *et al.* corroborated the longer urinary HDA excretion half-life calculated by Tinnerberg *et al.* (Tinnerberg *et al.*, 1995, Liu *et al.*, 2004, Budnik *et al.*, 2011). Liu *et al.* exposed 23 volunteers to HDI biuret aerosols [geometric mean (GM) = 98.7 $\mu g/m^3$] containing HDI monomer (GM = 53.8 $\mu g/m^3$) and trace levels of HDI uretdione (included in HDI biuret concentration) with a closed-circuit breathing apparatus, and calculated a urinary HDA excretion half-life of 2.8 h (Liu *et al.*, 2004). Budnik *et al.* exposed 55 volunteers to HDI monomer in an exposure chamber or with a closed-circuit breathing apparatus, and calculated a urinary HDA excretion half-life of 2.5 h (Budnik *et al.*, 2011). A closed-circuit breathing apparatus has an inherent advantage over an exposure chamber for an inhalation challenge because it removes the possible contribution of HDI monomer skin exposure to metabolism and excretion of HDA in urine. Budnik *et al.* did not specify how many volunteers each were exposed to HDI monomer in a closed-circuit breathing apparatus or in an exposure chamber (Budnik *et al.*, 2011). Thus, the 2.8 h half-life calculated by Liu *et al.* after inhalation exposure to HDI biuret aerosols with low levels of HDI monomer may be the best estimate of excretion half-life after inhalation exposure to complex mixtures of HDI monomer and HDI oligomers in occupational settings (Liu *et al.*, 2004).

1.7.2. Urine HDA levels in occupationally exposed populations

HDA has also been measured in urine collected from workers exposed to HDI monomer and/or HDI oligomers. Maitre *et al.* observed that post-shift urine HDA levels were significantly correlated with HDI monomer inhalation exposures monitored during 8-h work-shifts in HDI

production and manufacturing facilities (Maitre *et al.*, 1996). Pronk *et al.* collected urine for 24 h from employees in automotive refinishing shops and industrial painting companies potentially exposed to HDI and detected HDA in urine collected from spray-painters, technicians, and office workers (Pronk *et al.*, 2006b). The odds ratios for occurrence of HDA in urine samples collected from all automotive spray-painters were approximately 1.1 for samples collected between 08:00 – 15:00, and >2.0 for samples collected between 15:00 until 08:00 the next morning (Pronk *et al.*, 2006b). Gaines *et al.* observed that both HDI monomer breathing-zone and skin exposures were significantly associated with unadjusted and creatinine-adjusted urine HDA levels in automotive spray-painters (Gaines *et al.*, 2010a, Gaines *et al.*, 2011). Other studies have also measured HDA in urine samples collected from workers in vehicle manufacturing and refinishing industries but associations with HDI monomer, HDI oligomers, and/or total NCO concentrations were not reported (Rosenberg and Savolainen, 1986, Williams *et al.*, 1999, Creely *et al.*, 2006, Jones *et al.*, 2013, Ceballos *et al.*, 2017, Jones *et al.*, 2017).

1.7.3. Plasma HDA levels in populations exposed to HDI

HDI monomer-specific IgE and IgG have been identified in serum of workers exposed to HDI. An isocyanate-specific IgE is strongly predictive of isocyanate-induced asthma, while isocyanate-specific IgG is not predictive of adverse health effects but is frequently identified in workers exposed to isocyanates (Wisnewski, 2007, Wisnewski *et al.*, 2012). HDI monomer-specific IgG is readily detected in serum of workers and has been significantly associated with exposures to HDI (Wisnewski *et al.*, 2004, Pronk *et al.*, 2007, Wisnewski *et al.*, 2012). Analysis of HDA in blood as a biomarker of short-term HDI monomer exposure is less common. Flack *et al.* measured HDA in plasma of automotive spray-painters and observed significant associations with both short-term and past HDI monomer exposures (Flack *et al.*, 2010b). In a follow up

study, Flack *et al.* quantified HDA-hemoglobin adducts and found stronger associations with past HDI monomer exposures compared to short-term exposures (Flack *et al.*, 2011). Although research on biomarkers of HDI monomer exposures in blood is less common than in urine, these studies show metabolites in blood are significantly associated with short-term and past HDI monomer exposures and may also be indicative of sensitization and isocyanate-induced asthma.

1.7.4. Quantification of hydrolyzed HDA

Sample treatment steps to extract HDA from urine and plasma normally involve hydrolysis, liquid extraction, and derivatization prior to quantitative analysis (Flack *et al.*, 2010a). Strong acids such as HCl and H_2SO_4 are routinely used to hydrolyze biological samples to release conjugated HDI monomer and HDA from conjugates prior to extraction (Rosenberg and Savolainen, 1986, Brorson *et al.*, 1990a, Dalene *et al.*, 1990, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Williams *et al.*, 1999, Rosenberg *et al.*, 2002, Liu *et al.*, 2004, Creely *et al.*, 2006, Pronk *et al.*, 2006b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Jones *et al.*, 2013, Hu *et al.*, 2017, Jones *et al.*, 2017). The HDA level measured in acid-hydrolyzed samples represents a combination of free HDA, HDI monomer or HDA conjugated with macromolecules, and partially or fully acetylated HDA and conjugates (Flack *et al.*, 2010a). This hydrolysis method is used for non-specific quantification of total HDA to yield the highest measurable concentrations in biological samples and will mask different biomarkers resulting from multiple exposure routes and rapid or slow metabolism and excretion (Flack *et al.*, 2010a). Base hydrolysis with NaOH has also been used to measure free and acetylated HDA in biological samples (Skarping *et al.*, 1994b, Tinnerberg *et al.*, 1995, Flack *et al.*, 2010a). Base hydrolysis will release HDA conjugated with macromolecules by amide linkages but will not convert other conjugates to the amine form, thus the yield of HDA species will be lower than with acidic hydrolysis (Flack *et*

al., 2010a). However, the levels of free and acetylated HDA in base-hydrolyzed samples compared to acid-hydrolyzed total HDA may provide insight on the *N*-acetyltransferase (*NAT*) enzyme status of each individual (Flack *et al.*, 2010a). *NAT* acetylates amino groups of the toxicant promoting rapid excretion in urine (Flack *et al.*, 2010a). Studies have shown that polymorphisms to the slow acetylator *NAT1* and *NAT2* genotypes are associated with an increased risk of isocyanate-induced asthma (Wikman *et al.*, 2002, Yucesoy *et al.*, 2015). Alternatively, low concentrations of acetylated HDA may indicate the majority of biologically available HDI monomer and HDA are conjugated with macromolecules.

Liquid-liquid extraction (LLE) with nonpolar organic solvents is the standard method for extracting liberated HDA from hydrolyzed biological samples (Flack *et al.*, 2010a). Toluene is the most commonly used extraction solvent (Rosenberg and Savolainen, 1986, Brorson *et al.*, 1990a, Dalene *et al.*, 1990, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Rosenberg *et al.*, 2002, Liu *et al.*, 2004, Pronk *et al.*, 2006b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Hu *et al.*, 2017), although dichloromethane (Flack *et al.*, 2010a) and diethyl ether have also been used for extraction (Williams *et al.*, 1999, Creely *et al.*, 2006, Jones *et al.*, 2013, Jones *et al.*, 2017). Extracted amines are typically derivatized with polyfluorinated acid anhydrides such as heptafluorobutyric anhydride (HFBA) or pentafluoropropionic anhydride (PFPA) for quantitative analysis by gas chromatography-mass spectrometry (GC-MS) with negative chemical ionization (NCI) (Rosenberg and Savolainen, 1986, Brorson *et al.*, 1990a, Dalene *et al.*, 1990, Maitre *et al.*, 1996, Williams *et al.*, 1999, Rosenberg *et al.*, 2002, Liu *et al.*, 2004, Creely *et al.*, 2006, Pronk *et al.*, 2006b, Jones *et al.*, 2013). GC-MS analysis of HDA-HFBA or HDA-PFPA derivatives is specific and sensitive with limits of detection (LOD) commonly below 0.1 µg/L (Tinnerberg *et al.*, 1995, Flack *et al.*, 2010a, Flack *et al.*, 2010b, Gaines *et al.*, 2010a). However, GC-MS

analysis is hindered by its inability to monitor large molecular weight semi-volatile or non-volatile compounds including amines and polyfluorinated derivatives of HDI oligomers. Liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been sparingly used to quantify HDA in hydrolyzed biological samples (Skarping *et al.*, 1994b, Marand *et al.*, 2004a, Hu *et al.*, 2017). Because LC-MS with electrospray ionization (ESI) induces multiple charge states, it extends the detectible mass range for analytes, and possesses the ability to quantify derivatized biomarkers of oligomeric isocyanates or macromolecule conjugates of monomeric isocyanates and amines.

1.7.5. Urine HDA biomarker limits

Recommended biomarker limits for hydrolyzed HDA levels in post-shift urine samples have been established by the ACGIH and the UK HSE. The ACGIH Biological Exposure Index (BEI) recommends a maximum HDA concentration of 15 μg/g creatinine in a post-shift urine sample (ACGIH, 2015). This BEI corresponds to the absorbed and excreted dose of HDI monomer after a full-shift exposure to the recommended 8-h TWA of 34 $\mu g/m^3$ (ACGIH, 2015). The UK HSE established a Biological Monitoring Guidance Value (BMGV) for post-shift urine HDA of 1 μmol/mol creatinine (approximately 1.03 μg/g creatinine) (UK HSE, 2005b). The HDA BMGV is determined as the 90^{th} percentile of the measured HDA levels in workers exposed to HDI monomer in UK workplaces (Cocker *et al.*, 2007). An HDA concentration exceeding the BMGV does not necessarily mean adverse health outcomes will occur, but it does indicate exposures may need to be reduced (UK HSE, 2005b). There are no established limits for biomarkers of oligomeric isocyanates because these biomarkers have not previously been measured in human urine.

1.7.6. Biomonitoring of oligomeric isocyanate exposures

Biomonitoring of HDI oligomer exposures and other oligomeric isocyanate exposures has been primarily limited to measurement of corresponding amines of the monomers. Liu *et al.* measured HDA in urine of exposed volunteers as a biomarker of short-term exposure to inhaled aerosols of HDI biuret containing HDI monomer and trace levels of HDI uretdione (included in HDI biuret concentration) (Liu *et al.*, 2004). Urine HDA levels were significantly correlated with HDI monomer inhalation exposures, however, urine HDA levels were not significantly correlated with either HDI biuret or total NCO inhalation exposures (Liu *et al.*, 2004). This controlled exposure study demonstrates that urinary HDA is not a suitable biomarker of HDI oligomer inhalation exposures. In other studies where HDA was measured in urine or plasma as a biomarker of HDI monomer exposure in occupational settings, associations with concomitantly monitored HDI oligomers were not reported (Pronk *et al.*, 2006b, Flack *et al.*, 2010a, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Gaines *et al.*, 2011).

Studies identifying biomarkers of oligomeric isocyanate exposures are lacking. To our knowledge, biomarkers of oligomeric IPDI or TDI have not been identified. Biomarkers of short-term inhalation and skin exposures to polymeric 3-core MDI have been quantified in rats (Pauluhn, 2002a, Pauluhn and Lewalter, 2002). Low levels of 3-core methylenedianiline (3-core MDA) were measured in rat urine collected in both studies, however, hemoglobin adducts with 3-core MDA were not detected (Pauluhn, 2002a, Pauluhn and Lewalter, 2002). HDI biuret- and HDI isocyanurate-specific IgE and IgG in human serum have been identified in epidemiologic studies of workers exposed to HDI (Campo *et al.*, 2007, Pronk *et al.*, 2007). These biomarkers of HDI biuret and HDI isocyanurate exposures were primarily used to associate exposures with respiratory symptoms and isocyanate-induced asthma. Pronk *et al.* observed that HDI biuret-

specific IgE was significantly associated with estimated HDI biuret exposures, however, HDI isocyanurate-specific IgE or IgG were not significantly associated with estimated HDI isocyanurate exposures (Pronk *et al.*, 2007).

1.8. Automotive spray-painters in North Carolina and Washington State

An exposure assessment of 48 automotive spray-painters employed in auto body shops located in North Carolina and Washington State was conducted during 2005 – 2007 (NIOSH R01-OH007598). Inhalation exposures were monitored by PBZ sampling and skin exposures were monitored by tape-strip sampling for 47 spray-painters during 115 sampling visits (Fent *et al.*, 2009a, Fent *et al.*, 2009b). Hydrolyzed HDA levels were monitored in the urine of 48 spray-painters during 120 sampling visits (Gaines *et al.*, 2010a) and in the plasma of 46 spray-painters during 112 sampling visits (Flack *et al.*, 2010b).

A summary of HDI monomer and HDI isocyanurate exposures measured in this worker population is shown in **Table 1.2** (Fent *et al.*, 2009a, Fent *et al.*, 2009b). Spray-painters had higher inhalation and skin exposures to HDI isocyanurate compared to other HDI species during each task and during the full work-shift. HDI monomer and HDI isocyanurate PBZs were measured above the LOD in 279 (91%) and in 303 (99%) of 307 total tasks, respectively (Fent *et al.*, 2009a). However, PBZs and PBZ-APFs for HDI isocyanurate were significantly higher for task and visit measurements than HDI monomer (Fent *et al.*, 2009a). HDI monomer and HDI isocyanurate PBZs for tasks and visits frequently exceeded occupational exposure limits (**Table 1.3**). After APF adjustment, HDI isocyanurate PBZ-APF exceeded the STELs for Oregon OSHA, UK HSE, Swedish WEA, and California OEHHA during 8 (3%), 42 (14%), 108 (35%), and 172 (56%) tasks, respectively. HDI isocyanurate PBZ-APF exceeded the California OEHHA full-shift exposure limit during 79 of 115 (69%) visits. By contrast, the HDI monomer PBZ-APF

did not exceed the UK HSE or Swedish WEA exposure limits during any task, and only exceeded the California OEHHA exposure limits during 29 of 307 (9%) tasks and during 19 of 115 (17%) visits.

The tape-stripping method developed by Fent *et al.* was utilized in this study to measure skin exposures to HDI monomer and HDI oligomers (Fent *et al.*, 2006, Fent *et al.*, 2009b). HDI isocyanurate skin exposures were significantly higher than HDI monomer skin exposures. HDI monomer was only measured above the LOD in 101 of 276 (37%) tasks and in 61 of 115 (53%) visits, while HDI isocyanurate was measured above the LOD in 262 of 276 (95%) tasks and in 113 of 115 (98%) visits. The oligomers HDI uretdione and HDI biuret were also monitored in this study cohort by PBZ and tape-strip sampling (data not shown), however, HDI isocyanurate was the predominant HDI oligomer exposure in this worker population (Fent *et al.*, 2009a, Fent *et al.*, 2009b).

Hydrolyzed HDA was previously quantified in urine and plasma samples collected from this worker population by GC-MS with method detection limits (MDL) of 0.04 µg/L for urine and 0.02 µg/L for plasma (Flack *et al.*, 2010b, Gaines *et al.*, 2010a). Hydrolyzed HDA was detected in 259 of 417 (62%) urine samples in 47 of 48 (98%) workers and had an arithmetic mean (AM) ± arithmetic standard deviation (SD) of 0.53 ± 3.32 µg/L (Gaines *et al.*, 2010a). Hydrolyzed HDA was detected in 82 of 112 (73%) plasma samples in 45 of 46 (98%) workers and had an AM ± SD of 0.10 ± 0.14 µg/L (Flack *et al.*, 2010b). Flack *et al.* observed that plasma HDA levels were significantly correlated with both HDI monomer inhalation and skin exposures, and were also significantly correlated with HDI monomer skin exposures when inhalation exposure levels were below the LOD (Flack *et al.*, 2010b). HDI monomer PBZ with APF adjustment and HDI monomer skin exposure were both significantly associated with unadjusted

and creatinine-adjusted urine HDA levels by multiple linear regression analyses (Gaines *et al.*, 2010a, Gaines *et al.*, 2011). Additionally, painting in downdraft booths or wearing coveralls were both significantly associated with lower urine and plasma HDA levels in this study cohort (Flack *et al.*, 2010b, Gaines *et al.*, 2011).

For the purpose of this dissertation research, urine and plasma samples collected from the 48 automotive spray-painters were available for further analysis. The inhalation and skin exposures to HDI monomer and HDI isocyanurate and HDA levels in urine and plasma were previously characterized (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a). Because HDI isocyanurate was the predominant inhalation and skin exposure in this worker population and HDA was readily measured despite low HDI monomer exposures, biomarkers of HDI isocyanurate exposures were anticipated to be identifiable and quantifiable in the biological samples collected from these workers.

Table 1.2. Summary statistics by task and visit of personal breathing-zone, inhalation, and skin exposures to HDI monomer and HDI isocyanurate for 47 automotive spray-painters in North Carolina (n = 15) and Washington State (n = 32).

	Summary Statistics by Task					Summary Statistics by Visit				
	Range	Mean	SD	GM	GSD	Range	Mean	SD	GM	GSD
HDI monomer										
PBZ (μg/m^3)	<LOD – 178.6	10.9	16.6	3.3	12.0	<LOD – 178.6	13.2	22.8	5.8	5.1
PBZ-APF (μg/m^3)	<LOD – 53.0	1.0	3.4	0.08	21.8	<LOD – 53.0	1.5	5.4	0.2	11.4
INH (μg)	<LOD – 31.5	1.9	3.1	0.5	14.8	<LOD – 59.7	5.2	8.5	1.9	6.0
INH-APF (μg)	<LOD – 3.2	0.1	0.3	0.01	24.2	<LOD – 6.0	0.4	0.8	0.06	11.4
Skin (μg)	<LOD – 211.9	1.5	13.8	0.001	199.1	<LOD – 211.9	3.7	21.3	0.005	331.2
HDI isocyanurate										
PBZ (μg/m^3)	<LOD – 20313.9	3035.5	3355.4	1543.2	5.6	14.6 – 14565.7	3052.8	2949.7	1979.4	2.9
PBZ-APF (μg/m^3)	<LOD – 13951.1	268.3	856.2	39.2	13.7	0.3 – 13951.1	352.6	1313.6	65.7	9.2
INH (μg)	<LOD – 12094.5	567.2	1056.5	215.2	6.7	3.2 – 18284.7	1512.6	2664.5	658.5	3.9
INH-APF (μg)	<LOD – 1209.4	43.0	108.2	5.5	14.4	0.1 – 1828.5	114.7	262.5	21.9	9.5
Skin (μg)	<LOD – 17979.4	495.0	1475.1	46.8	32.5	<LOD – 17979.4	1187.9	2581.4	174.4	15.2
Paint time (min)	1.0 – 53.0	7.5	5.7	6.0	2.0	1.5 – 98.0	20.3	20.0	14.3	2.3

Mean = arithmetic mean; SD = arithmetic standard deviation; GM = geometric mean; GSD = geometric standard deviation; PBZ = personal breathing-zone concentration (μg/m^3); PBZ-APF = APF adjusted personal breathing-zone concentration (μg/m^3); INH = inhalation exposure (μg); INH-APF = APF adjusted inhalation exposure (μg); <LOD = below the limit of detection.

Table 1.3. Number of tasks and visits where the measured personal breathing-zone concentrations were above short-term or full-shift exposure limits for 47 automotive spray-painters in North Carolina (n = 15) and Washington State (n = 32).

Agency		*NIOSH*	*ACGIH*	*Oregon OSHA*	*UK HSE*[a]	*Swedish WEA*[b]	*California OEHHA*
Short-term	Tasks						
HDI PBZ	307	0 (0%)	**	**	0 (0%)	7 (2%)	205 (67%)
HDI PBZ-APF	307	0 (0%)	**	**	0 (0%)	0 (0%)	29 (9%)
ISO PBZ	307	**	**	135 (44%)	256 (83%)	282 (92%)	298 (97%)
ISO PBZ-APF	307	**	**	8 (3%)	42 (14%)	108 (35%)	172 (56%)
Full-shift	Visits						
HDI PBZ	115	0 (0%)	0 (0%)	**	0 (0%)	0 (0%)	92 (80%)
HDI PBZ-APF	115	0 (0%)	0 (0%)	**	0 (0%)	0 (0%)	19 (17%)
ISO PBZ	115	**	**	5 (4%)	40 (35%)	40 (35%)	114 (99%)
ISO PBZ-APF	115	**	**	0 (0%)	3 (3%)	3 (3%)	79 (69%)

HDI = HDI monomer; ISO = HDI isocyanurate; PBZ = personal breathing-zone concentration; PBZ-APF = APF adjusted personal breathing-zone concentration; ** = Agency does not have occupational exposure limit for this chemical; [a]HDI monomer or HDI isocyanurate equivalent concentration (µg/m^3) of the UK HSE total NCO exposure limit; [b]HDI monomer or HDI isocyanurate equivalent concentration (µg/m^3) of the Swedish WEA total NCO exposure limit.

1.9. Specific aims

The objective of this research was to develop a sample treatment and analytical method to quantify trisaminohexyl isocyanurate (TAHI), a hydrolysis product of HDI isocyanurate, in urine and plasma and evaluate its potential as a biomarker of exposure in workers exposed to HDI-containing paints in the automotive refinishing industry. Little is known about the contribution of HDI isocyanurate to the development of adverse health effects associated with exposures to complex mixtures of HDI. It is not yet understood whether inhalation and skin provide equally important exposure pathways for rapid systemic availability and blood circulation of HDI isocyanurate. Therefore, the identification and quantification of a biomarker of HDI isocyanurate exposure represents a key step for understanding the fate of this toxicant in workers employed in

the automotive refinishing industry. The following specific aims were proposed to accomplish the objectives of this dissertation research:

Aim 1: Develop a sample treatment and analytical method to quantify hydrolyzed TAHI in urine and plasma.

Aim 2: Quantify hydrolyzed TAHI in urine and plasma collected from 48 automotive spray-painters occupationally exposed to HDI-containing paints.

Aim 3: Investigate the relationships between HDI isocyanurate and HDI monomer inhalation and skin exposures and urine and plasma TAHI and HDA levels.

The development of a sample treatment and analytical method to quantify hydrolyzed TAHI in urine is detailed in Chapter 2 to fulfill Aims 1 and 2. Chapter 3 describes the adaptation of the sample treatment and analytical method described in Chapter 2 to quantify hydrolyzed TAHI in plasma to fulfill Aims 1 and 2. The associations between HDI isocyanurate and HDI monomer exposures, and TAHI and HDA biomarker levels measured in automotive spray-painters exposed to HDI-containing paints are presented in Chapter 4 to fulfill Aim 3. Chapter 5 summarizes the findings of Chapters 2 – 4 and presents the strengths and limitations of the research and proposes future research objectives for studying biomarkers of exposure to HDI isocyanurate and other oligomeric isocyanates.

CHAPTER 2: TRISAMINOHEXYL ISOCYANURATE, A URINARY BIOMARKER OF HDI ISOCYANURATE EXPOSURE[1]

2.1. Introduction

Aromatic and aliphatic isocyanates are highly reactive, low-molecular-weight compounds included in the 187 hazardous air pollutants of the Clean Air Act Amendments of 1990 (U.S. EPA, 1990). They are used in the manufacturing of many common products containing polyurethane such as adhesives, spray-paints, foams, insulation, resins, sealants, and surface coatings (NIOSH, 1996, NIOSH, 2004). One of the most commonly used isocyanates is 1,6-hexamethylene diisocyanate (HDI), comprised of its monomer and oligomers (**Figure 2.1**) (NIOSH, 1978). Occupational exposure occurs during industrial production or during spray-painting operations such as auto body refinishing or application of marine coatings (NIOSH, 1996). Exposures in the general population can occur from contact with isocyanate-containing consumer goods, from slow-curing isocyanate coatings or materials used in housing construction, in outdoor areas near industrial sites where isocyanates are used in manufacturing, or in neighborhoods surrounding auto-refinishing businesses (Kelly *et al.*, 1999, Darcey *et al.*, 2002, Jarand *et al.*, 2002, Bello *et al.*, 2007a, Bello *et al.*, 2007b, Geddie *et al.*, 2011, Wilder *et al.*, 2011).

[1]This chapter previously appeared as an article in the Journal of Chromatography B. The original citation is as follows: Robbins, Z., Bodnar, W., Zhang, Z., Gold, A. & Nylander-French, L.A., 2018. Trisaminohexyl isocyanurate, a urinary biomarker of HDI isocyanurate exposure. *J Chromatogr B Analyt Technol Biomed Life Sci,* 1076**,** 117-129.

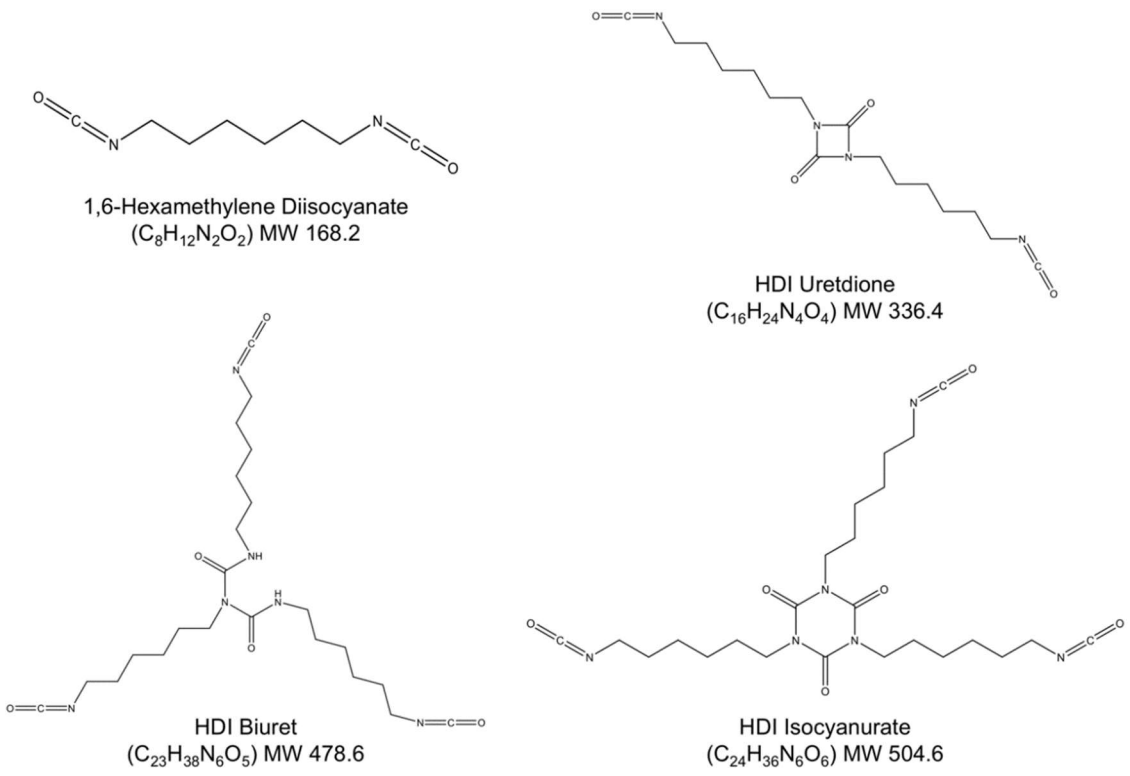

Figure 2.1. Chemical structures of 1,6-hexamethylene diisocyanate monomer and its oligomers uretdione, biuret, and isocyanurate.

Exposures to aerosols and vapors of HDI monomer and oligomers, including HDI isocyanurate, are associated with a high risk of contact dermatitis and asthma (Vandenplas *et al.*, 1993c, Chan-Yeung and Malo, 1995, Bernstein, 1996, Piirila *et al.*, 2000, Aalto-Korte *et al.*, 2010). Acute exposure can cause shortness of breath, rhinitis, irritation of the skin, eyes, and mucous membranes, and pulmonary edema (Bernstein, 1996, Goossens *et al.*, 2002, Bello *et al.*, 2007a, Bello *et al.*, 2007b).

Significant levels of inhalation and skin exposure to HDI monomer and its oligomers have been reported in spray-painters (Maitre *et al.*, 1996, Pronk *et al.*, 2006b, Fent *et al.*, 2009a, Fent *et al.*, 2009b, Reeb-Whitaker *et al.*, 2012). The predominant inhalation and skin exposure in automotive spray-painting is to HDI isocyanurate (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Reeb-Whitaker *et al.*, 2012), but the relative contributions of exposure to the HDI monomer and

isocyanurate in the etiology of immune sensitization and disease is currently unknown. The skin sensitization capacity of HDI isocyanurate has been indicated to be greater than the HDI monomer and HDI biuret in both humans and animals (Zissu *et al.*, 1998, Aalto-Korte *et al.*, 2010), and occupational asthma has been linked to HDI oligomer exposure without an immune response to the monomer (Vandenplas *et al.*, 1993a). Furthermore, it has been shown that HDI isocyanurate also penetrates skin at much faster rates (approximately 300 to 700 times) than HDI monomer (Thomasen and Nylander-French, 2012).

Biological monitoring to estimate the systemic doses of HDI monomer and oligomers through exposure has been limited primarily to 1,6-diaminohexane (HDA), the hydrolysis product of HDI monomer, in urine and blood (Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Rosenberg *et al.*, 2002, Liu *et al.*, 2004, Pronk *et al.*, 2006b, Flack *et al.*, 2010a, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Flack *et al.*, 2011). However, it has been shown that measured biomarker levels of HDI monomer exposure do not correlate with HDI oligomer exposure (Liu *et al.*, 2004). Until now a method has not existed to detect biomarkers of HDI isocyanurate exposure in urine or blood. Therefore, to investigate the relationship between external exposure, exposure routes, and biomarker levels, it is imperative that a biomarker for HDI isocyanurate exposure be established. This biomarker assay is also critical for investigation of relative potency and dose-response relationships of HDI monomer and oligomer exposures, to establish causality for associated health effects from monomer and/or oligomer exposures, and, thus, to improve exposure and risk assessment for isocyanates. Towards this end, our goals were to: (i) design an extraction and derivatization protocol and liquid chromatography-mass spectrometry (LC-MS) method for analysis of trisaminohexyl isocyanurate (TAHI), a hydrolysis product and novel urine

biomarker of HDI isocyanurate, and (ii) apply this method to quantify TAHI in urine collected from workers exposed to HDI isocyanurate during automotive spray-painting operations.

2.2. Experimental

2.2.1. Instrumentation

Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were acquired on a Varian INOVA 400 (Palo Alto, CA) at 400 MHz for ^{1}H NMR spectra and 100 MHz for ^{13}C NMR spectra. Mass spectra were acquired on a TSQ Quantum Ultra triple-quadrupole mass spectrometer with an electrospray ionization (ESI) source (Thermo Scientific, Waltham, MA) coupled to an Acquity ultra-performance liquid chromatography (UPLC) system (UPLC-ESI-MS/MS) (Waters Corp., Milford, MA), and a TSQ Quantum Ultra triple-quadrupole mass spectrometer with a nano-electrospray ionization source coupled to a NanoAcquity UPLC system (nano-UPLC-ESI-MS/MS) (Waters Corp.).

2.2.2. Synthesis of standards

The analytical standards required for sample processing and quantitative analysis were not available commercially, therefore, they were synthesized in-house. The synthesis and purification was a labor-intensive process and yielded limited quantities of the following four standards: 1,3,5-Tris(6-aminohexyl)-1,3,5-triazinane-2,4,6-trione (trisaminohexyl isocyanurate; TAHI), *N,N',N''*-((2,4,6-trioxo-1,3,5-triazinane-1,3,5-triyl)tris(hexane-6,1-diyl))triacetamide (trisacetamidohexyl isocyanurate; TAAHI), 1,3,5-tris(7-aminoheptyl)-1,3,5-triazinane-2,4,6-trione (trisaminoheptyl isocyanurate; TAHpI), and *N,N',N''*-((2,4,6-trioxo-1,3,5-triazinane-1,3,5-triyl)tris(heptane-7,1-diyl))triacetamide (trisacetamidoheptyl isocyanurate; TAAHpI). The chemical structures are shown in **Figure 2.2**. Composition and purity of the four standards were confirmed by NMR and LC-MS/MS in-house (see below in Section 2.2.2.6).

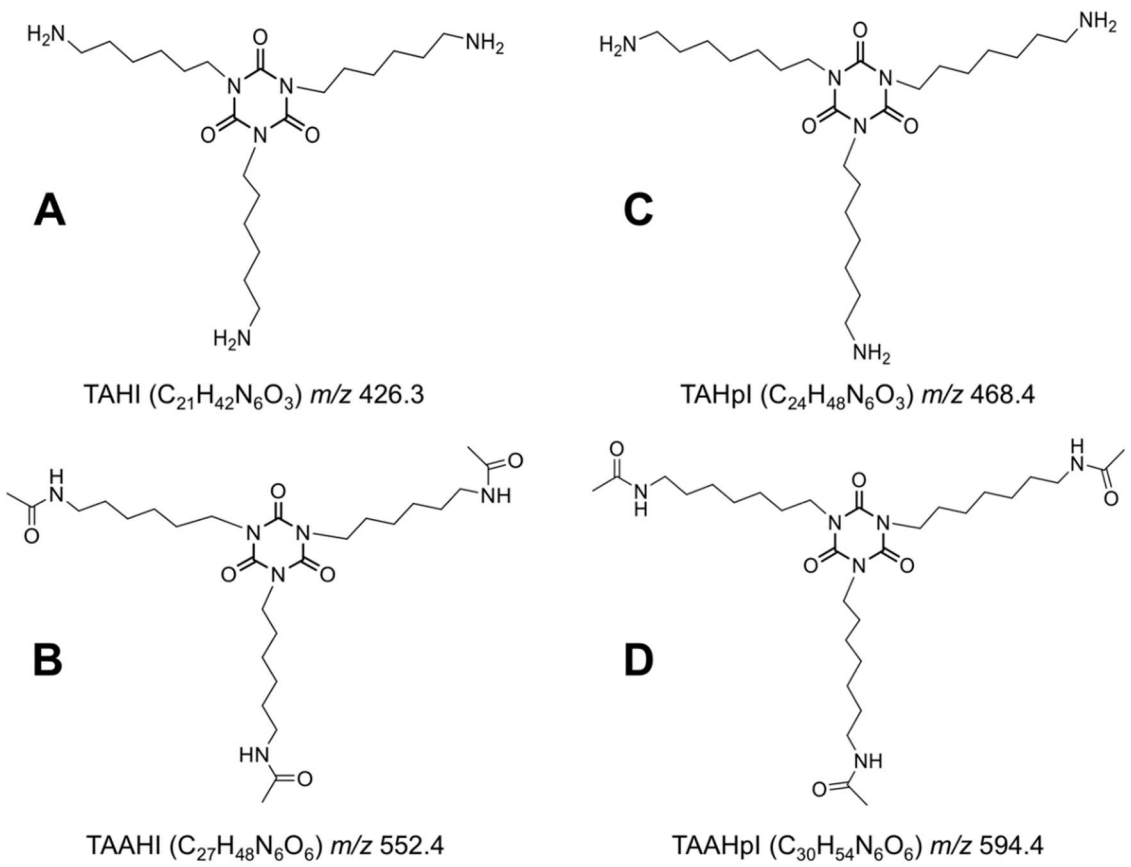

Figure 2.2. Chemical structures of [A] trisaminohexyl isocyanurate (TAHI), [B] trisacetamidohexyl isocyanurate (TAAHI), [C] trisaminoheptyl isocyanurate (TAHpI), and [D] trisacetamidoheptyl isocyanurate (TAAHpI).

2.2.2.1. Trisaminohexyl isocyanurate (M = 426.3 g/mol; ***Figure 2.2A)***

Commercially available Desmodur® N 3300 (Bayer MaterialScience, Pittsburgh, PA) was mixed with concentrated HCl and refluxed for 30 min, during which time the initially heterogeneous mixture became homogeneous. The solvent was removed under vacuum to afford a trichloride salt. ^{1}H NMR: (400 MHz, D_2O) 1.51-1.60 (12H), 1.73-1.85 (12H), 3.14 (t, J = 7.2 Hz, 6H), 3.98 (t, J = 7.2 Hz, 6H) ppm (**Figure A.1**). Fragmentation spectra of precursor ion $[M + H]^+$ for TAHI (*m/z* 427.3) were obtained by nano-UPLC-ESI-MS/MS at collision energies 25 eV (**Figure A.2**) and 35 eV (**Figure A.3**).

2.2.2.2. Trisacetamidohexyl isocyanurate (M = 552.4 g/mol; ***Figure 2.2B****)*

TAHI trichloride was mixed with triethylamine in tetrahydrofuran and excess acetic anhydride was added and the mixture stirred overnight. Tetrahydrofuran was removed under vacuum and the residue partitioned between water and dichloromethane. The organic extract was washed with brine, dried over Na_2SO_4, and concentrated. Pure product was isolated by chromatography (silicon dioxide, dichloromethane/methanol, 20:1). ^{1}H NMR: (400 MHz, $CDCl_3$) 1.33-1.35 (12H), 1.45-1.48 (6H), 1.60-1.65 (6H), 1.96 (s, 9H), 3.20 (q, J = 6.4 Hz, 6H), 3.86 (t, J = 7.6 Hz, 6H) (**Figure A.4**). ^{13}C NMR: (100 MHz, $CDCl_3$) 170.1, 149.0, 42.7, 39.3, 29.3, 27.6, 26.2, 26.1, 23.2 ppm (**Figure A.5**). Fragmentation spectra of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) were obtained by direct injection on ESI-MS/MS at collision energies 25 eV (**Figure 2.3**) and 50 eV (**Figure 2.4**).

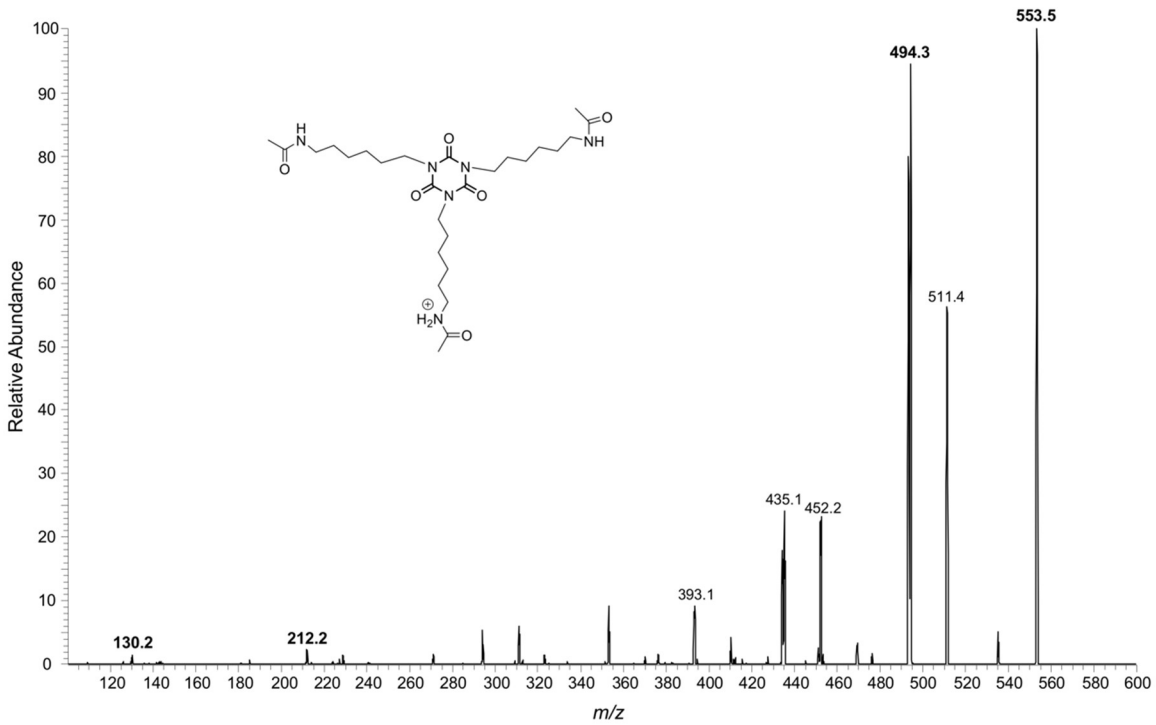

Figure 2.3. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) obtained by direct injection on ESI-MS/MS operated in positive ion-mode with electrospray ionization (scan range, *m/z* 100-600; collision energy, 25 eV).

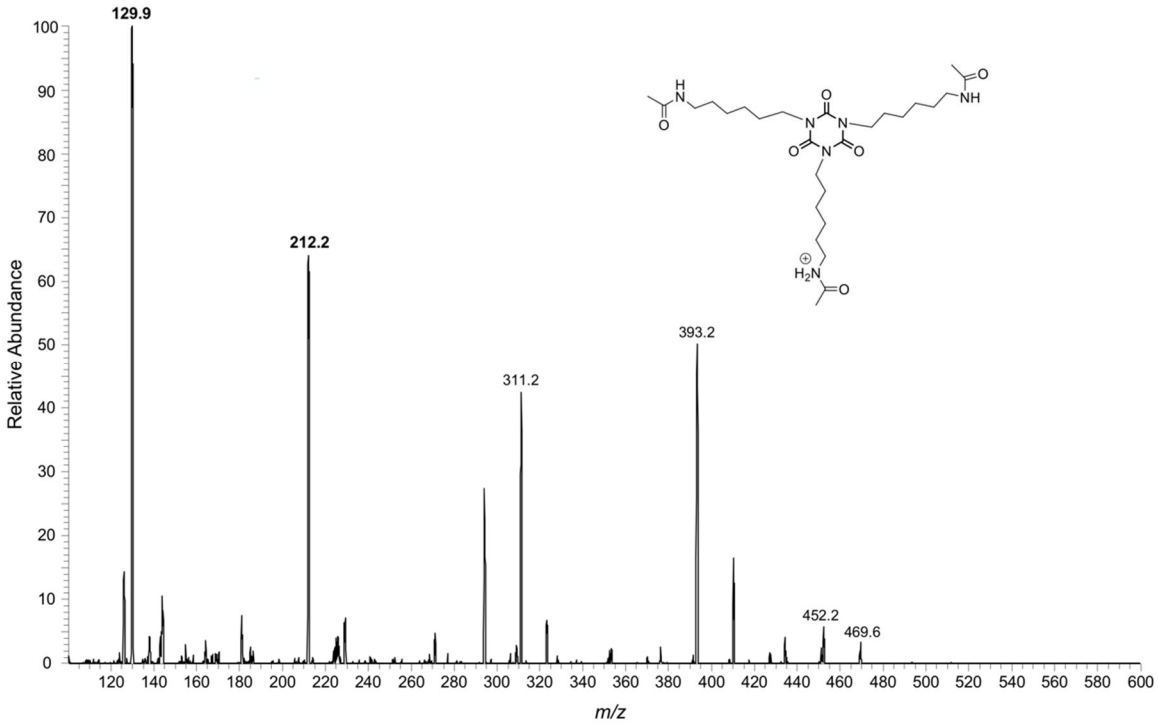

Figure 2.4. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) obtained by direct injection on ESI-MS/MS operated in positive ion-mode with electrospray ionization (scan range, *m/z* 100-600; collision energy, 50 eV).

2.2.2.3. 7,7',7''-(2,4,6-trioxo-1,3,5-triazinane-1,3,5-triyl)triheptanenitrile

To a mixture of potassium isocyanate (492 mg, 6 mmol) in dimethylformamide (1 mL), 7-bromoheptanenitrile (550 mg, 4 mmol) was added dropwise at 125°C. After heating for 2 h followed by cooling to room temperature, the mixture was partitioned between water and ethyl acetate and the organic layer was separated and washed with 0.3 N HCl, dried over Na_2SO_4, and distilled under vacuum to remove solvent. The residue was then purified by column chromatography (silicon dioxide, dichloromethane/methanol, 20:1) to afford the product. ^{1}H NMR: (400 MHz, $CDCl_3$) 1.35-1.39 (6 H), 1.47-1.50 (6 H), 1.62-1.68 (m, 12 H), 2.33 (t, J = 7.0 Hz, 6H), 3.86 (t, J = 7.8 Hz, 6H) ppm (**Figure A.6**). ^{13}C NMR, (100 MHz, $CDCl_3$), 149.1, 119.8, 43.0, 28.4, 27.7, 26.1, 25.4, 17.3 ppm (**Figure A.7**).

2.2.2.4. Trisaminoheptyl isocyanurate (M = 468.4 g/mol; ***Figure 2.2C****)*

7,7',7''-(2,4,6-Trioxo-1,3,5-triazinane-1,3,5-triyl)triheptanenitrile was hydrogenated (60 PSI) in the presence of platinum dioxide in methanol and concentrated HCl overnight, the reaction was filtered and distilled under vacuum to remove methanol. The residue was portioned between water and diethyl ether and the aqueous layer was washed further with ether and then lyophilized to afford a trichloride salt. ^{1}H NMR: (400 MHz, D_2O) 1.40-1.50 (18H), 1.67-1.73 (12H), 2.85 (t, J = 7.2 Hz, 6H), 3.97 (t, J = 7.2 Hz, 6H) ppm (**Figure A.8**). Fragmentation spectra of precursor ion $[M + H]^+$ for TAHpI (*m/z* 469.3) were obtained by nano-UPLC-ESI-MS/MS at collision energies 25 eV (**Figure A.9**) and 35 eV (**Figure A.10**).

2.2.2.5. Trisacetamidoheptyl isocyanurate (M = 594.4 g/mol; ***Figure 2.2D****)*

TAHpI trichloride was acetylated with *N,N'*-dicyclohexylcarbodiimide and acetic acid. Fragmentation spectra of precursor ion $[M + H]^+$ for TAAHpI (*m/z* 595.3) were obtained by nano-UPLC-ESI-MS/MS at collision energies 25 eV (**Figure 2.5**) and 50 eV (**Figure 2.6**).

2.2.2.6. Mass spectrometric characterization of standards

Stock solutions were analyzed with ESI-MS/MS by direct injection with isocratic flow (0.5 mL/min; 50:50 water:acetonitrile) and with nano-UPLC-ESI-MS/MS. Nanoflow chromatographic separations were carried out using the parameters described below. Instrument parameters were optimized for each precursor ion $[M + H]^+$ and fragmentation spectra were obtained for TAHI (*m/z* 427.3) and TAHpI (*m/z* 469.3) at collision energies 25 and 35 eV (scan range, *m/z* 100 – 500), and for TAAHI (*m/z* 553.3) and TAAHpI (*m/z* 595.3) at collision energies 25 and 50 eV (scan range, *m/z* 100 – 600).

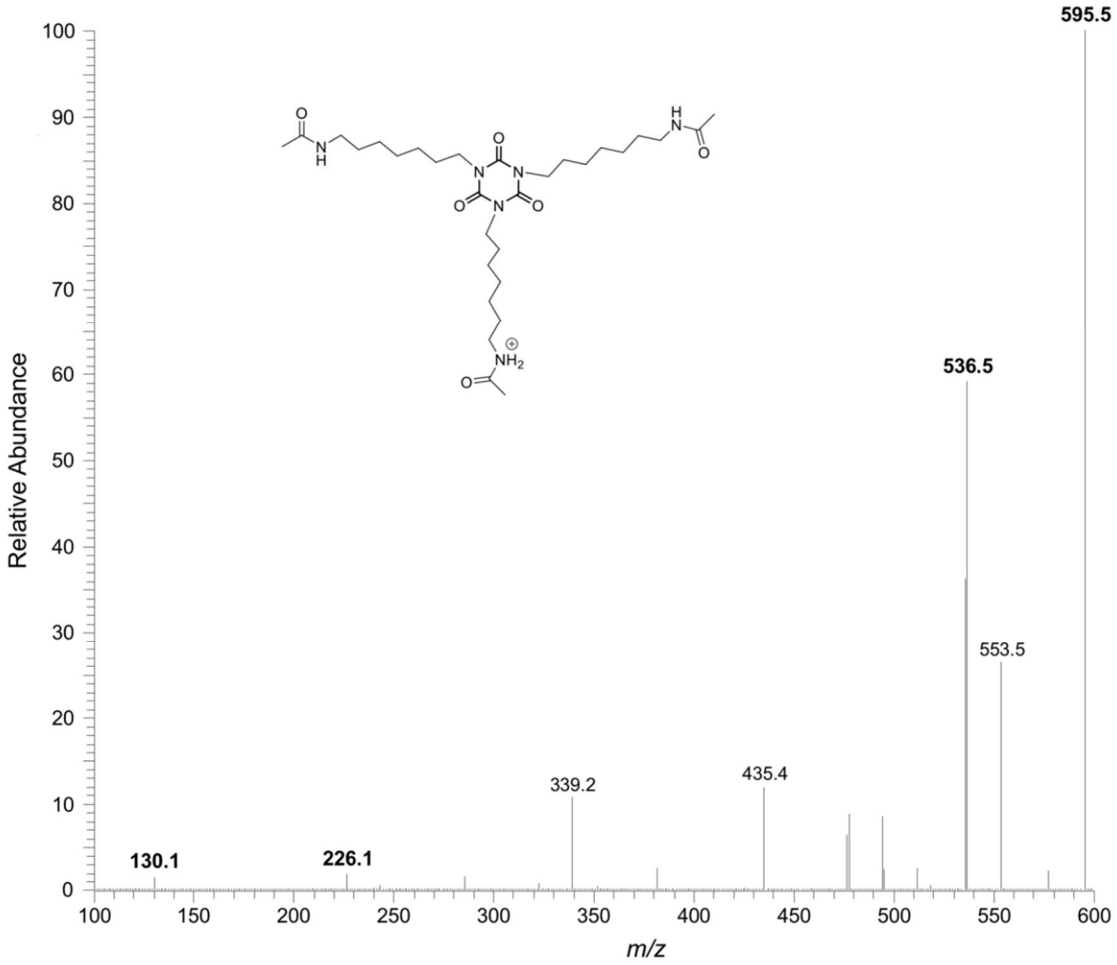

Figure 2.5. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHpI (*m/z* 595.3) obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-600; collision energy, 25 eV).

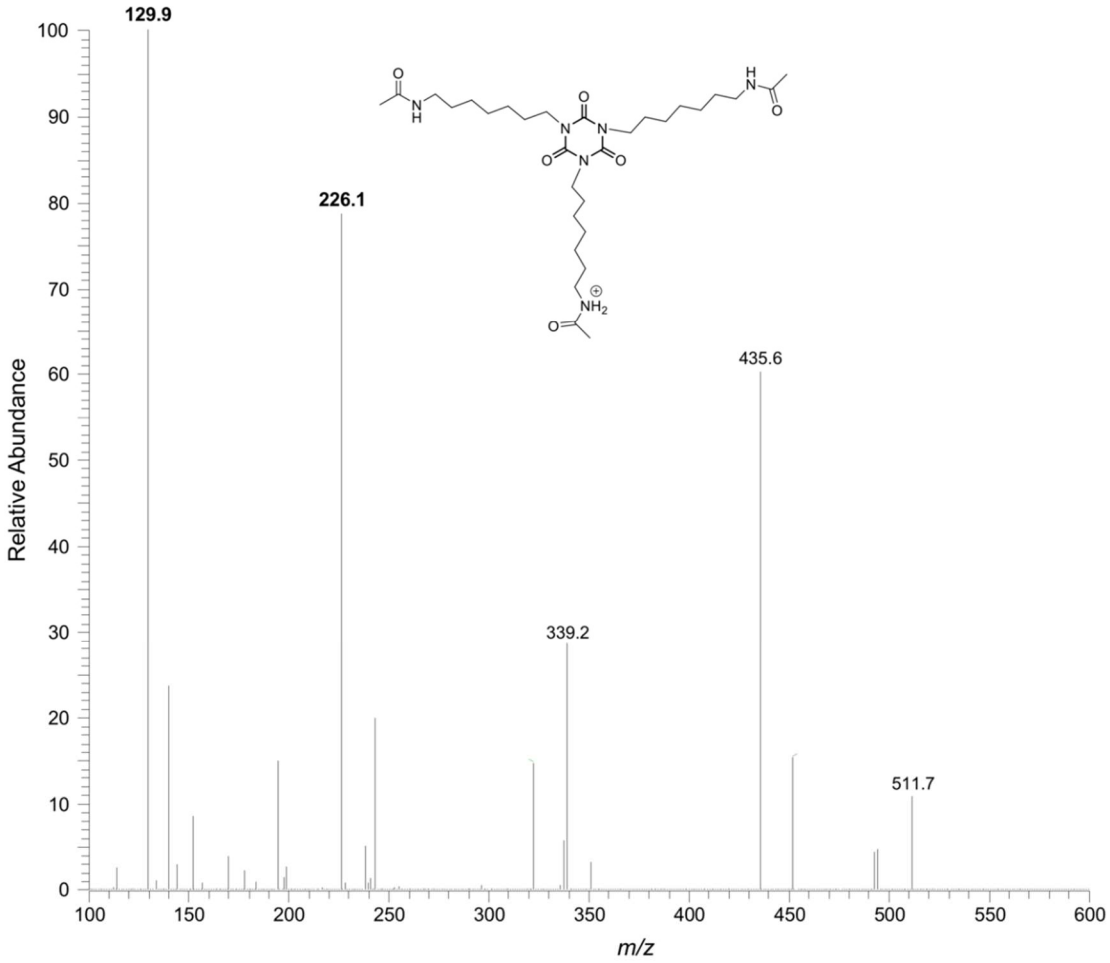

Figure 2.6. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHpI (*m/z* 595.3) obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-600; collision energy, 50 eV).

2.2.3. Study population

Urine samples were collected from 15 male spray-painters ($N = 111$) in 11 auto body shops in North Carolina with workers' consent and by approval of the Institutional Review Board in the Office of Human Research Ethics at the University of North Carolina at Chapel Hill. Spot urine samples were obtained from each participating painter before the start of work and during the workday each time he urinated. At a minimum, one pre-exposure sample and one end-of-day sample were collected. An average of 3.4 urine samples were obtained per worker per day. Exposure assessment for this worker cohort, which is a part of a larger spray-painter study

cohort, has been described previously (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a). HDI monomer and oligomer exposures were quantified using personal breathing-zone and skin tape-strip sampling (Fent *et al.*, 2009a, Fent *et al.*, 2009b), and HDA levels were quantified in plasma and urine (Flack *et al.*, 2010b, Gaines *et al.*, 2010a).

2.2.4. Sample preparation

The work-up procedure for TAHI analysis in urine involved acid hydrolysis, dichloromethane extraction, and derivatization with acetic anhydride prior to analysis by nano-UPLC-ESI-MS/MS. In a round-bottom borosilicate-glass centrifuge tube, an aliquot of urine (1 mL) was spiked with 10 µL of TAHpI (0.2 µg/mL) internal standard and hydrolyzed with sulfuric acid (100 µL) by heating at 100°C for 16 h. The sample was then adjusted to pH 14 with 25 M sodium hydroxide (2 mL) prior to liquid-liquid extraction with dichloromethane (3 x 2 mL). For each extraction step, dichloromethane (2 mL) was added to the aqueous layer, the sample was vortexed, and the tubes centrifuged at 1200 RCF for 20 min. The pooled dichloromethane extracts were then derivatized with acetic anhydride (100 µL) by heating at 55°C for 16 h on an orbital shaker. Following derivatization, excess acetic anhydride was removed by extraction with 4 mL of 1 M monobasic potassium phosphate (pH 7). The sample was vortexed, centrifuged at 500 RCF for 20 min, and then 4 mL of the dichloromethane layer was transferred to a new tube. Remaining water was removed by absorption with anhydrous sodium sulfate (500 mg). The sample was vortexed, centrifuged at 500 RCF for 10 min, and the organic layer transferred into a round-bottom borosilicate-glass culture tube and dried under a gentle flow of nitrogen gas (2 psi increasing to 5 psi) in a water bath (32°C). The dried sample was reconstituted in 200 µL of 0.1% formic acid in acetonitrile, sonicated, and transferred to a plastic autosampler vial (300 µL limited volume). The sample was dried by vacuum

centrifugation and reconstituted in 50 µL of 0.1% formic acid in water prior to nano-UPLC-ESI-MS/MS analysis.

2.2.5. Chromatographic and mass spectrometric conditions

Urine samples were analyzed with nano-UPLC-ESI-MS/MS. Reversed phase separations were carried out using a Symmetry C18 trapping column (5 µm, 180 µm × 20 mm; Waters Corp.) coupled with an Atlantis dC18 analytical column (3 µm, 100 µm × 100 mm; Waters Corp.). Mobile phase A consisted of 0.1% formic acid in deionized water and mobile phase B consisted of 0.1% formic acid in acetonitrile. Samples (2 µL) were trapped at 10 µL/min with 95% A for 1.5 min then eluted at 0.6 µL/min through the analytical column with the linear gradient program: 95% A to 10% A over 17 min (**Table A.1**). Precursor ions $[M + H]^+$ were generated by electrospray in the positive-ion mode and detected by selected reaction monitoring (SRM). Three reactions were monitored for TAAHI: *m/z* 553.3 → 494.4 (24eV), *m/z* 553.3 → 212.1 (46 eV), and *m/z* 553.3 → 130.0 (52 eV) (**Figure 2.7A**), and for TAAHpI: *m/z* 595.3 → 536.4 (24 eV), *m/z* 595.3 → 226.1 (45 eV), and *m/z* 595.3 → 130.0 (55 eV) (**Figure 2.7B**).

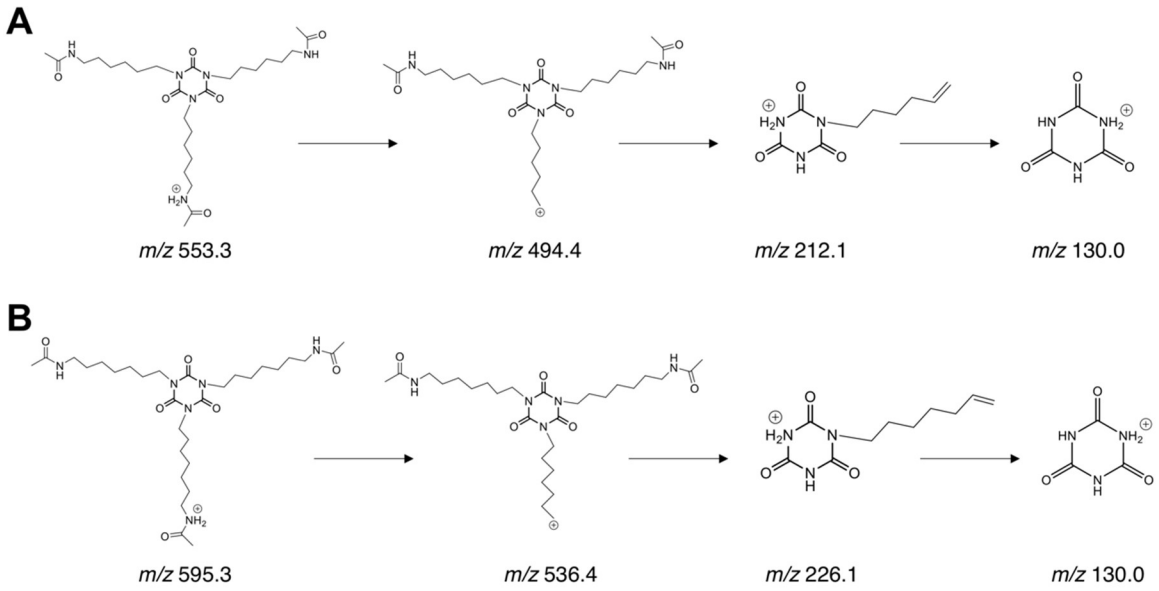

Figure 2.7. [A] TAAHI mass spectral fragments and [B] TAAHpI mass spectral fragments.

2.2.6. Preparation of standard curve and determination of method detection limit

Standard curves were prepared as follows. Stock solutions were prepared in 1 M H_2SO_4 using the trichloride salts of TAHI (1 mg/mL, equivalent to 0.80 mg/mL free amine) and TAHpI (1 mg/mL, equivalent to 0.81 mg/mL free amine). Excess stock solutions were stored at -20°C until further use. Dilutions of the TAHI and TAHpI stocks were prepared at 3-month intervals and stored at 4°C. Control urine used for calibration curves was collected from a non-exposed volunteer and processed by the experimental protocol without standard additions to verify the absence of interferences with the product ions of TAAHI and TAAHpI. Calibration standards were created by spiking 20 µL of TAHI at 13 different levels and 10 µL of TAHpI (0.2 µg/mL) into control urine (1 mL) prior to hydrolysis. Calibration standards (N = 14) included TAHpI internal standard at 2.0 µg/L and TAHI at the following concentrations: 0, 0.06, 0.09, 0.13, 0.19, 0.25, 0.37, 0.50, 0.75, 1.00, 2.00, 2.99, 3.99, and 7.98 µg/L.

Calibration curves were generated using the TAAHI/TAAHpI instrument response ratio and were linear from 0.06 to 7.98 µg/L (N = 13) with correlation coefficients $r \geq 0.995$ (CORREL

function in Microsoft Excel 2016). TAAHI fragments *m/z* 130.0 and *m/z* 494.4 (*m/z* 212.1 for analyte confirmation only) and all three TAAHpI fragments were included in the TAAHI/TAAHpI instrument response ratio. Weighted linear regression was used to fit the calibration curves according to Almeida *et al.* (Almeida *et al.*, 2002). CurveExpert 1.4 for Windows was used to evaluate linear regression weighting factors ($w = x^{-1}, x^{-2}, y^{-1}, y^{-2}$; where x = TAAHI/TAAHpI instrument response ratio and y = TAHI concentration). The mean absolute percentage error (MAPE) for the experimental concentrations was calculated in Excel to choose the best weighting scheme (MAPE < 10%). The weighting scheme ($w = x^{-2}$) was determined to have the lowest MAPE for all calibration curves. For quality control of sample treatment, a control urine sample with TAHpI (2.0 µg/L) was prepared with each batch of workers' urine samples to verify that no TAHI contamination was present from sample treatment or LC-MS/MS analysis. TAHI standards at three levels (0.06, 0.37, and 0.50 µg/L) were processed and analyzed in parallel with workers' urine samples for quality control. The analytical error was less than 15% for each quality control standard. The method detection limit (MDL) was calculated using the procedure established by the U.S. EPA (U.S. EPA, 2016). Ten control urine samples were spiked with the lowest calibration standard (0.06 µg/L TAHI; 2.0 µg/L TAHpI). Based on values in our study (s = 11.8 ng/L, N = 10, and t = 2.821 at α = 0.1), the MDL was calculated to be 0.03 µg/L.

2.3. Results

2.3.1. Verification of TAAHI fragments in treated urine

Extracted ion chromatograms acquired by selected reaction monitoring for three TAAHI fragments (*m/z* 553.3 → 130.0, *m/z* 553.3 → 212.1, and *m/z* 553.3 → 494.4) and the total ion chromatogram for TAAHpI (combined *m/z* 595.3 → 130.0, 226.1, and 536.4) are displayed in **Figure 2.8**. Control urine from a non-exposed volunteer (**Figure 2.8A**) and urine sample 8 from worker #7 (**Figure 2.8B**) were each spiked with TAHpI (2.0 µg/L) prior to sample processing. All three fragments of TAAHI were detected by SRM in treated samples using nano-UPLC-ESI-MS/MS when TAHI was present while no TAAHI fragments were observed in the treated control urine. For mass spectral confirmation, fragmentation spectra were obtained for TAAHI (*m/z* 553.3) at collision energies 25 and 50 eV (scan range, *m/z* 100 – 600) in urine sample 2 from worker #13 (**Figure 2.9** and **Figure 2.10**) and urine sample 3 from worker #14 (**Figure 2.11** and **Figure 2.12**). The fragmentation spectra for both spray-painters' urine samples closely resembled the spectra obtained from the purified standard (**Figure 2.3** and **Figure 2.4**). TAAHI fragments *m/z* 130.0 and *m/z* 494.4 and all three TAAHpI fragments were included in the TAAHI/TAAHpI instrument response ratio to create calibration curves for TAHI quantification. The total ion chromatograms used for quantification are displayed in **Figure 2.13** for control urine from a non-exposed volunteer spiked with 0.25 µg/L TAHI and 2.0 µg/L TAHpI (**Figure 2.13A**) and urine sample 8 from worker #7 spiked with 2.0 µg/L TAHpI (**Figure 2.13B**). In both the control urine spiked with TAHI and urine sample 8 from worker #7, internal standard TAAHpI peaks are produced with minimal to no signal interference and TAAHI peaks are sensitive and specific well above background noise from the biological matrix.

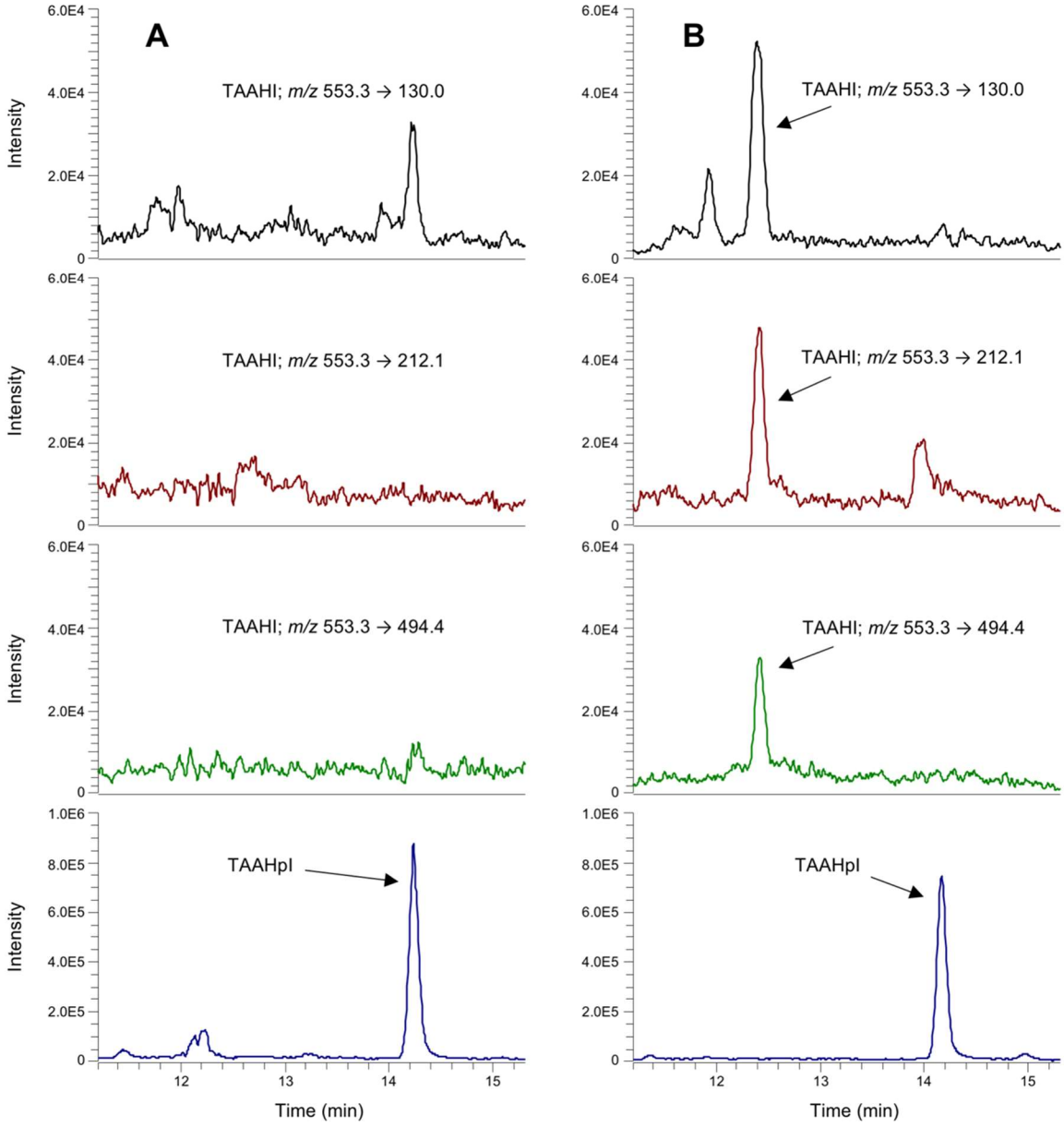

Figure 2.8. Extracted ion chromatograms acquired by selected reaction monitoring for TAAHI: *m/z* 553.3 → 130.0, *m/z* 553.3 → 212.1, and *m/z* 553.3 → 494.4, and total ion chromatogram for TAAHpI (added intensities of mass transitions *m/z* 595.3 → 130.0, 226.1, and 536.4); obtained for [A] control urine spiked with TAHpI (2.0 µg/L), and [B] urine sample 8 from worker #7 spiked with TAHpI (2.0 µg/L) and a calculated concentration of 0.36 µg/L for TAHI.

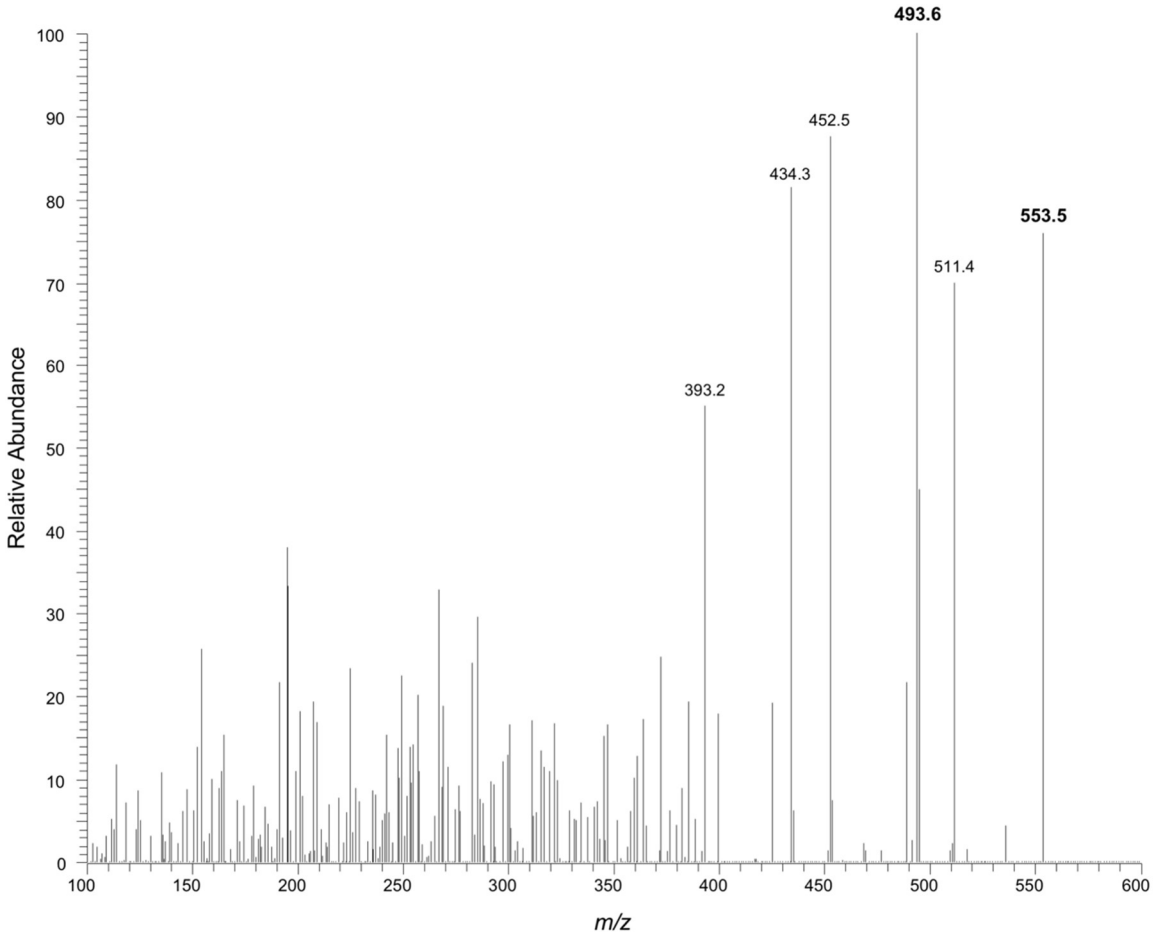

Figure 2.9. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) in urine sample 2 from worker #13 (TAHI 3.98 µg/L). Spectrum was obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-600; collision energy, 25 eV).

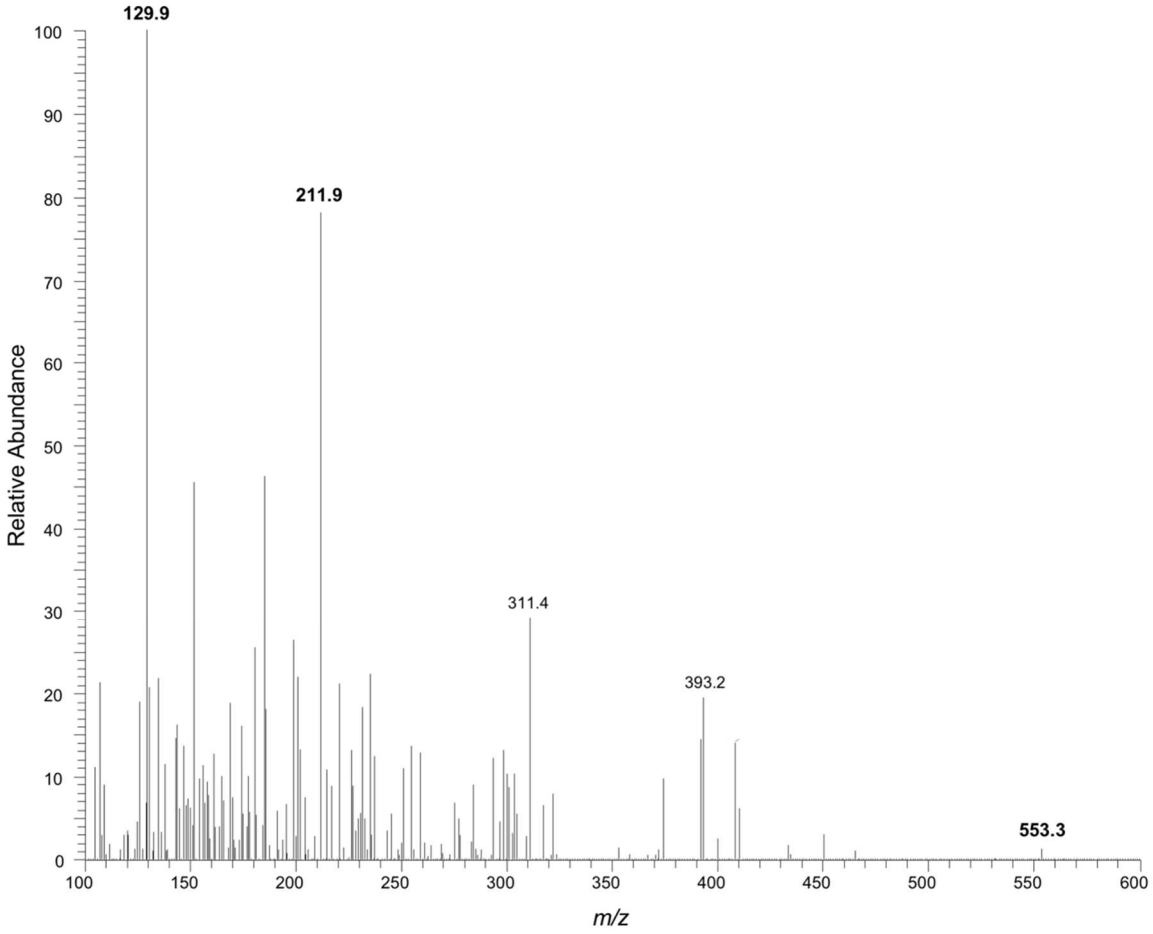

Figure 2.10. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) in urine sample 2 from worker #13 (TAHI 3.98 µg/L). Spectrum was obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-600; collision energy, 50 eV).

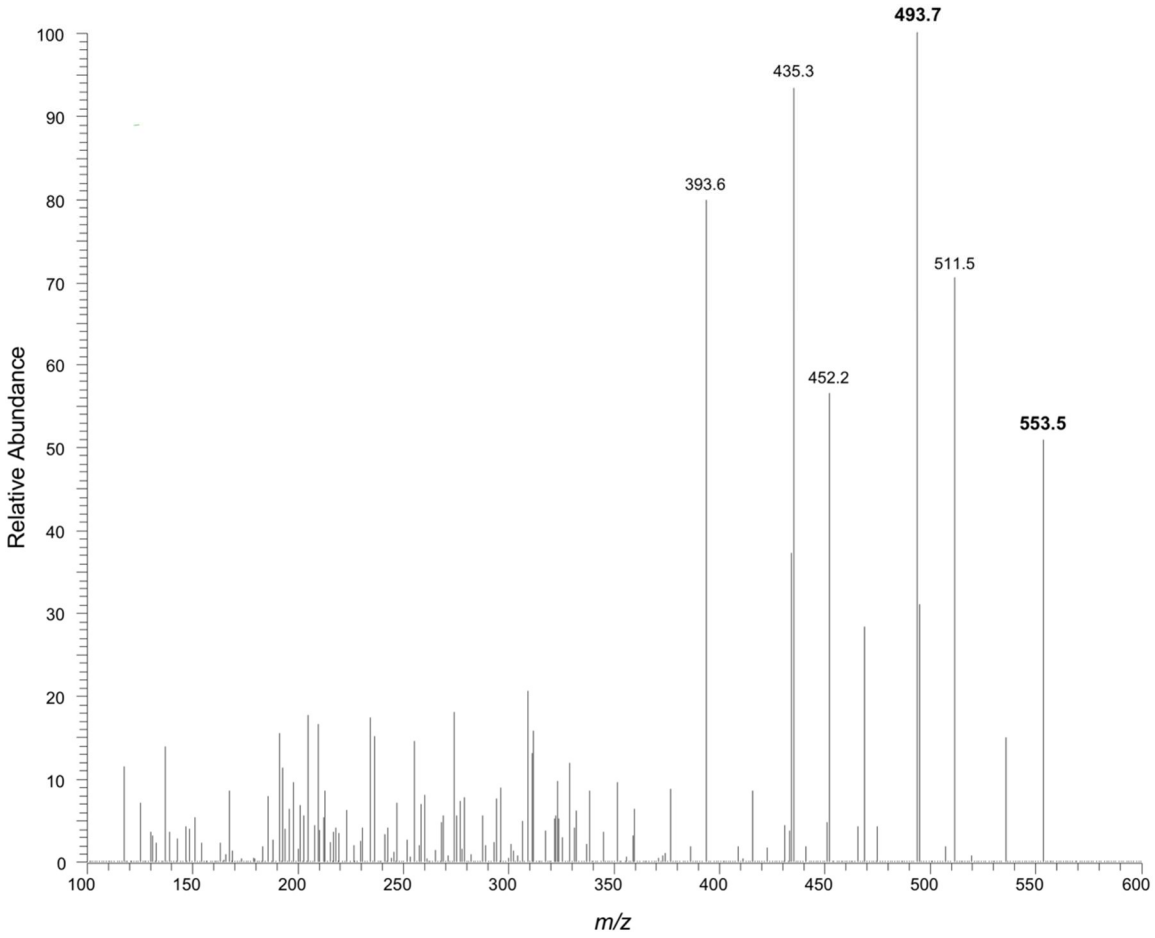

Figure 2.11. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) in urine sample 3 from worker #14 (TAHI 9.89 µg/L). Spectrum was obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-600; collision energy, 25 eV).

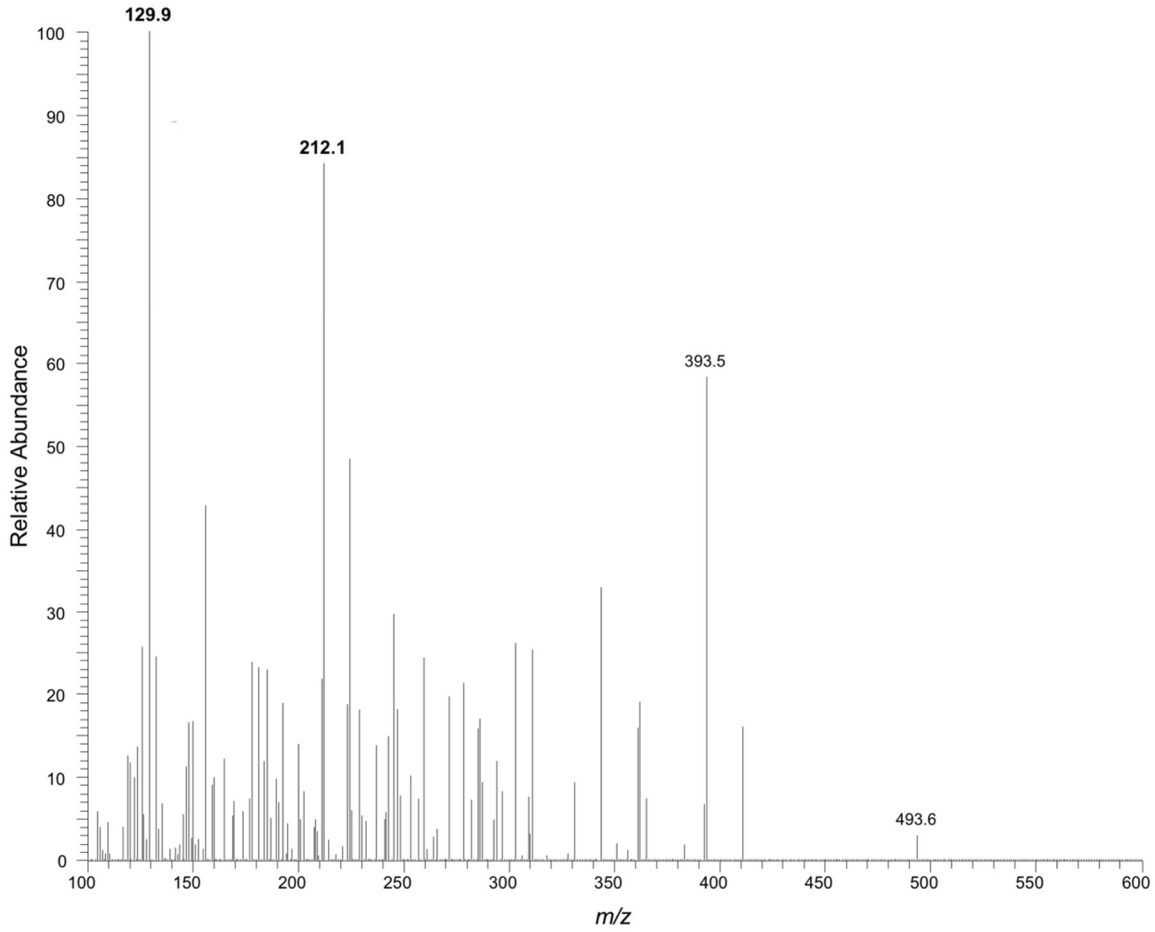

Figure 2.12. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) in urine sample 3 from worker #14 (TAHI 9.89 µg/L). Spectrum was obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-600; collision energy, 50 eV).

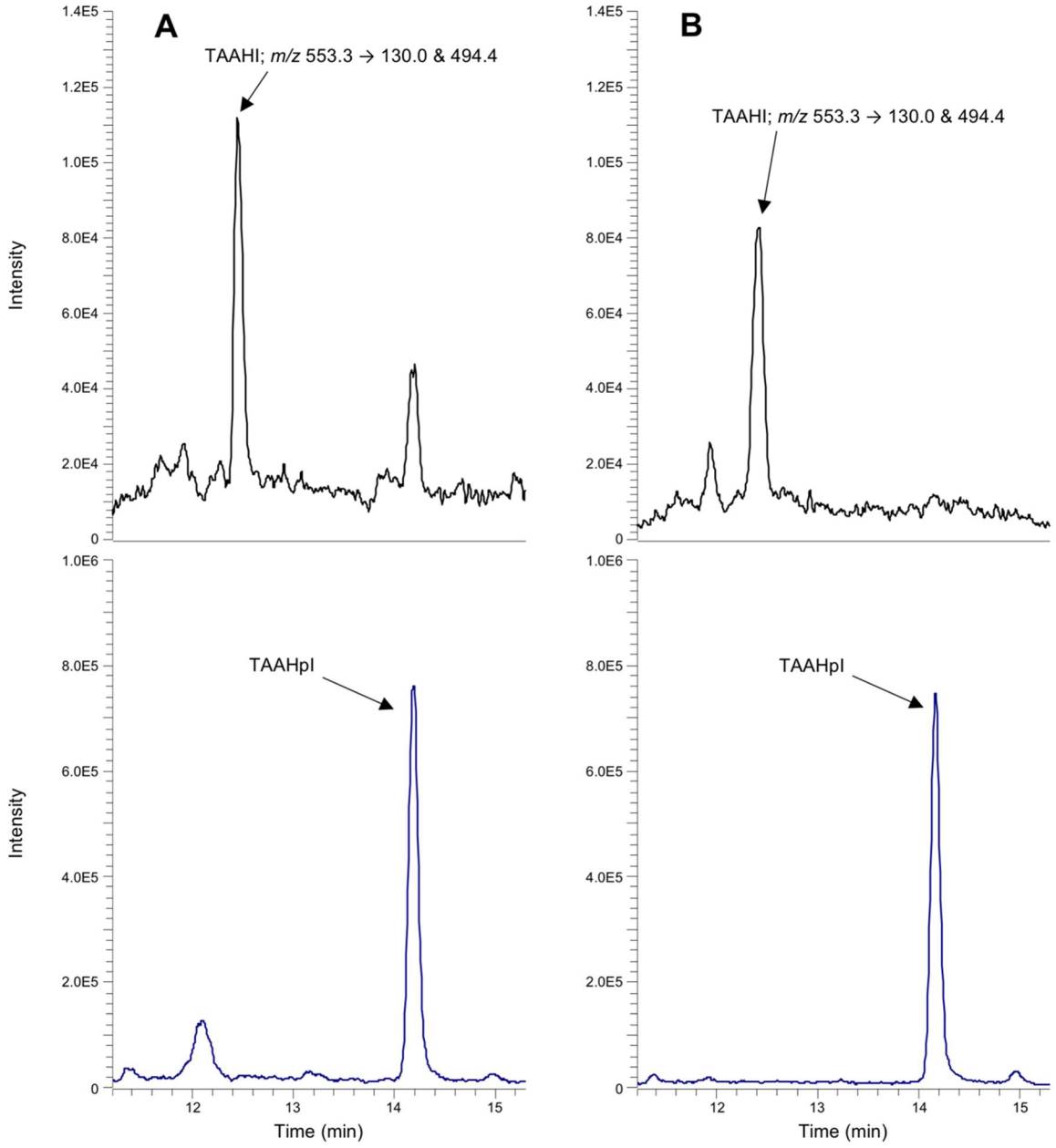

Figure 2.13. Total ion chromatograms acquired by selected reaction monitoring for TAAHI (added intensities of mass transitions *m/z* 553.3 → 130.0 and 494.4) and TAAHpI (added intensities of mass transitions *m/z* 595.3 → 130.0, 226.1, and 536.4); obtained for [A] control urine spiked with TAHI (0.25 µg/L) and TAHpI (2.0 µg/L), and [B] urine sample 8 from worker #7 spiked with TAHpI (2.0 µg/L) and a calculated concentration of 0.36 µg/L for TAHI.

2.3.2. TAHI in urine of spray-painters

Table 2.1 summarizes the mean paint-time adjusted breathing-zone and skin concentrations of HDI isocyanurate and urine levels of HDA and TAHI measured in 15 spray-painters during 1 – 3 exposure monitoring visits. The spray-painter's breathing-zone and skin HDI isocyanurate exposure was measured previously (Fent *et al.*, 2009a, Fent *et al.*, 2009b). The mean and standard deviation for the paint-time adjusted breathing-zone exposure ranged from 70 ± 39 to 34304 ± 27191 μg/m^3 and for the skin exposure from 3 ± 4 to 3857 ± 3882 μg/mm^3. TAHI was detected in the urine of 11 workers in concentrations up to 9.89 μg/L, with 33 of 111 urine samples above the MDL of 0.03 μg/L. A positive linear correlation was observed between the measured paint-time adjusted daily total breathing-zone HDI isocyanurate concentration and the daily total urine TAHI concentration (r = 0.28 without creatinine adjustment; r = 0.14 with creatinine adjustment), while the respective correlation for HDI monomer and creatinine-adjusted HDA in urine was r = 0.06.

Table 2.1. Mean ± standard deviation for paint-time adjusted breathing-zone and skin HDI isocyanurate levels and urine HDA and TAHI levels for 15 spray-painters.

Worker	Number of Visits	Number of Paint Tasks	Mean Paint Time (min)	Mean Air Isocyanurate (μg/m^3)	Mean Skin Isocyanurate (μg/mm^3)	Number of Urine Samples	Mean HDA (μg/L)	Mean TAHI (μg/L)
1	3	10	5.3 ± 3.6	11802 ± 11460	887 ± 1189	10	1.72 ± 3.01	<MDL[a]
2	3	5	7.9 ± 3.5	3656 ± 1820	204 ± 181	7	0.23 ± 0.27	<MDL
3	3	3	3.8 ± 1.0	10232 ± 6570	313 ± 277	12	0.22 ± 0.38	0.32 ± 0.24
4	1	2	8.0 ± 1.4	10752 ± 12539	1387 ± 1816	3	0.06 ± 0.06	<MDL
5	1	1	19.5	21931	637	3	0.07 ± 0.12	0.04 ± 0.07
6	2	6	6.2 ± 2.5	34304 ± 27191	1181 ± 570	8	0.34 ± 0.42	0.14 ± 0.18
7	3	12	5.2 ± 3.3	17101 ± 14805	730 ± 502	19	0.42 ± 0.68	0.14 ± 0.23
8	2	3	5.8 ± 2.8	16418 ± 4785	676 ± 520	5	0.27 ± 0.15	0.06 ± 0.13
9	2	4	9.0 ± 5.4	12870 ± 20501	635 ± 836	6	0.19 ± 0.12	<MDL
10	2	5	3.4 ± 1.7	18970 ± 29236	207 ± 426	6	0.55 ± 0.70	0.02[b] ± 0.06
11	1	2	4.8 ± 1.8	70 ± 39	3 ± 4	3	5.96 ± 1.84	0.11 ± 0.10
12	3	3	4.5 ± 2.5	27618 ± 32774	3857 ± 3882	6	0.32 ± 0.13	0.34 ± 0.55
13	1	1	1.5	20927	339	2	0.18 ± 0.26	1.99 ± 2.81
14	3	10	7.6 ± 3.1	20435 ± 22563	258 ± 228	12	0.10 ± 0.14	0.87 ± 2.84
15	3	8	5.6 ± 2.1	8306 ± 8268	10 ± 7	9	0.08 ± 0.09	0.08 ± 0.24

[a]<MDL = all samples below method detection limit; [b]5 of 6 urine samples were below MDL

2.4. Discussion

Inhalation and skin exposure to HDI monomer, isocyanurate, and other oligomers have been well characterized in the automotive refinishing industry using breathing-zone sampling and skin tape-strip sampling (Pronk *et al.*, 2006b, Fent *et al.*, 2009a, Fent *et al.*, 2009b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Flack *et al.*, 2011, Reeb-Whitaker *et al.*, 2012). However, biological monitoring has been limited to the metabolites of HDI monomer exposure (Brorson *et al.*, 1990a, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Williams *et al.*, 1999, Rosenberg *et al.*, 2002, Pronk *et al.*, 2006b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Flack *et al.*, 2011) even though HDI isocyanurate constitutes the largest portion of isocyanate exposure for spray-painters (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Reeb-Whitaker *et al.*, 2012). With increasing concern over spray-painters' predominant HDI isocyanurate exposures, it is critical to develop a method to quantitate HDI isocyanurate biomarkers in urine in order to delineate the biological availability of both HDI monomer and isocyanurate. This will allow a more informed investigation of the relative potency and dose-response relationships for HDI monomer and oligomer exposures, to establish causality for associated health effects from monomer and/or oligomer exposures, and, thus, to improve exposure and risk assessment for isocyanate exposures.

Gas chromatography-mass spectrometry (GC-MS) is commonly used for HDA analysis in urine, plasma, and hemoglobin of workers or human volunteers exposed to HDI monomer (Brorson *et al.*, 1990a, Brorson *et al.*, 1990b, Dalene *et al.*, 1990, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Skarping *et al.*, 1996, Williams *et al.*, 1999, Rosenberg *et al.*, 2002, Liu *et al.*, 2004, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Flack *et al.*, 2011). However, the derivatizing agents commonly used for HDA analysis, heptafluorobutyric anhydride (HFBA) or pentafluoropropionic anhydride (PFPA), would yield an HDI isocyanurate product above the

mass limit of most GC-MS systems. Therefore, we selected LC-MS as the analytical method for quantitating the amine metabolite of HDI isocyanurate. LC-MS analysis has been used to analyze HDA as a free amine as well as HDA derivatized with HFBA or PFPA (Skarping *et al.*, 1994b, Tinnerberg *et al.*, 1995, Littorin *et al.*, 2000, Marand *et al.*, 2004a). LC-MS has also been used in analysis of biomarkers of exposure to methylene diphenyl diisocyanate (MDI) (Skarping *et al.*, 1994a, Robert *et al.*, 2007) and toluene diisocyanate (TDI) (Carbonnelle *et al.*, 1996, Sakai *et al.*, 2002, Marand *et al.*, 2004b). Three methods were investigated for clean-up and concentration of the target analyte TAHI: (1) liquid-liquid extraction (LLE), (2) solid-phase extraction (SPE), and (3) HPLC. Significant interferences present in the urine matrix were not removed by SPE or HPLC extraction. Based on exploratory analyses, LLE was adopted for further method development. In addition to limiting confounding matrix effects, LLE has the advantages of low-cost, short procedural time, and low MDL.

The experimental protocol was based on previous studies for HDI, MDI, and TDI biomarkers in urine and plasma (Brorson *et al.*, 1990a, Brorson *et al.*, 1990b, Dalene *et al.*, 1995, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Skarping *et al.*, 1996, Rosenberg *et al.*, 2002, Sennbro *et al.*, 2003, Liu *et al.*, 2004, Pronk *et al.*, 2006b, Sabbioni *et al.*, 2007, Flack *et al.*, 2010b, Gaines *et al.*, 2010a). Acid hydrolysis is non-selective with a higher yield of total amine from acetylated, protein-conjugated, as well as unconjugated species (Brorson *et al.*, 1990b, Flack *et al.*, 2010a, Flack *et al.*, 2010b, Gaines *et al.*, 2010a) and is preferable to alkaline hydrolysis which selectively releases mono- and di-acetylated HDA (Brorson *et al.*, 1990b, Sepai *et al.*, 1995a, Sepai *et al.*, 1995b, Pauluhn, 2002a, Flack *et al.*, 2010a). Dichloromethane, an extraction solvent reported in the analytical literature (Sepai *et al.*, 1995a, Sepai *et al.*, 1995b, Kaaria *et al.*, 2001, Sakai *et al.*, 2005, Sabbioni *et al.*, 2007, Flack *et al.*, 2010a, Flack *et al.*,

2011), was observed to be the most suitable solvent in our exploratory analysis for LLE, combining low matrix effects with high sensitivity. Sakai *et al.* reported dichloromethane was the most efficient extraction solvent for isomeric diaminotoluenes 2,4- and 2,6-TDA (Sakai *et al.*, 2002). Three additional extraction solvents reported in the analytical literature were also investigated in this study for analysis by nano-UPLC-ESI-MS/MS analysis: toluene, the most commonly used solvent (Brorson *et al.*, 1990a, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Skarping *et al.*, 1996, Rosenberg *et al.*, 2002, Sennbro *et al.*, 2003, Liu *et al.*, 2004, Marand *et al.*, 2004a, Pronk *et al.*, 2006b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a), hexane, and ethyl acetate (Bailey *et al.*, 1990, Sepai *et al.*, 1995b, Sakai *et al.*, 2002). No analyte could be detected by extraction with hexane or toluene, and confounding matrix effects persisted with ethyl acetate.

MDLs for nano-UPLC-ESI-MS/MS analysis were determined for the free amine, and the acetyl and HFBA derivatives. MDLs for TAHI and TAHI-HFBA were poor, ranging from 0.6 to 2.0 µg/L following work-up by LLE, SPE, or HPLC. By contrast, the MDL of the acetylated derivative generated by treatment of the free amine with acetic anhydride was 20- to 60-fold lower than that of TAHI or TAHI-HFBA. Acetylated amines (acetamides) protonate well with positive electrospray ionization under acidic conditions and are highly sensitive with LC-MS analysis. The MDL (0.03 µg/L) and the calibration curve range 0.06 to 7.98 µg/L ($w = x^{-2}$, R^2 = 0.995) determined for TAHI are similar to those recently reported in the literature for HDA analysis in urine by GC-MS (0.04 µg/L and 0.08 to 20.0 µg/L; $w = y^{-2}$, R^2 = 0.98, respectively) (Gaines *et al.*, 2010a).

This new method for analysis of TAHI is key to understanding the toxicokinetics of this biomarker and to establish the urinary half-life of TAHI. Currently, it is unknown whether the

metabolism and excretion of HDI isocyanurate follows a pattern similar to that of HDI monomer. The observed difference between the number of urine samples with detectable HDA and TAHI cannot be solely explained by the breathing-zone and skin exposure levels to HDI monomer and HDI isocyanurate. HDI monomer comprised <1% of total HDI species (monomer, uretdione, biuret, and isocyanurate) while HDI isocyanurate comprised >90% of all HDI species quantified in the breathing-zone, skin tape-stripping, and spray-paint mixtures. The mean HDI isocyanurate concentration in the spray-paint mixtures used was 66637 mg/L compared to 196 mg/L for HDI monomer, which is reflected in the significant differences observed between the mean paint-time adjusted breathing-zone concentration for HDI isocyanurate and HDI monomer (15946 $\mu g/m^3$ and 65 $\mu g/m^3$, respectively) and the mean skin concentration (670 $\mu g/mm^3$ and 3 $\mu g/mm^3$, respectively). Despite the greater exposures to HDI isocyanurate, TAHI was detected in 11 of 15 workers' urine samples while HDA was detected in all 15 workers' urine samples. However, the maximum concentration detected for both biomarkers was comparable (9.89 µg/L for TAHI; 10.11 µg/L for HDA). In this study, urine samples were collected during the same day that the exposure monitoring was conducted and, thus, limited our ability to determine the exact half-life of urinary TAHI, which may be longer than the half-life of 2.9 h for HDA (Gaines *et al.*, 2010a). The HDA and TAHI biomarker analyses developed in our laboratory can be applied in future studies to discern the metabolism and elimination of TAHI to inform the toxicokinetics of HDI isocyanurate exposure.

2.5. Conclusions

This is the first report of an LC-MS determination (nano-UPLC-ESI-MS/MS) and quantification of a biomarker, TAHI, in the urine of HDI isocyanurate exposed workers. As is the case for the urine biomarker HDA, the urine biomarker TAHI quantified in our analysis is the

sum of free, acetylated, and protein-conjugated metabolites. In the short term, measurement of TAHI as a biomarker for HDI isocyanurate exposure allows investigation of the relationship between inhalation and skin exposure, work practices and work environment, and the source of variance in biomarker levels in the spray-painter cohort. It is noteworthy that the positive linear correlation observed between the measured paint-time adjusted daily total breathing-zone HDI isocyanurate concentration and the daily total urine TAHI concentration ($r = 0.28$ without creatinine adjustment; $r = 0.14$ with creatinine adjustment) was much stronger than the respective correlation for HDI monomer and creatinine-adjusted HDA in urine ($r = 0.06$) in this study population of North Carolina automotive spray-painters ($n = 15$). Measurement of HDA in urine of spray-painters has established a biphasic urinary half-life (Gaines *et al.*, 2010a). This new method for biomarker analysis of TAHI will allow us to determine whether urinary TAHI follows a similar pattern in future studies. Such studies will improve isocyanate exposure assessment through characterization of exposure-dose relationships for both HDI monomer and HDI isocyanurate in occupationally exposed populations. Additional studies will be necessary to apportion the individual monomer and oligomer contributions to total dose. Since HDI isocyanurate inhalation and skin exposure levels are significantly higher than levels of HDI monomer in the spray-painting environment and HDI isocyanurate is potentially more potent sensitizing agent (Zissu *et al.*, 1998, Aalto-Korte *et al.*, 2010), the measurement of TAHI as a direct biomarker of HDI isocyanurate dose will be critical in evaluating the potency and role of HDI isocyanurate exposure in the development of sensitization and adverse respiratory effects. The widespread occupational exposure to HDI isocyanurate makes research of uptake and metabolism imperative. In the long term, the utility of TAHI as a biomarker will be important in toxicological studies directed at establishing the mode of action of HDI isocyanurate.

CHAPTER 3: TRISAMINOHEXYL ISOCYANURATE (TAHI) QUANTIFICATION IN PLASMA AS A BIOMARKER OF EXPOSURE TO HDI ISOCYANURATE[2]

3.1. Introduction

Exposure to isocyanates is a leading cause of occupationally-induced asthma and is also associated with a multitude of adverse health effects including irritation of the upper respiratory system, hypersensitivity pneumonitis, and allergic contact dermatitis (Vandenplas *et al.*, 1993c, Chan-Yeung and Malo, 1995, Bernstein, 1996, Piirila *et al.*, 2000, Bello *et al.*, 2007a, Aalto-Korte *et al.*, 2010). In the automotive refinishing industry, spray-painters are exposed to high levels of 1,6-hexamethylene diisocyanate (HDI) monomer and its oligomers (uretdione, biuret, and isocyanurate) during application of polyurethane clearcoat paints (Janko *et al.*, 1992, Maitre *et al.*, 1996, Sparer *et al.*, 2004, Pronk *et al.*, 2006a, Pronk *et al.*, 2006b, Fent *et al.*, 2008, Fent *et al.*, 2009a, Fent *et al.*, 2009b, Reeb-Whitaker *et al.*, 2012). HDI isocyanurate exposures are of increasing concern in the workplace due to its greater skin sensitization capacity (Zissu *et al.*, 1998, Aalto-Korte *et al.*, 2010) and faster skin penetration rate (Thomasen and Nylander-French, 2012) compared to HDI monomer and HDI biuret. Despite these concerns, biomarkers of HDI exposure have been limited to unconjugated plasma and urine biomarkers such as 1,6-diaminohexane (HDA), the hydrolysis product of HDI monomer (Dalene *et al.*, 1994, Skarping *et al.*, 1994b, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Liu *et al.*, 2004, Pronk *et al.*, 2006b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Flack *et al.*, 2011).

In previous studies, hydrolyzed HDA levels in plasma and urine were significantly correlated with HDI monomer inhalation and skin exposures (Flack *et al.*, 2010b, Gaines *et al.*, 2010a). However, in a controlled inhalation challenge study where volunteers were exposed to HDI biuret aerosols containing HDI monomer and trace levels of HDI uretdione, hydrolyzed HDA levels in urine were not significantly correlated with either HDI biuret or total NCO inhalation exposures (Liu *et al.*, 2004). Recently, we published a method to detect hydrolyzed trisaminohexyl isocyanurate (TAHI) in processed urine as a biomarker of HDI isocyanurate exposure (Robbins *et al.*, 2018). We have now adapted this method to measure hydrolyzed TAHI in plasma to improve exposure assessment for oligomeric isocyanates.

3.2. Experimental

3.2.1. Standards and materials

High-performance liquid chromatography (HPLC) grade dichloromethane was obtained from Sigma-Aldrich (St. Louis, MO, USA). American Chemical Society (ACS) grade acetic anhydride was obtained from Acros Organics (Fair Lawn, NJ, USA). ACS grade sodium hydroxide (pellets) was obtained from VWR International, LLC (Solon, OH, USA). Laboratory grade monobasic potassium phosphate, ACS grade anhydrous sodium sulfate and sulfuric acid, and Optima® LC-MS grade acetonitrile and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Both K_3EDTA and Li-Heparin anticoagulated plasma from healthy human volunteers were purchased from Biological Specialty Corporation (Colmar, PA).

The following analytical standards were synthesized in house: 1,3,5-Tris(6-aminohexyl)-1,3,5-triazinane-2,4,6-trione (trisaminohexyl isocyanurate, **TAHI**); *N,N',N''*-((2,4,6-trioxo-1,3,5-triazinane-1,3,5-triyl)tris(hexane-6,1-diyl))triacetamide (trisacetamidohexyl isocyanurate, **TAAHI**); 1,3,5-tris(7-aminoheptyl)-1,3,5-triazinane-2,4,6-trione (trisaminoheptyl isocyanurate,

TAHpI); and *N,N',N''*-((2,4,6-trioxo-1,3,5-triazinane-1,3,5-triyl)-tris(heptane-7,1-diyl))triacetamide (trisacetamidoheptyl isocyanurate, **TAAHpI**). Composition and purity of the synthesized standards were confirmed by proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy and by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Robbins *et al.*, 2018).

3.2.2. Study population and sample collection

Blood samples ($N = 112$) were collected from automotive spray-painters in North Carolina ($n = 14$) and Washington State ($n = 32$) during 1 – 3 visits to 35 automotive repair shops according to a protocol approved by the Institutional Review Boards at the University of North Carolina at Chapel Hill and Washington State (Flack *et al.*, 2010b). Blood was drawn from workers near the end of the work-shift and collected in separate tubes containing K_3EDTA or Li-Heparin anticoagulants. Exposure assessment for this worker cohort has been described previously (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a). HDI monomer and oligomer exposures were quantified by personal breathing-zone and skin tape-strip sampling (Fent *et al.*, 2009a, Fent *et al.*, 2009b). Hydrolyzed HDA levels in plasma were quantified as described previously (Flack *et al.*, 2010b).

3.2.3. Instrumental analysis

Mass spectra were acquired on a TSQ Quantum Ultra triple-quadrupole mass spectrometer with a nano-electrospray ionization (ESI) source (Thermo Scientific, Waltham, MA) coupled to a NanoAcquity UPLC system (Waters Corporation, Milford, MA). Reverse phase separations were carried out on a Waters' Symmetry C18 trapping column (5 µm, 180 µm × 20 mm) coupled to a Waters' Atlantis dC18 analytical column (3 µm, 100 µm × 100 mm). Mobile phase A consisted of 0.1% formic acid in deionized water and mobile phase B consisted

of 0.1% formic acid in acetonitrile. Xcalibur 3.0 software (Thermo Scientific, Waltham, MA) was utilized to create the following nano-UPLC-ESI-MS/MS method and to acquire and process the chromatographic data. Samples (2 µL) were injected and trapped with 10 µL/min of 95% A for 1.5 min then eluted at 0.6 µL/min through the analytical column (maintained at 35°C) with the linear gradient program: 95% A to 10% A over 17 min. Precursor ions $[M + H]^+$ were generated by electrospray (2000 V) in the positive-ion mode and detected by selected reaction monitoring (SRM) with 1.5 mTorr argon as the collision gas. Five mass transitions were monitored for TAAHI (collision energies denoted in parentheses): *m/z* 553.3 → 494.4 (24 eV), *m/z* 553.3 → 452.3 (32 eV), *m/z* 553.3 → 393.3 (38 eV), *m/z* 553.3 → 212.1 (46 eV), and *m/z* 553.3 → 130.0 (52 eV), and three transitions were monitored for the internal standard TAAHpI: *m/z* 595.3 → 536.4 (24 eV), *m/z* 595.3 → 226.1 (45 eV), and *m/z* 595.3 → 130.0 (55 eV). Structures of the TAAHI mass spectral fragments and the fragmentation spectra at collision energies 50 eV (A) and 25 eV (B) are shown in **Figure 3.1**.

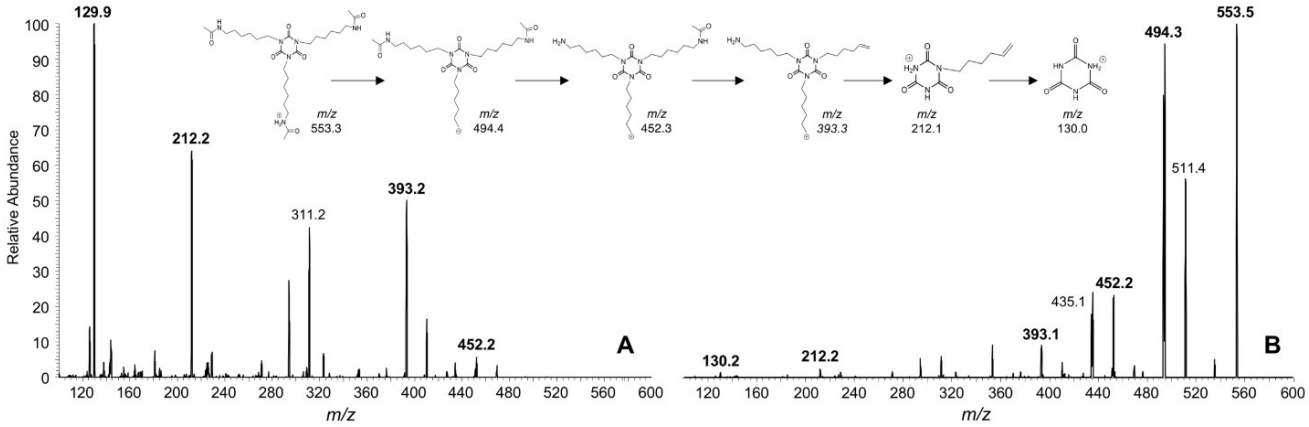

Figure 3.1. TAAHI mass transitions: *m/z* 553.3 → 494.4 (24eV), *m/z* 553.3 → 452.3 (32 eV), *m/z* 553.3 → 393.3 (38 eV), *m/z* 553.3 → 212.1 (46 eV), and *m/z* 553.3 → 130.0 (52 eV). Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) obtained by direct injection into ESI-MS/MS operated in positive ion-mode with electrospray ionization (scan range, *m/z* 100-600) at [A] collision energy 50 eV and [B] collision energy 25 eV.

3.2.4. Plasma sample preparation

The work-up procedure for analysis of hydrolyzed TAHI in plasma was adapted from Robbins *et al.* (Robbins *et al.*, 2018). Briefly, an aliquot of plasma (1 mL) was spiked with 10 µL of TAHpI (0.2 µg/mL) and hydrolyzed with sulfuric acid (100 µL) by heating at 100°C for 16 h. Sodium hydroxide (2 mL of 25 M) was added to the hydrolyzed plasma to raise the pH above the amine pKa prior to liquid-liquid extraction with dichloromethane (3 x 2 mL). For each extraction step, the sample was vortexed and then centrifuged at 1200 RCF for 20 min. The pooled dichloromethane extracts were then derivatized with acetic anhydride (100 µL) by heating at 55°C for 16 h. Following derivatization, excess acetic anhydride was removed by extraction with 4 mL of 1 M monobasic potassium phosphate (pH 7). The sample was vortexed and centrifuged at 500 RCF for 20 min, and then the dichloromethane layer (4 mL) was transferred to a new tube and remaining water was removed with anhydrous sodium sulfate (500 mg). The sample was vortexed, centrifuged at 500 RCF for 10 min, and the organic layer was transferred to a new tube and taken to dryness under a gentle flow of nitrogen gas in a water bath (32°C). The dried sample was reconstituted in 200 µL of 0.1% formic acid in acetonitrile, sonicated, and transferred to an autosampler vial. The sample was dried by vacuum centrifugation and reconstituted in 50 µL of 0.1% formic acid in water prior to nano-UPLC-ESI-MS/MS analysis.

3.2.5. Standard curve and method detection limit

Previously described preparation of hydrolyzed TAHI standard curves in urine (Robbins *et al.*, 2018) were modified for analysis of hydrolyzed TAHI in plasma. Standard curves were prepared using K_3EDTA or Li-Heparin control plasma for matrix matching with samples of workers' plasma. Limited volume of 112 worker plasma samples (81 plasma samples were collected with K_3EDTA and 31 plasma samples were collected with Li-Heparin) was available

from prior analysis (Flack *et al.*, 2010b). Calibration standards were generated by spiking 10 µL of TAHI at 11 different levels and 10 µL of TAHpI (0.2 µg/mL) into control plasma (1 mL) and hydrolyzed following the same protocol used for samples. Calibration standards ($N = 12$) included TAHpI internal standard at 2.0 µg/L and TAHI at the following concentrations: 0, 0.03, 0.06, 0.09, 0.12, 0.19, 0.25, 0.37, 0.50, 0.75, 1.00, and 3.99 µg/L. Standard curves were generated using the TAAHI/TAAHpI instrument response ratio and were linear with correlation coefficients $r \geq 0.995$ in both K_3EDTA and Li-Heparin plasma from 0.03 to 3.99 µg/L ($N = 11$). CurveExpert 1.4 (Hyams Development) was used for weighted linear regression ($w = x^{-1}$, x^{-2}, y^{-1}, y^{-2}; where x = TAAHI/TAAHpI instrument response ratio and y = TAHI concentration) to fit the calibration curves according to Almeida *et al.* (Almeida *et al.*, 2002). The weighting scheme was determined using the lowest mean absolute percentage error (MAPE < 10%) of calibration standards, and subsequently $w = y^{-2}$ was chosen for K_3EDTA curves and $w = x^{-2}$ was chosen for Li-Heparin. The experimental method detection limit (MDL) was calculated using the procedure established by the U.S. EPA (U.S. EPA, 2016) using data from ten control plasma samples spiked with the lowest calibration standard (0.03 µg/L TAHI; 2.0 µg/L TAHpI). The TAAHI signal-to-noise (s/n) ratio of each MDL sample was calculated by averaging the baseline noise 60 s before the TAAHI peak and 30 s after the TAAHI peak. The geometric mean (GM) and geometric standard deviation (GSD) of the TAAHI s/n ratio for all ten MDL samples was GM = 20.3 s/n ratio (GSD = 1.4) with a range of 13 - 37 s/n ratio. Based on values in our study [standard deviation (SD) = 6.2 ng/L, $N = 10$, and $t = 2.821$ at $\alpha = 0.1$], the experimental MDL was calculated to be 0.02 µg/L. Analytical carryover was evaluated by injecting mobile phase blanks (50:50, mobile phase A:B) after the highest calibration standard (3.99 µg/L) and the highest observed carryover was inconsequential (0.8% abundance).

3.3. Results and discussion

Figure 3.2 displays representative extracted ion chromatograms for TAAHI (analyte) and TAAHpI (internal standard) mass transitions in (**3.2A**) control plasma spiked with 2.0 µg/L TAHpI, (**3.2B**) control plasma spiked with 0.06 µg/L TAHI and 2.0 µg/L TAHpI, and (**3.2C**) a plasma sample collected from a worker spiked with 2.0 µg/L TAHpI (calculated concentration of 0.10 µg/L TAHI). For the five TAAHI mass transitions monitored (*m/z* 553.3 → 494.4, *m/z* 553.3 → 452.3, *m/z* 553.3 → 393.3, *m/z* 553.3 → 212.1, and *m/z* 553.3 → 130.0), signals were detected in the control plasma spiked with TAHI and also in the worker sample, however no TAAHI signals were observed in the control plasma sample spiked with TAHpI internal standard only.

The SRM method previously developed for analysis of TAAHI in processed urine included three mass transitions (*m/z* 553.3 → 494.4, *m/z* 553.3 → 212.1, and *m/z* 553.3 → 130.0) and three mass transitions for TAAHpI internal standard *m/z* 595.3 → 536.4, *m/z* 595.3 → 226.1, and *m/z* 595.3 → 130.0) (Robbins *et al.*, 2018). TAAHI mass transitions *m/z* 553.3 → 494.4 and *m/z* 553.3 → 130.0 were combined for quantification, while *m/z* 553.3 → 212.1 was used as a qualifier. For plasma samples, higher background and lower analyte signal intensity were observed for TAAHI mass transitions (*m/z* 553.3 → 212.1 and *m/z* 553.3 → 130.0) at the highest collision energies. Therefore, the method was optimized by replacing those two mass transitions with *m/z* 553.3 → 452.3 and *m/z* 553.3 → 393.3, resulting in lower background and higher s/n ratios. **Figure 3.3** displays this difference in background noise and s/n ratio for TAAHI extracted ion chromatograms used for hydrolyzed TAHI quantification in a control urine sample (**3.3A**) and control plasma sample (**3.3B**), both spiked with 0.06 µg/L TAHI (and 2.0 µg/L TAHpI). Modification of the urine SRM analytical method improved sensitivity of the MS assay for

plasma by extending the lower range of the plasma calibration curve and MDL (0.03 – 3.99 µg/L; 0.02 µg/L) relative to the TAHI urine calibration curve (0.06 – 7.99 µg/L; 0.03 µg/L) (Robbins *et al.*, 2018).

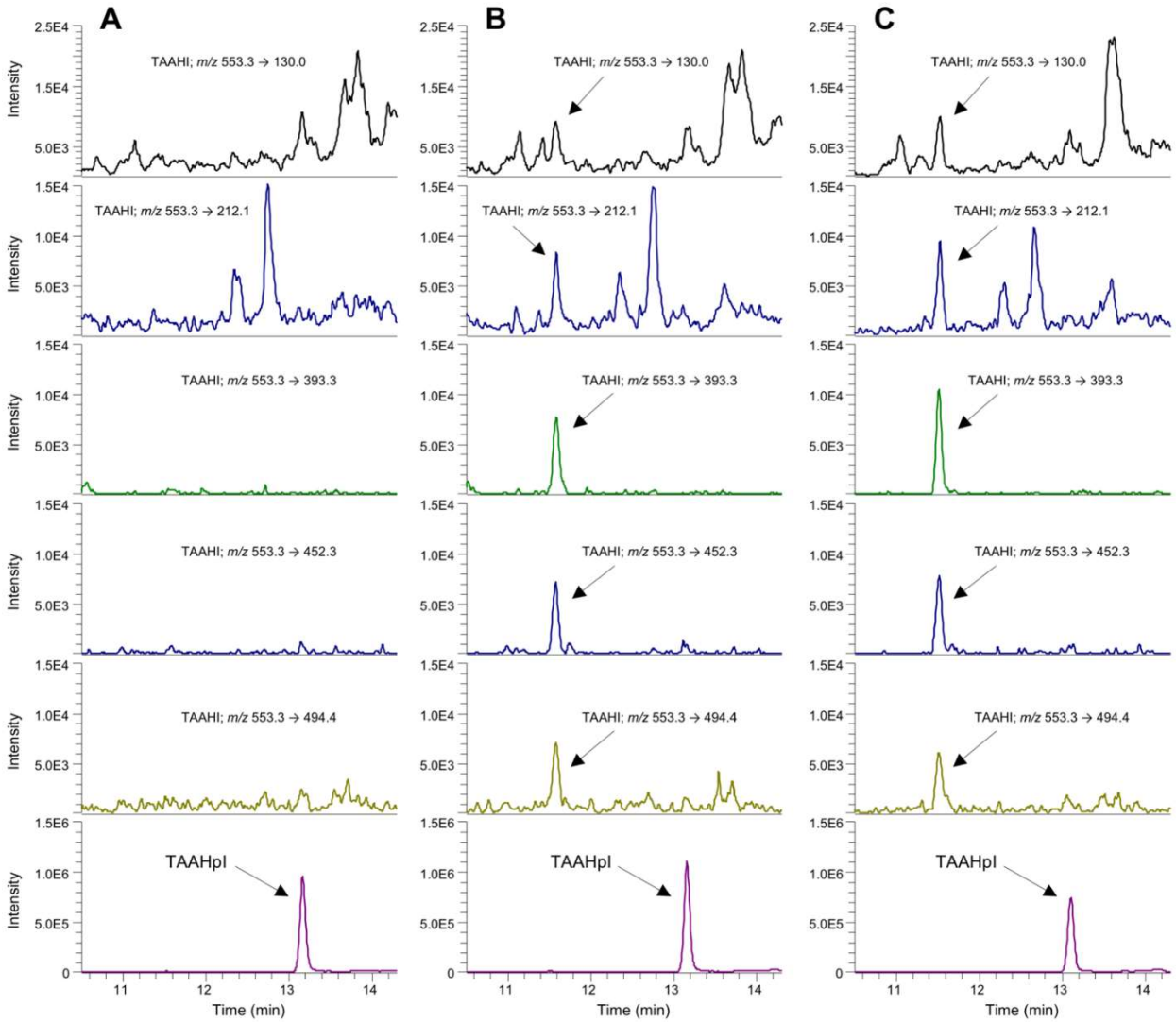

Figure 3.2. Individual extracted ion chromatograms acquired by selected reaction monitoring for TAAHI: *m/z* 553.3 → 130.0, *m/z* 553.3 → 212.1, *m/z* 553.3 → 393.3, *m/z* 553.3 → 452.3, and *m/z* 553.3 → 494.4, and total ion chromatogram for TAAHpI (summed intensities of mass transitions *m/z* 595.3 → 130.0, 226.1, and 536.4); obtained for [A] control plasma spiked with TAHpI (2.0 µg/L), [B] control plasma spiked with TAHI (0.06 µg/L) and TAHpI (2.0 µg/L), and [C] a plasma sample collected from a worker spiked with TAHpI (2.0 µg/L) and a calculated concentration of 0.10 µg/L for TAHI.

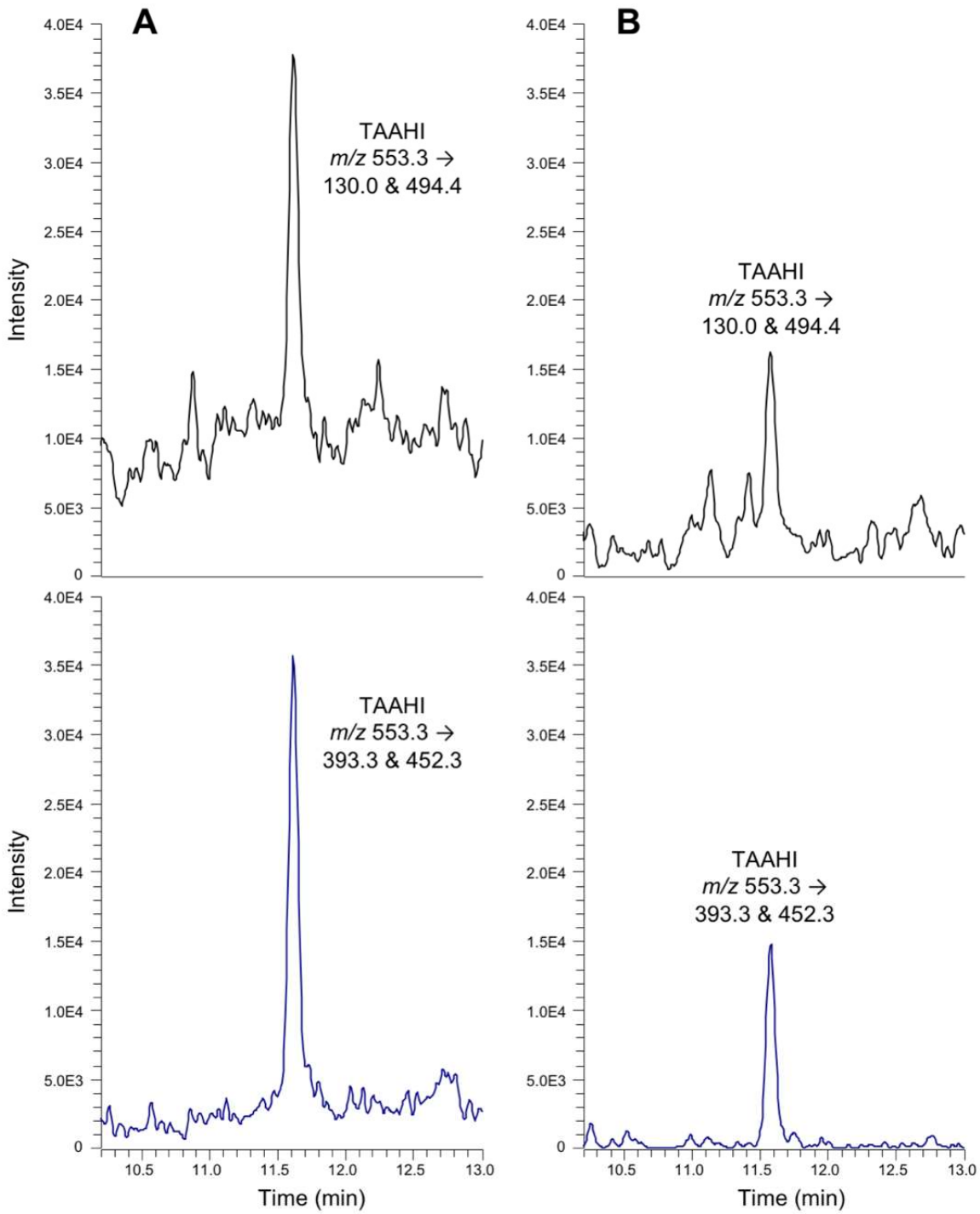

Figure 3.3. Comparison of extracted ion chromatograms for TAAHI in [A] control urine and [B] control plasma, both spiked with TAHI (0.06 µg/L) and TAHpI (2.0 µg/L). TAAHI ion chromatograms are displayed as summed intensities of mass transitions *m/z* 553.3 → 130.0 and 494.4 and summed intensities of mass transitions *m/z* 553.3 → 393.3 and 452.3.

Hydrolyzed TAHI was observed in 24 of 112 (21%) plasma samples in 14 of 46 (30%) workers and had a concentration range of <MDL to 0.32 µg/L [arithmetic mean (AM) and SD of <MDL ± 0.04 µg/L and GM (GSD) of <MDL µg/L (GSD = 12.03)]. In the same population, hydrolyzed HDA was reported in 82 of 112 (73%) plasma samples in 45 of 46 (98%) workers and had a concentration range of <MDL to 0.92 µg/L [AM and SD of 0.10 ± 0.14 µg/L and GM (GSD) of <MDL µg/L (GSD = 24.98)] where the MDL was 0.02 µg/L (Flack *et al.*, 2010b).

3.4. Conclusions

The sensitive method we developed for analysis of urine biomarker TAHI by nano-UPLC-ESI-MS/MS was successfully adapted and further optimized for the analysis of this biomarker in plasma of automotive spray-painters occupationally exposed to HDI isocyanurate. No changes in sample treatment were required for plasma samples, however, two additional TAAHI mass transitions (*m/z* 553.3 → 452.3 and *m/z* 553.3 → 393.3) were included in the SRM method. These two mass transitions were utilized for quantification due to better s/n ratio compared to mass transitions *m/z* 553.3 → 494.4, *m/z* 553.3 → 212.1, and *m/z* 553.3 → 130.0 previously monitored for urine samples. The modified protocol improved sensitivity of the MS method, decreasing the MDL from 0.03 µg/L in urine to 0.02 µg/L in plasma. The measurement of hydrolyzed TAHI in plasma in combination with analysis of hydrolyzed TAHI in urine will be key in future development of toxicokinetic models for HDI isocyanurate exposure. In addition, these methods will improve exposure assessment and evaluation of the relative effectiveness of workplace safety measures such as personal protective equipment for reducing occupational exposures to oligomeric HDI isocyanates. While hydrolyzed HDA is the currently established biomarker for exposure to HDI monomer, continued monitoring of HDA and the addition of a marker for oligomeric HDI isocyanates is important because relative and cumulative contributions of HDI isocyanurate and HDI monomer to adverse health outcomes have not been assessed.

CHAPTER 4: BIOMARKERS TO DIFFERENTIATE AND DEFINE EXPOSURE TO 1,6-HEXAMETHYLENE DIISOCYANATE (HDI) MONOMER AND HDI ISOCYANURATE

4.1. Introduction

Spray-painters in vehicle manufacturing and refinishing industries during clearcoat applications are commonly exposed to higher levels of HDI isocyanurate than HDI monomer and HDI biuret (Janko *et al.*, 1992, Carlton and England, 2000, Bello *et al.*, 2004, Pronk *et al.*, 2006a, Pronk *et al.*, 2006b, Fent *et al.*, 2008, Fent *et al.*, 2009a, Fent *et al.*, 2009b, Reeb-Whitaker *et al.*, 2012, Ceballos *et al.*, 2017). HDI isocyanurate is a respiratory irritant (Ferguson *et al.*, 1987, Pauluhn, 2000, Pauluhn and Mohr, 2001, Pauluhn, 2002b, Pauluhn, 2004, Ma-Hock *et al.*, 2007) and a respiratory sensitizer that is linked to the development of isocyanate-induced asthma (Vandenplas *et al.*, 1993a, Pronk *et al.*, 2007). HDI isocyanurate also exhibits skin sensitizing capacity without inhalation exposure and without concomitant exposure to HDI monomer or HDI biuret (Zissu *et al.*, 1998, Pauluhn, 2002b, Pauluhn *et al.*, 2002, Aalto-Korte *et al.*, 2010).

Because HDI isocyanurate exposure markedly overshadows exposure to other isocyanate species in vehicle refinishing industries, it is important to understand the magnitude and variability of inhalation and skin exposures to this compound as well as to gauge the effectiveness of workplace safety measures in order effectively to mitigate exposure.

Exposure assessment of HDI oligomers in occupational settings is challenged by sampling methods required for multiple exposure routes, diverse work environments, inconsistent use of personal protective equipment (PPE), and inter-individual differences in physical health, metabolism, and genetics (Sparer *et al.*, 2004, Bello *et al.*, 2007a, Henneken *et al.*, 2007, Whittaker and Reeb-Whitaker, 2009). Sampling methods to measure personal breathing-zone HDI oligomers exposures are well-established (Bello *et al.*, 2002, Pronk *et al.*, 2006b, Henneken *et al.*, 2007, Fent *et al.*, 2008, Reeb-Whitaker *et al.*, 2012), but measurements are potentially confounded by respirator use, smoking, preexisting respiratory conditions (*e.g.*, asthma, COPD), and breathing rate (Sparer *et al.*, 2004, Woskie *et al.*, 2004, Liu *et al.*, 2006, Fent *et al.*, 2009a). By contrast, isocyanate skin sampling is a nascent field lacking standardized methods. SWYPE™ and tape-strip sampling have been previously used to measure HDI oligomer skin exposures in automotive refinishing shops after painting or after unrelated tasks (*e.g.*, buffing, sanding, compounding, mechanical work, taping and untaping) (Liu *et al.*, 2007, Bello *et al.*, 2008, Fent *et al.*, 2009b), yet these removal techniques are prone to underestimate exposure due to absorption, physical removal, or chemical reactions (Wisnewski *et al.*, 2000, Bello *et al.*, 2006, Thomasen and Nylander-French, 2012). Interception techniques have been developed to capture skin exposures during spray applications, however, these techniques have either not been replicated in a large study cohort (Thomasen *et al.*, 2011) or have not been utilized to measure HDI exposures (*i.e.*, both HDI monomer and its oligomers) (Blake *et al.*, 2012, Harari *et al.*, 2016, Bello *et al.*, 2019). [Note: from hereon, concomitant exposure to HDI monomer and its oligomers is referred to as “HDI exposure”.]

Biomonitoring of HDI exposure complements exposure assessment by conveying knowledge of how chemical mixtures, workplace safety measures, PPE use, as well as intra- and

inter-individual physical characteristics may modify exposures and subsequent systemic availability of the toxicant (Flack *et al.*, 2010a, Flack *et al.*, 2010b, Gaines *et al.*, 2010a). Biomonitoring of HDI exposure has been primarily limited to quantification of 1,6-diaminohexane (HDA), the hydrolysis product of HDI monomer, in urine and plasma as a marker for a short-term or cumulative exposure (Brorson *et al.*, 1990a, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Liu *et al.*, 2004, Creely *et al.*, 2006, Pronk *et al.*, 2006b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Jones *et al.*, 2013). Although significant associations between HDI monomer exposures and hydrolyzed HDA levels in urine and plasma have been observed in automotive spray-painters (Maitre *et al.*, 1996, Pronk *et al.*, 2006b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Gaines *et al.*, 2011), hydrolyzed HDA levels have not been found to be associated with HDI oligomer or total NCO exposures (Liu *et al.*, 2004). HDI isocyanurate-specific immunoglobulin E (IgE) and immunoglobulin G (IgG) in human serum have been identified in workers exposed to HDI (Campo *et al.*, 2007, Pronk *et al.*, 2007), however, neither were significantly associated with estimated HDI isocyanurate exposures (Pronk *et al.*, 2007).

Recently, we published a method to measure a biomarker of HDI isocyanurate exposure in urine (Robbins *et al.*, 2018). Because toxicokinetics of oligomeric isocyanates in humans may differ from the corresponding monomers, methods to monitor biomarkers of oligomer exposures are vital to understanding how oligomeric isocyanate exposures contribute to the development of adverse health effects observed in exposed workers. In this study, we analyzed levels of HDI isocyanurate biomarker, trisaminohexyl isocyanurate (TAHI), in urine and plasma of automotive spray-painters and investigated the associations between HDI isocyanurate exposure and these biomarker levels. Concurrently, we also investigated whether biomarkers of HDI isocyanurate

and HDI monomer exposures can be used interchangeably to estimate exposure to either compound.

4.2. Experimental

4.2.1. Study population and sample collection

Exposure assessment and biological sample collection was conducted at automotive refinishing shops with the participation of 47 spray-painters in North Carolina (n = 15) and Washington (n = 32) according to a protocol approved by the Institutional Review Boards at the University of North Carolina at Chapel Hill and Washington State (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a). Automotive refinishing shops were visited 1 to 3 times with a minimum of 3 weeks between visits for a total of 115 sampling visits (mean 2.4 visits per worker). Inhalation exposures to HDI monomer and HDI isocyanurate were monitored during 307 paint tasks (mean 2.7 tasks per worker per visit; range 1 – 8 tasks) by personal breathing-zone sampling. Skin exposures were monitored after 276 paint tasks (mean 2.4 tasks per worker per visit; range 1 – 5 tasks) by skin tape-stripping (Fent *et al.*, 2009a, Fent *et al.*, 2009b). Urine samples (N = 400; mean 3.5 samples per worker per visit; range 2 – 9 samples) were collected during the work-shift with at least one urine sample collected before the first paint task (Gaines *et al.*, 2010a). A blood sample was drawn near the end of the work-shift from each of the 46 workers who consented to blood biomarker analysis (N = 108). Blood samples were collected into separate tubes containing K_3EDTA or Li-Heparin anticoagulants (Flack *et al.*, 2010b). Inhalation and skin exposure measures of HDI monomer and HDI isocyanurate, urine and plasma HDA levels, and plasma TAHI levels in the whole study cohort and urine TAHI levels from workers in North Carolina have been published previously (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Robbins *et al.*, 2018). For this study, we also

analyzed urine TAHI levels in the workers from Washington State who were not part of the previously published method development study of HDI isocyanurate urine biomarker (Robbins *et al.*, 2018).

4.2.2. Sample treatment and quantitative analysis

Treatment for urine and plasma samples has been described previously (**Sections 2.2.4 and 3.2.4**) (Robbins *et al.*, 2018). Briefly, aliquots of urine and plasma (1 mL) were spiked with TAHpI (2.0 µg/L) and hydrolyzed with sulfuric acid (100 µL) by heating (100°C) for 16 h. Two milliliters of sodium hydroxide (25 M) was added and the samples were extracted with dichloromethane (3 x 2 mL). Each time dichloromethane was added, the sample was vortexed and then centrifuged at 1200 RCF for 20 min. Pooled dichloromethane extracts were derivatized with acetic anhydride (100 µL) and heated (55°C) for 16 h. The sample was washed with 4 mL monobasic potassium phosphate (1 M), vortexed, and then centrifuged at 500 RCF for 20 min. The dichloromethane layer (4 mL) was transferred then anhydrous sodium sulfate (500 mg) was added to remove remaining water. The sample was vortexed, centrifuged at 500 RCF for 10 min, the dichloromethane layer was transferred to a new vial and taken to dryness under nitrogen gas in a heated water bath (32°C). The dried sample was reconstituted with 0.1% formic acid in acetonitrile (200 µL), sonicated, and transferred to an autosampler vial and dried by vacuum centrifugation. The sample was then reconstituted with 0.1% formic acid in water (50 µL) for nano-UPLC-ESI-MS/MS analysis.

Preparation of standard curves with control urine and control plasma has been described previously (**Sections 2.2.6 and 3.2.5**) (Robbins *et al.*, 2018). Briefly, control urine and control plasma (1 mL) were spiked with internal standard TAHpI (2.0 µg/L) and with TAHI at the concentration ranges 0.06 – 7.99 µg/L and 0.03 – 3.99 µg/L, respectively. Standard curves were

generated using the TAAHI/TAAHpI instrument response ratio and were linearly correlated ($r \geq 0.995$). Weighting schemes for weighted linear regression of standard curves were chosen based on the lowest mean absolute percentage error (Almeida *et al.*, 2002), and subsequently, $w = y^{-2}$ was selected for urine and K_3EDTA plasma, and $w = x^{-2}$ was selected for Li-Heparin plasma. The method detection limit (MDL) was generated by spiking 10 control samples with the same level of the internal standard (2.0 µg/L) and the lowest standard used in calibration curves (0.06 µg/L for urine; 0.03 µg/L for plasma) in accordance with the procedure established by the U.S. EPA (U.S. EPA, 2016). The calculated MDLs for TAHI analysis in urine (**Section 2.2.6**) (Robbins *et al.*, 2018) and plasma were 0.03 µg/L and 0.02 µg/L (**Section 3.2.5**), respectively.

Urine and plasma samples were analyzed using nano-UPLC-ESI-MS/MS. Mass spectra were acquired on a TSQ Quantum Ultra triple-quadrupole mass spectrometer with a nano-electrospray ionization (nano-ESI) source (Thermo Scientific, Waltham, MA) coupled to a NanoAcquity UPLC system (nano-UPLC-ESI-MS/MS) (Waters Corp.). Reverse phase separations were carried out on a Waters' Symmetry C18 trapping column (5 µm, 180 µm × 20 mm) coupled to a Waters' Atlantis dC18 analytical column (3 µm, 100 µm × 100 mm). Mobile phase A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Samples were injected (2 µL) and trapped with 10 µL/min of 95% A for 1.5 min then eluted at 0.6 µL/min through the analytical column (35°C) with the gradient program 95% A to 10% A over 17 min. Precursor ions were generated by positive electrospray and detected in the selected reaction monitoring (SRM) mode. Three mass transitions were monitored for TAAHI (collision energies denoted in parentheses) in processed urine: *m/z* 553.3 → 494.4 (24 eV), *m/z* 553.3 → 212.1 (46 eV), and *m/z* 553.3 → 130.0 (52 eV). Two additional mass transitions for TAAHI were monitored in processed plasma [*m/z* 553.3 → 452.3 (32 eV) and *m/z*

553.3 → 393.3 (38 eV)] due to matrix effects and lower signal intensity for the three mass transitions monitored in urine. Three mass transitions were monitored for TAAHpI in processed urine and plasma: *m/z* 595.3 → 536.4 (24 eV), *m/z* 595.3 → 226.1 (45 eV), and *m/z* 595.3 → 130.0 (55 eV). For quantification of TAHI in processed urine, the signal intensities of TAAHI mass transitions *m/z* 553.3 → 494.4 and *m/z* 553.3 → 130.0 (52 eV) were summed and then divided by the summed intensities of all three TAAHpI mass transitions (Robbins *et al.*, 2018). For quantification of TAHI in processed plasma, the signal intensities of TAAHI mass transitions *m/z* 553.3 → 452.3 and *m/z* 553.3 → 393.3 were summed and then divided by the summed intensities of all three TAAHpI mass transitions. The TAAHI mass transitions that were not included in quantification were used as qualifiers.

4.2.3. Statistical analysis

Daily personal breathing-zone concentration (PBZ) was calculated as a time-weighted average (μg/m^3) using the sum of HDI monomer or HDI isocyanurate mass (μg) collected on air sampling filters from all tasks divided by the summation of paint time (min). Daily inhalation exposure (INH; μg) was calculated by multiplying PBZ by the summed paint time (min) and the average male breathing rate (0.0232 m^3/min) (Adams, 1993). PBZ and INH were also adjusted for respirator use by dividing by the OSHA assigned protection factor (APF) based on respirator type [none = 1; half-face negative-pressure air purifying = 10; full-face negative-pressure air purifying = 50; full-face powered air purifying (PAPR) = 1000; full-face continuous flow supplied-air = 1000] (OSHA, 2009). Daily skin exposure (μg) was calculated by summing the mass of HDI monomer or HDI isocyanurate collected on three consecutive tape strips (10 cm^2) applied to six different sites on the worker's body (*e.g.*, right and left forearms, hands, neck) after each paint task (Fent *et al.*, 2009b). When a paint task was performed after blood

withdrawal or after post-shift urine collection, those tasks were excluded from calculating daily inhalation (N = 20 tasks post-plasma; N = 7 tasks post-urine) and skin (N = 18 tasks post-plasma; N = 6 tasks post-urine) exposure measures.

Urine samples collected before the first paint task (N = 117) were included in descriptive statistics but were excluded for exposure-biomarker analysis because these biomarker levels reflect exposures received prior to the monitored spray-painting task and sampling visit. Daily mean urine levels were calculated by averaging urine samples without creatinine adjustment (µg/L) and with creatinine adjustment (µg/g creatinine). A total of 283 urine samples from 47 workers were available to calculate daily mean urine levels for 115 visits (mean 2.5 samples per worker per visit). Plasma levels (µg) were calculated by multiplying the plasma concentration (µg/L) by the plasma volume estimated using individual's estimated body surface area ($\text{Haycock BSA} = 0.024265 \times \text{height(cm)}^{0.3964} \times \text{weight(kg)}^{0.5378}$) (Hurley, 1975, Haycock *et al.*, 1978).

Data analysis was carried out using Microsoft Excel and SAS statistical software (SAS 9.4, SAS Institute Cary, NC). Prior to natural log-transformation, all exposure and biological samples measured below the MDL or limit of detection (LOD) were imputed to non-zero values using equations $(\text{MDL}/\sqrt{2})/100$ or $(\text{LOD}/\sqrt{2})/100$. The PROC UNIVARIATE, PROC CORR, PROC GLM, and PROC MIXED procedures and natural log-transformed exposure measures and biomarker levels were used in SAS analyses (SAS codes for each procedure can be found in Appendix B). The Shapiro-Wilks test ($W > 0.95$; PROC UNIVARIATE) for normality were statistically significant ($p < 0.05$) for natural log-transformed urine and plasma TAHI and HDA levels due to the large number of samples below the MDL. However, upon visual inspection, the

natural log-transformed data for non-zero urine and plasma TAHI and HDA levels appeared to be normally distributed.

Correlations between natural log-transformed exposure measures and biomarker levels were investigated by linear regression (PROC CORR) to calculate Pearson correlation coefficients (r) and p values. The associations between workplace factors (*e.g.*, respirator type, glove type, coverall use, booth type) and biomarker levels were evaluated by Tukey-Kramer multiple comparisons tests at α-level 0.05 (PROC GLM TUKEY). Workplace factors were combined to create dichotomous variables based on less protective and more protective groupings. Dichotomous respirator type was grouped as follows: 0 = no respirator or half-face air purifying; 1 = full-face air purifying, PAPR, or supplied-air. Dichotomous glove type was grouped as follows: 0 = no gloves or latex gloves; 1 = nitrile or neoprene gloves. Two workers during five visits wore gloves but the glove type was not recorded, consequently, these visits were included in glove use (0 = no gloves; 1 = gloves) but were excluded from dichotomous glove type analyses. Dichotomous booth type was grouped as follows: 0 = crossdraft or semi-downdraft booths; 1 = downdraft booth.

Multiple linear regression by linear mixed-effects modeling was used to investigate the relative influences of observed fixed effects (*e.g.*, exposure measures, creatinine level, workplace factors) and random effects associated with the i^{th} individual on the j^{th} visit on biomarker level. Mixed models were constructed (PROC MIXED) with compound symmetry as the covariance structure for repeated measurements. The general form of the mixed model used to investigate the influences of fixed and random effects on biomarker level was:

$$Y_{ij} = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \beta_3 X_{3ij} + \beta_4 X_{4ij} + \alpha_i + \varepsilon_{ij}$$

where Y_{ij} represents the natural logarithm of the urine biomarker level (with or without creatinine adjustment) or the plasma biomarker level for the i^{th} individual on the j^{th} visit, β_0 is the intercept, X_{1ij} represents the natural logarithm of the air exposure level (with or without APF adjustment), X_{2ij} represents the natural logarithm of the skin exposure level, X_{3ij} represents the natural logarithm of the creatinine level if it is included as an explanatory variable, X_{4ij} represents the workplace factors (*e.g.*, coverall use, respirator type), α_i represents the random effects associated with the i^{th} individual, and ε_{ij} represents the random errors associated with the j^{th} visit for the i^{th} individual. This approximate mixed model structure has been used previously to evaluate the associations between HDI monomer breathing-zone and skin exposures, urine HDA levels, and plasma HDA levels in this study population (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Gaines *et al.*, 2011).

With biomarker level as the response variable, base mixed models were constructed with air and skin exposure as explanatory variables. Initially, air exposure was included in base mixed models as a PBZ or INH variable, with or without APF adjustment (data not shown). Although PBZ and INH provided similar model fit statistics, PBZ was chosen for further base mixed model analyses because paint time could be included as an explanatory variable with PBZ or PBZ-APF in mixed models. Urine level as the response variable was modeled both with and without creatinine adjustment. A stepwise model selection was used by introducing continuous and categorical variables into base mixed models to estimate significance (p value cutoff < 0.10) and model fit by the Akaike's Information Criterion (AIC) and marginal R^2 statistic. We also evaluated model fit with the marginal R^2 statistic for the goodness-of-fit of fixed effects (Vonesh, 1997, Orelien and Edwards, 2008). The marginal R^2 statistic was determined by replicating the mixed model structure in the PROC GLM procedure.

4.3. Results

4.3.1. Biomarker levels in urine and plasma

Table 4.1 displays the descriptive statistics of the measured urine and plasma TAHI and HDA levels. TAHI was measured above the MDL in 127 of 400 (32%) urine samples in 35 of 47 (74%) workers and had a mean and standard deviation of 0.22 ± 0.92 µg/g creatinine. HDA was measured above the MDL in 252 of 400 (63%) urine samples in all 47 workers and had a mean and standard deviation of 0.29 ± 1.20 µg/g creatinine. The maximum urine level was 12.91 µg/g creatinine for TAHI and 21.58 µg/g creatinine for HDA. TAHI was measured in 19% of pre-shift urine samples, 37% of samples collected after the first task, and 32% of post-shift samples. By contrast, HDA was measured in 56% of pre-shift urine samples, 66% of samples collected after the first task, and 71% of post-shift samples.

TAHI was measured above the MDL in 24 of 108 (22%) plasma samples in 14 of 46 (30%) workers and had a mean and standard deviation of <MDL ± 0.14 µg. HDA was measured above the MDL in 80 of 108 (74%) plasma samples in 45 of 46 (98%) workers and had a mean and standard deviation of 0.35 ± 0.50 µg. The maximum plasma level was 1.12 µg for TAHI and 3.25 µg for HDA.

4.3.2. Linear regression analysis

Pearson correlation coefficients (r) and significance of the correlations (p value) between exposure measures and biomarker levels are shown in **Table 4.2**. Creatinine-adjusted urine TAHI levels were significantly correlated with both HDI isocyanurate PBZ ($r = 0.27$, $p = 0.0038$) and INH ($r = 0.34$, $p = 0.0002$), but not after APF adjustment (for both PBZ-APF and INH-APF $p \geq 0.1536$). To the contrary, APF-adjusted HDI monomer inhalation exposure levels were significantly correlated with creatinine-adjusted urine HDA levels (PBZ-APF $r = 0.23$, $p =$

0.0132 and INH-APF $r = 0.27$, $p = 0.0038$) while correlation with unadjusted INH exposure level was much weaker ($r = 0.18$, $p = 0.0485$) and PBZ was not significantly correlated ($r = 0.14$, $p = 0.1229$). HDI isocyanurate and HDI monomer skin exposures were significantly correlated with creatinine-adjusted urine TAHI ($r = 0.22$, $p = 0.0162$) and HDA ($r = 0.29$, $p = 0.0016$) levels, respectively.

Unlike for urine, plasma TAHI levels were not significantly correlated with either HDI isocyanurate PBZ or PBZ-APF (for both $p \geq 0.0824$). However, plasma TAHI levels were significantly correlated with all other HDI isocyanurate exposure measures (for all $p \leq 0.0223$) as well as paint time ($r = 0.43$, $p < 0.0001$). By contrast, none of HDI monomer exposure measures or paint time correlated with plasma HDA levels (for all $p \geq 0.1526$).

Table 4.1. Summary statistics of TAHI and HDA levels in urine (µg/g creatinine) collected pre-shift, after the first paint task, and post-shift as well as in plasma samples (µg) collected from automotive spray-painters.

	Workers		Samples		Urine (µg/g creatinine) and plasma (µg) levels				
	n	*n* > MDL (%)	*N*	*N* > MDL (%)	Range	Mean	SD	GM	GSD
Urine TAHI									
Pre-shift	47	16 (34%)	117	22 (19%)	<MDL – 1.57	0.08	0.22	<MDL	20.61
After first task	47	33 (70%)	283	105 (37%)	<MDL – 12.91	0.28	1.08	<MDL	42.76
Mean after first task	47	33 (70%)	115	51 (44%)	<MDL – 4.30	0.24	0.61	<MDL	22.69
Post-shift	47	25 (53%)	115	37 (32%)	<MDL – 2.95	0.18	0.44	<MDL	41.05
Total	47	35 (75%)	400	127 (32%)	<MDL – 12.91	0.22	0.92	<MDL	36.97
Plasma TAHI	46	14 (30%)	108	24 (22%)	<MDL – 1.12	<MDL	0.14	<MDL	12.53
Urine HDA									
Pre-shift	47	36 (77%)	117	66 (56%)	<MDL – 6.07	0.22	0.61	<MDL	29.85
After first task	47	47 (100%)	283	186 (66%)	<MDL – 21.58	0.32	1.37	<MDL	24.47
Mean after first task	47	45 (96%)	115	87 (76%)	<MDL – 11.16	0.34	1.12	<MDL	16.28
Post-shift	47	42 (89%)	115	82 (71%)	<MDL – 21.58	0.46	2.09	0.03	22.94
Total	47	47 (100%)	400	252 (63%)	<MDL – 21.58	0.29	1.20	<MDL	26.23
Plasma HDA	46	45 (98%)	108	80 (74%)	<MDL – 3.25	0.35	0.50	<MDL	25.05

n = number of workers; *N* = number of samples; MDL = method detection limit; Mean = arithmetic mean; SD = arithmetic standard deviation; GM = geometric mean; GSD = geometric standard deviation.

Table 4.2. Pearson correlations between HDI isocyanurate and HDI monomer exposure measures and levels of TAHI and HDA in urine and plasma.

HDI isocyanurate	**Exposure-Urine Correlations**						**Exposure-Plasma Correlations**					
	Exposure level		TAHI (μg/g)		HDA (μg/g)		Exposure level		TAHI (μg)		HDA (μg)	
Explanatory	GM	GSD	*r*	*p* value[a]	*r*	*p* value[a]	GM	GSD	*r*	*p* value[a]	*r*	*p* value[a]
PBZ (μg/m^3)	1969.2	2.9	0.27	**0.0038**	-0.07	0.4775	1801.4	4.6	0.12	0.2223	0.02	0.8784
PBZ-APF (μg/m^3)	65.3	9.4	0.05	0.5622	0.12	0.2210	62.6	11.4	0.17	0.0824	0.01	0.9450
INH (μg)	635.0	3.9	0.34	**0.0002**	0.02	0.8382	556.9	6.0	0.31	**0.0013**	0.07	0.4652
INH-APF (μg)	21.1	9.9	0.13	0.1536	0.15	0.0990	19.4	12.1	0.31	**0.0011**	0.05	0.6191
Skin (μg)	170.2	15.4	0.22	**0.0162**	0.26	**0.0045**	152.2	15.9	0.22	**0.0223**	0.09	0.3407
HDI monomer	**Exposure-Urine Correlations**						**Exposure-Plasma Correlations**					
	Exposure level		TAHI (μg/g)		HDA (μg/g)		Exposure level		TAHI (μg)		HDA (μg)	
Explanatory	GM	GSD	*r*	*p* value[a]	*r*	*p* value[a]	GM	GSD	*r*	*p* value[a]	*r*	*p* value[a]
PBZ (μg/m^3)	5.7	5.1	0.22	**0.0182**	0.14	0.1229	5.3	6.4	0.10	0.3271	0.09	0.3789
PBZ-APF (μg/m^3)	0.2	11.5	0.08	0.3876	0.23	**0.0132**	0.2	12.4	0.16	0.0958	0.06	0.5336
INH (μg)	1.9	6.1	0.30	**0.0011**	0.18	**0.0485**	1.6	7.5	0.27	**0.0049**	0.13	0.1791
INH-APF (μg)	0.1	11.8	0.16	0.0973	0.27	**0.0038**	0.1	12.5	0.31	**0.0013**	0.10	0.2954
Skin (μg)	0.005	331.7	0.18	0.0535	0.29	**0.0016**	0.003	337.3	0.21	**0.0285**	0.14	0.1526
Paint time (min)	13.9	2.4	0.22	**0.0194**	0.12	0.2191	13.3	2.4	0.43	**<0.0001**	0.12	0.2206

μg/g = μg/g creatinine; GM = geometric mean; GSD = geometric standard deviation; *r* = Pearson correlation coefficient ([a]significance was determined at α-level 0.05); PBZ = personal breathing-zone (μg/m^3); PBZ-APF = APF adjusted personal breathing-zone (μg/m^3); INH = inhalation (μg); INH-APF = APF adjusted inhalation (μg).

4.3.3. Influences of workplace factors

Summaries of TAHI and HDA biomarker data stratified twice by workplace factors and the significant associations determined by multiple comparisons tests are shown in **Tables 4.3 and 4.4**. The biomarker data were first stratified by booth type, respirator type, coverall use, or glove type, and then followed by a second stratification by these variables. Other workplace factors (*e.g.*, shop location, glove use, hat use, weekday) were also investigated but no significant associations were observed (data not shown). Creatinine-adjusted urine TAHI levels were significantly higher in workers who painted in crossdraft or semi-downdraft booths (mean and SD of 0.34 ± 0.55 μg/g creatinine) than workers who painted in downdraft booths (mean and SD of 0.19 ± 0.63 μg/g creatinine) before stratification by another workplace factor ($p = 0.0004$; data not shown). When urine biomarker data were stratified by booth type and then stratified a second time by workplace factors, painting in crossdraft or semi-downdraft booths remained significantly associated with increased creatinine-adjusted urine TAHI levels. For example, workers who painted in crossdraft or semi-downdraft booths and wore coveralls (TAHI > MDL in 65% of visits) had significantly higher creatinine-adjusted urine TAHI levels ($p = 0.0048$; **Table 4.3**) than workers who painted in downdraft booths and wore coveralls (TAHI > MDL in 35% of visits). Workers who painted in crossdraft or semi-downdraft booths also had significantly higher creatinine-adjusted urine TAHI levels than workers who painted in downdraft booths when coveralls were not worn, when no gloves or latex gloves were worn, when no respirator or half-face air purifying respirators were worn, and when full-face air purifying, PAPR, or supplied-air respirators were worn (for all $p \leq 0.0423$). Interestingly, no significant differences were observed in creatinine-adjusted urine HDA levels with any stratification of workplace factors.

Table 4.3. Associations between workplace factors and the mean creatinine-adjusted urine TAHI or HDA levels (µg/g creatinine) by stratification. General linear modeling was used to evaluate the significance of the compared variable in predicting urine biomarker levels given the indicated workplace condition.

Workplace Condition		Compared Variable	Compared Categories	*N*	TAHI (µg/g creatinine)		HDA (µg/g creatinine)	
					N > MDL (%)	*p* value[a]	*N* > MDL (%)	*p* value[a]
Booth type	Cross/Semi	Respirator	None/Half-face	24	14 (58%)	0.5313	22 (92%)	0.4749
			Full-face[b]	13	9 (69%)		10 (77%)	
Booth type	Downdraft	Respirator	None/Half-face	61	23 (38%)	0.7842	45 (74%)	0.1563
			Full-face[b]	17	5 (29%)		10 (59%)	
Respirator	None/Half-face	Glove type	None/Latex	50	24 (48%)	0.1670	41 (82%)	0.9191
			Nitrile/Neoprene	30	11 (37%)		22 (73%)	
Respirator	Full-face[b]	Glove type	None/Latex	14	7 (50%)	0.8687	10 (71%)	0.9251
			Nitrile/Neoprene	16	7 (44%)		10 (63%)	
Coverall	No	Glove type	None/Latex	28	14 (50%)	0.5699	25 (89%)	0.9094
			Nitrile/Neoprene	5	2 (40%)		4 (80%)	
Coverall	Yes	Glove type	None/Latex	36	17 (47%)	0.4191	26 (72%)	0.7100
			Nitrile/Neoprene	41	16 (39%)		28 (68%)	
Respirator	None/Half-face	Booth type	Cross/Semi	24	14 (58%)	**0.0113**	22 (92%)	0.1946
			Downdraft	61	23 (38%)		45 (74%)	
Respirator	Full-face[b]	Booth type	Cross/Semi	13	9 (69%)	**0.0191**	10 (77%)	0.2016
			Downdraft	17	5 (29%)		10 (59%)	
Coverall	No	Booth type	Cross/Semi	17	10 (59%)	**0.0423**	15 (88%)	0.9199
			Downdraft	21	8 (38%)		18 (86%)	
Coverall	Yes	Booth type	Cross/Semi	20	13 (65%)	**0.0048**	17 (85%)	0.0902
			Downdraft	57	20 (35%)		37 (65%)	
Glove type	None/Latex	Booth type	Cross/Semi	22	15 (68%)	**0.0040**	20 (91%)	0.2601
			Downdraft	42	16 (38%)		31 (74%)	
Glove type	Nitrile/Neoprene	Booth type	Cross/Semi	15	8 (53%)	0.0707	12 (80%)	0.2096
			Downdraft	31	10 (32%)		20 (65%)	

N = number of samples (mean urine level by visit); MDL = method detection limit; [a]Significance was determined by Tukey-Kramer multiple comparisons tests at α-level 0.05; [b]Full-face variable includes full-face air purifying, PAPR, or supplied-air respirators.

Table 4.4. Associations between workplace factors and the plasma TAHI and HDA levels (µg) by stratification. General linear modeling was used to evaluate the significance of the compared variable in predicting plasma biomarker levels given the indicated workplace condition.

Workplace Condition		Compared Variable	Compared Categories	*N*	TAHI (µg)		HDA (µg)	
					N > MDL (%)	*p* value[a]	*N* > MDL (%)	*p* value[a]
Booth type	Cross/Semi	Respirator	None/Half-face	22	5 (23%)	0.8059	19 (86%)	0.7039
			Full-face[b]	12	3 (25%)		10 (83%)	
Booth type	Downdraft	Respirator	None/Half-face	59	16 (27%)	**0.0211**	42 (71%)	0.5436
			Full-face[b]	15	0 (0%)		9 (60%)	
Respirator	None/Half-face	Glove type	None/Latex	46	12 (26%)	0.4351	37 (80%)	0.2708
			Nitrile/Neoprene	30	6 (20%)		21 (70%)	
Respirator	Full-face[b]	Glove type	None/Latex	11	3 (27%)	**0.0365**	7 (64%)	0.5196
			Nitrile/Neoprene	16	0 (0%)		12 (75%)	
Coverall	No	Glove type	None/Latex	25	5 (20%)	0.9956	20 (80%)	0.8367
			Nitrile/Neoprene	5	1 (20%)		4 (80%)	
Coverall	Yes	Glove type	None/Latex	32	10 (31%)	**0.0284**	24 (75%)	0.8408
			Nitrile/Neoprene	41	5 (12%)		29 (71%)	
Respirator	None/Half-face	Booth type	Cross/Semi	22	5 (23%)	0.8130	19 (86%)	0.1377
			Downdraft	59	16 (27%)		42 (71%)	
Respirator	Full-face[b]	Booth type	Cross/Semi	12	3 (25%)	**0.0320**	10 (83%)	0.1284
			Downdraft	15	0 (0%)		9 (60%)	
Coverall	No	Booth type	Cross/Semi	15	2 (13%)	0.1428	12 (80%)	0.8905
			Downdraft	20	7 (35%)		15 (75%)	
Coverall	Yes	Booth type	Cross/Semi	19	6 (32%)	0.0907	17 (89%)	**0.0153**
			Downdraft	54	9 (17%)		36 (67%)	
Glove type	None/Latex	Booth type	Cross/Semi	19	5 (26%)	0.7918	16 (84%)	0.3106
			Downdraft	38	10 (26%)		28 (74%)	
Glove type	Nitrile/Neoprene	Booth type	Cross/Semi	15	3 (20%)	0.2318	13 (87%)	0.0563
			Downdraft	31	3 (10%)		20 (65%)	

N = number of samples; MDL = method detection limit; [a]Significance was determined by Tukey-Kramer multiple comparisons tests at α-level 0.05; [b]Full-face variable includes full-face air purifying, PAPR, or supplied-air respirators.

The results obtained for TAHI levels in plasma are less clear than for urine and most likely because TAHI was detected in fewer plasma samples. TAHI was measured above the MDL in 16 of 59 (27%) plasma samples collected from workers who painted in downdraft booths and wore half-face air purifying respirators, while TAHI was not detected in any of the plasma samples ($N = 15$) collected from workers who painted in downdraft booths and wore full-face air purifying, PAPR, or supplied-air respirators ($p = 0.0211$; **Table 4.4**). Workers who did not wear gloves or who wore latex gloves had significantly higher plasma TAHI levels than workers who wore nitrile or neoprene gloves when coveralls were worn ($p = 0.0284$), or when full-face air purifying, PAPR, or supplied-air respirators were worn ($p = 0.0365$).

Stratification of plasma biomarker data by booth type and then a second stratification by respirator type, coverall use, or glove type did not clearly indicate that painting in crossdraft or semi-downdraft booths would lead to higher plasma TAHI or HDA levels (**Table 4.4**). Workers who wore full-face air purifying, PAPR, or supplied-air respirators and painted in crossdraft or semi-downdraft booths had significantly higher plasma TAHI levels ($p = 0.0320$) than workers with similar respirator protection and who painted in downdraft booths. However, no significant difference was observed in plasma TAHI levels between booth types when workers wore no respirator or half-face air purifying respirators ($p = 0.8130$), or after both stratifications of coverall use and glove type (for all $p \geq 0.0907$). Significantly higher plasma HDA levels ($p = 0.0153$) were only observed in workers who painted in crossdraft or semi-downdraft booths compared to those who painted in downdraft booths when coveralls were worn. For any other stratification of workplace factors, plasma HDA levels were not significantly different (for all $p \geq 0.0563$).

4.3.4. Linear mixed models

The results from the linear mixed models for urine TAHI and HDA levels are displayed in **Table 4.5**. The final model (Model 1-C) for urine TAHI levels included HDI isocyanurate PBZ ($p = 0.0123$) and booth type ($p = 0.0068$) as significant variables and paint time ($p = 0.0591$) as a borderline significant variable with model fit statistics AIC = 554.9 and marginal $R^2 = 0.21$. It is noteworthy that HDI isocyanurate skin exposure or creatinine level were not significant predictors of TAHI levels in either base model ($p \geq 0.0712$ and $p \geq 0.8220$, respectively). HDI isocyanurate skin exposure remained non-significant when paint time and booth type were added to Model 1-A, and therefore, skin exposure was removed from the final model (Model 1-C). Respirator type was not significant when it was added to Model 1-A (data not shown), which was in agreement with the non-significance of HDI isocyanurate PBZ-APF in Model 1-B ($p = 0.6833$). Other workplace factors were also introduced to Models 1-A and 1-B but were observed to be non-significant (data not shown).

The final model (Model 1-F) for urine HDA levels included creatinine level ($p < 0.0001$) and HDI monomer skin exposure ($p = 0.0344$) as significant variables and respirator type ($p = 0.0961$) as a borderline significant variable with model fit statistics AIC = 541.7 and marginal $R^2 = 0.29$ (**Table 4.5**). HDI monomer PBZ-APF was a significant predictor in Model 1-E ($p = 0.0237$). HDI monomer PBZ was not a significant predictor of urine HDA levels when respirator type was added to Model 1-D ($p = 0.3944$; data not shown) and, therefore, was removed from the final model (Model 1-F). Paint time and other workplace factors were also introduced to Models 1-D and 1-E but were non-significant (data not shown).

Table 4.6 displays the linear mixed model results for plasma TAHI and HDA levels. The final model (Model 2-C) for plasma TAHI levels included paint time ($p = 0.0019$) as a

significant variable and HDI isocyanurate PBZ-APF ($p = 0.0676$) as a borderline significant variable with model fit statistics AIC = 464.1 and marginal $R^2 = 0.30$. HDI isocyanurate exposure measures were not significant predictors of plasma TAHI levels in the base models (Model 2-A $p = 0.4209$; Model 2-B $p = 0.2893$). Workplace factors were added to both base models but were not observed to be significant predictors of plasma TAHI levels (data not shown).

HDI monomer skin exposure was a borderline significant predictor of plasma HDA levels in all three models (for all $p \leq 0.0871$; **Table 4.6**). HDI monomer PBZ and PBZ-APF were not significant predictors of plasma HDA levels (for both $p \geq 0.8026$) even when additional variables were included in the models (data not shown), and therefore, was removed from the final model (Model 2-F). Paint time and workplace factors were added to both base models but were not observed to be significant predictors of plasma HDA levels (data not shown). The final model for plasma HDA levels only included HDI monomer skin exposure as a borderline significant variable (Model 2-F, AIC = 551.0, marginal $R^2 = 0.05$), however, this model did not have better model fit statistics than either of the base models.

Table 4.5. Summary of linear mixed models for predicting mean urine TAHI and HDA levels (µg/L).

TAHI level	Explanatory	Estimate	Standard Error	*p* value	AIC	R^2	HDA level	Explanatory	Estimate	Standard Error	*p* value	AIC	R^2
Base	Intercept	-11.33	3.47	0.0021	561.7	0.07	Base	Intercept	-13.39	2.30	<0.0001	546.4	0.28
Model 1-A	ISO PBZ	0.58	0.29	**0.0467**			Model 1-D	HDI PBZ	0.15	0.17	0.3944		
	ISO Skin	0.13	0.11	0.2262				HDI Skin	0.09	0.05	0.0888		
	Creatinine	0.11	0.49	0.8220				Creatinine	2.08	0.44	**<0.0001**		
	Worker var	3.89	1.40	0.0054				Worker var	1.68	0.91	0.0659		
	Residual var	5.16	0.91	<0.0001				Residual var	5.29	0.92	<0.0001		
Base	Intercept	-6.70	2.67	0.0154	566.8	0.05	Base	Intercept	-13.43	2.14	<0.0001	542.7	0.29
Model 1-B	ISO PBZ-APF	0.06	0.16	0.6833			Model 1-E	HDI PBZ-APF	0.27	0.12	**0.0237**		
	ISO Skin	0.20	0.11	0.0712				HDI Skin	0.06	0.05	0.1737		
	Creatinine	-0.06	0.49	0.8962				Creatinine	2.21	0.43	**<0.0001**		
	Worker var	4.08	1.47	0.0055				Worker var	1.63	0.87	0.0612		
	Residual var	5.32	0.94	<0.0001				Residual var	5.07	0.87	<0.0001		
Final	Intercept	-11.07	2.24	<0.0001	554.9	0.21	Final	Intercept	-12.88	2.15	<0.0001	541.7	0.29
Model 1-C	ISO PBZ	0.65	0.25	**0.0123**			Model 1-F	HDI Skin	0.10	0.04	**0.0344**		
	Paint time	0.66	0.34	0.0591				Creatinine	2.10	0.43	**<0.0001**		
	Booth type	-1.95	0.69	**0.0068**				Respirator type	-1.12	0.66	0.0961		
	Worker var	2.58	1.19	0.0293				Worker var	1.74	0.91	0.0546		
	Residual var	5.29	0.94	<0.0001				Residual var	5.13	0.88	<0.0001		

AIC = Akaike's Information Criterion; R^2 = marginal R^2 statistic calculated with the PROC GLM procedure in SAS; ISO = HDI isocyanurate; HDI = HDI monomer; var = variance.

Table 4.6. Summary of linear mixed models for predicting plasma TAHI and HDA levels (µg).

TAHI level	Explanatory	Estimate	Standard Error	*p* value	AIC	R^2	HDA level	Explanatory	Estimate	Standard Error	*p* value	AIC	R^2
Base	Intercept	-7.71	1.08	<0.0001	468.4	0.09	Base	Intercept	-2.55	0.60	0.0001	547.7	0.05
Model 2-A	ISO PBZ	0.12	0.15	0.4209			Model 2-D	HDI PBZ	0.04	0.18	0.8026		
	ISO Skin	0.05	0.08	0.5622				HDI Skin	0.10	0.06	0.0871		
	Worker var	3.55	1.01	0.0004				Worker var	-1.34	1.02	0.1915		
	Residual var	2.61	0.47	<0.0001				Residual var	11.53	2.02	<0.0001		
Base	Intercept	-7.28	0.60	<0.0001	468.5	0.10	Base	Intercept	-2.42	0.41	<0.0001	548.5	0.05
Model 2-B	ISO PBZ-APF	0.12	0.11	0.2893			Model 2-E	HDI PBZ-APF	0.03	0.12	0.8226		
	ISO Skin	0.04	0.08	0.6058				HDI Skin	0.10	0.05	0.0712		
	Worker var	3.51	1.00	0.0005				Worker var	-1.29	1.03	0.2129		
	Residual var	2.60	0.47	<0.0001				Residual var	11.47	2.01	<0.0001		
Final	Intercept	-9.69	0.92	<0.0001	464.1	0.30	Final	Intercept	-2.45	0.40	<0.0001	551.0	0.05
Model 2-C	ISO PBZ-APF	0.18	0.10	0.0676			Model 2-F	HDI Skin	0.10	0.05	0.0528		
	Paint time	0.93	0.29	**0.0019**				Worker var	-1.19	0.99	0.2301		
	Worker var	2.45	0.80	0.0023				Residual var	11.24	1.94	<0.0001		
	Residual var	2.64	0.47	<0.0001									

AIC = Akaike's Information Criterion; R^2 = marginal R^2 statistic calculated with the PROC GLM procedure in SAS; ISO = HDI isocyanurate; HDI = HDI monomer; var = variance.

4.4. Discussion

Even though significant levels of inhalation and skin exposure to HDI isocyanurate have been measured concomitantly with HDI monomer exposure, validation of biomarkers of HDI isocyanurate exposure are lacking. Here, we observed that urine and plasma biomarkers of HDI isocyanurate exposures are readily detectable in automotive spray-painters. HDI isocyanurate inhalation exposure appears to be a significant source of TAHI in urine and plasma (**Tables 4.2, 4.5, and 4.6**). Significant correlations were observed between HDI isocyanurate skin exposure and TAHI levels in urine (creatinine-adjusted $p = 0.0162$) and plasma ($p = 0.0223$; **Table 4.2**) but, interestingly, skin exposure did not significantly predict TAHI levels in urine or plasma in the mixed model analyses (**Tables 4.5 and 4.6**). However, in concordance with our earlier reported findings (Gaines *et al.*, 2010a, Gaines *et al.*, 2011), HDI monomer skin exposure was significantly correlated with urine HDA levels (creatinine-adjusted $p = 0.0016$; **Table 4.2**) and was also a significant predictor of urine HDA levels in the mixed model analyses ($p = 0.0344$; **Table 4.5**). It is noteworthy that creatinine level, which was a highly significant predictor for urine HDA levels in this study ($p < 0.0001$; **Table 4.5**) as also reported in other studies (Gaines *et al.*, 2010a, Gaines *et al.*, 2011), was not a significant predictor of urine TAHI levels.

In this cohort, some type of respirator was used by every worker except one. Among workers who wore half-face air purifying respirators, the most commonly worn respirator in this study cohort, urine TAHI and HDA levels were measured above the MDL during 72% and 75% of the visits, respectively. Adjustment for the OSHA APF value has been used to estimate HDI exposure from breathing-zone measurements because it is not feasible to measure inhalation exposure due to respirator occlusion and intra- and inter-individual differences in breathing rate and pulmonary absorption (Liu *et al.*, 2006, OSHA, 2009). Unpredictably, we observed that APF

adjustment did not affect associations with urine and plasma TAHI and HDA levels in the same fashion. APF adjustment of HDI isocyanurate inhalation exposures weakened associations with creatinine-adjusted urine TAHI levels but strengthened associations between HDI monomer inhalation exposures and creatinine-adjusted urine HDA levels (**Table 4.2**). Previously, Gaines *et al.* reported that APF adjustment improved the associations between HDI monomer PBZ and creatinine-adjusted urine HDA levels (discrete spot sample concentration and average concentration between-task) in this same study cohort (Gaines *et al.*, 2010a, Gaines *et al.*, 2011). Additionally, respirator type was significantly associated with pooled between-tasks urine HDA levels (Gaines *et al.*, 2011). These trends were supported by the results observed in the mixed model analyses where HDI isocyanurate PBZ was a significant predictor of urine TAHI levels and HDI monomer PBZ-APF was a significant predictor of urine HDA levels (**Table 4.5**). INH and INH-APF variables were more strongly correlated with creatinine-adjusted urine TAHI and HDA levels than PBZ and PBZ-APF variables (**Table 4.2**), indicating that duration of exposure may be more consequential for systemic availability than high short-term airborne concentrations. This is also supported by the significant correlation of paint time with creatinine-adjusted urine TAHI levels ($p = 0.0194$; **Table 4.2**) and its borderline significance observed in the mixed model analyses ($p = 0.0591$; **Table 4.5**).

Our results clearly show that painting in downdraft booths is significantly associated with lower urine TAHI levels compared to painting in crossdraft or semi-downdraft booths, which is supported by previous studies that demonstrated downdraft booths significantly reduced HDI isocyanurate inhalation and skin exposures (Fent *et al.*, 2009a, Fent *et al.*, 2009b). Creatinine-adjusted urine TAHI levels were significantly higher in workers who painted in crossdraft or semi-downdraft booths than in workers who painted in downdraft booths after urine biomarker

data were stratified by respirator type and coverall use, and when workers wore no gloves or wore latex gloves (for all $p \leq 0.0423$; **Table 4.3**). Additionally, booth type was a significant predictor of urine TAHI levels by mixed model analyses ($p = 0.0068$; **Table 4.5**). We previously reported that booth type was significantly associated with HDA levels in pooled urine samples (*i.e.*, spot urine samples collected during the work-shift), but not with HDA levels in single post-shift urine samples (Gaines *et al.*, 2011). The calculated geometric mean urine HDA level (both unadjusted and creatinine-adjusted) was not significantly associated with booth type after urine biomarker data were stratified by respirator type, coverall use, or glove type (for all $p \geq 0.0902$; **Table 4.3**) or in mixed model analyses (data not shown). Based on these results, painting in downdraft booths significantly reduces exposure and urine TAHI levels, but it is unclear whether reduced HDI monomer exposures from painting in downdraft booths is associated with lower urine HDA levels.

The differences between the levels of TAHI and HDA measured above the MDL (**Table 4.1**) was more pronounced in plasma samples (22% and 74%, respectively) than in urine samples (32% and 63%, respectively) despite equivalent MDLs for the analytical protocols (Flack *et al.*, 2010b). Although HDI monomer skin exposure was a significant predictor of plasma HDA levels, the AIC (range 547.7 – 551.0) and marginal R^2 (0.05) indicated weaker model fit than for plasma TAHI levels (AIC range 464.1 – 468.5 and marginal R^2 range 0.09 – 0.30) in mixed model analyses (**Table 4.6**). The weak associations between plasma HDA levels and HDI monomer exposures may be a result of significant covalent binding of HDI monomer and/or partially hydrolyzed HDA to albumin and other long-lived macromolecules in plasma (Wisnewski *et al.*, 2000, Flack *et al.*, 2010b, Wisnewski *et al.*, 2013). Thus, plasma HDA levels may not be a suitable biomarker of same-day HDI monomer exposure due to significant

contributions from past HDI monomer exposures to hydrolyzed HDA levels. By contrast, plasma TAHI levels were significantly associated with HDI isocyanurate inhalation exposures in this cohort (**Table 4.2**), suggesting that HDI isocyanurate remain unreacted longer (*i.e.*, not readily available for binding with macromolecules) or is hydrolyzed more readily than HDI monomer in plasma.

The results indicate that half-face air purifying respirators did not adequately reduce HDI isocyanurate inhalation exposures in this study cohort, which led to increased levels of TAHI in plasma. Among workers who painted in downdraft booths, TAHI was measured above the MDL in 27% of plasma samples collected from workers wearing half-face air purifying respirators while no TAHI was detected in the plasma samples collected from workers wearing full-face air purifying, PAPR, or supplied-air respirators ($p = 0.0211$; **Table 4.4**). On the other hand, HDI isocyanurate PBZ with APF adjustment was borderline significantly associated with plasma TAHI levels in linear regression ($p = 0.0824$; **Table 4.2**) and in mixed model analyses ($p = 0.0676$; **Table 4.6**). Because paint time was significantly associated with plasma TAHI levels in linear regression ($p < 0.0001$; **Table 4.2**) and mixed model analyses ($p = 0.0019$; **Table 4.6**), it is probable that the protection provided by a half-face air purifying respirator decreases with a longer paint task, thus, leading to increased exposure and plasma TAHI levels.

We observed that wearing no gloves or wearing latex gloves was significantly associated with increased plasma TAHI levels when workers wore coveralls ($p = 0.0284$; **Table 4.4**). This is in accordance with previous studies in which little to no permeation of HDI isocyanurate through gloves or coveralls was reported (Ceballos *et al.*, 2014b, Mellette *et al.*, 2019). The relationships between booth type and plasma TAHI levels are more difficult to interpret due to the low prevalence of TAHI above the MDL concentration, which likely contributed to the lack of

associations observed for booth type after plasma biomarker data was stratified by coverall use and glove type (for all $p \geq 0.0907$), and for workers who wore no respirators or wore half-face air purifying respirators ($p = 0.8130$; **Table 4.4**).

Evaluation of HDA as a biomarker of HDI isocyanurate exposures has been largely ignored and associations between HDI isocyanurate exposures and urine or plasma HDA levels have not been previously reported. Significant associations between HDI monomer inhalation or skin exposures and urine or plasma HDA levels have been reported in numerous studies (Brorson *et al.*, 1990a, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Liu *et al.*, 2004, Pronk *et al.*, 2006b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Budnik *et al.*, 2011, Gaines *et al.*, 2011). Urine HDA levels were not significantly associated with HDI oligomer exposures in a controlled inhalation study (Liu *et al.*, 2004). Our linear regression analyses indicate that urine HDA level is not a suitable biomarker of HDI isocyanurate inhalation exposures (for all $p \geq 0.0990$; **Table 4.2**), and plasma HDA level is not a suitable biomarker of HDI isocyanurate inhalation or skin exposures (for all $p \geq 0.3407$). By contrast, urine TAHI levels were significantly correlated with HDI monomer inhalation exposures without APF adjustment (PBZ $p = 0.0182$ and INH $p = 0.0011$). Additionally, plasma TAHI levels were significantly correlated with HDI monomer inhalation (paint time-adjusted) and skin exposures (for all three $p \leq 0.0285$). These results show TAHI levels in urine and plasma are stronger biomarkers of HDI exposures than HDA biomarker levels.

The following issues that may have affected the measured biomarker levels are important to acknowledge. Previous studies have shown that when half- or full-face air purifying respirators are worn, the presence of facial hair decreases the respirator fit factor resulting in leakage (Skretvedt and Loschiavo, 1984, Stobbe *et al.*, 1988, Floyd *et al.*, 2018). We did not

record facial hair status for this study but did observe that workers were not always clean-shaven, and some had beards. It is unclear how respirator leakage may have affected inhalation exposures in this study because HDI isocyanurate is predominantly in the aerosol phase and HDI monomer partitions between the vapor and aerosol phases (Bello *et al.*, 2004). Bello *et al.* sampled skin surfaces occluded by half-face air purifying respirators after painting and detected HDI oligomers in 80% of samples and HDI monomer in 30% of samples (Bello *et al.*, 2008). The differences in volatility and phase between HDI isocyanurate and HDI monomer and the analyses presented in this paper suggest APF does not similarly adjust inhalation exposures to HDI isocyanurate and HDI monomer. It would be prudent in future surveys to record facial hair status to investigate its associations with biomarker levels when half- or full-face air purifying respirators are worn.

Tape-strip sampling utilized in this study may have underestimated HDI isocyanurate and HDI monomer skin exposures received during spray-painting (Fent *et al.*, 2009b, Thomasen *et al.*, 2011). Two previous studies of HDI penetration rates into excised skin demonstrated 10 – 25% of topical HDI isocyanurate or HDI monomer doses were absorbed during short and long exposures (Bello *et al.*, 2006, Thomasen and Nylander-French, 2012). These permeation studies suggest that a longer task would lead to higher absorptions of HDI isocyanurate and HDI monomer into layers of the skin beyond what is measured by tape-strips before skin exposure sampling could be performed, resulting in an underestimation of the worker's exposure. Contact with surfaces may be another source of skin exposure missed by tape-strip sampling. Unreacted HDI oligomers have been found on surfaces hours after painting, and workers are less likely to wear coveralls and gloves when performing other tasks (Pronk *et al.*, 2006b, Bello *et al.*, 2007b, Liu *et al.*, 2007, Bello *et al.*, 2008). However, skin exposures from direct contact with unreacted

HDI species on surfaces was minimal in a study of 18 workers in 5 auto body shops (De Vries *et al.*, 2012). Tape-strip sampling may underestimate HDI isocyanurate skin absorption during spray-painting, but it is uncertain whether exposures from unknown sources substantively contribute to overall dose. Underestimation of skin exposure by tape-strip sampling may partially explain weaker associations between HDI isocyanurate skin exposure and TAHI biomarker levels if significant levels of HDI isocyanurate were absorbed during spray-painting.

4.5. Conclusions

This study provides evidence that levels of hydrolyzed TAHI in urine and plasma are important biomarkers of HDI isocyanurate inhalation and skin exposures in the automotive refinishing industry. Although TAHI was measured in 32% of urine samples and 22% of plasma samples collected from occupationally exposed workers, exposures and workplace factors (*e.g.*, booth type, glove type) were more strongly associated with TAHI biomarker levels than with HDA biomarker levels. HDI isocyanurate inhalation exposure and paint time were significantly associated with urine and plasma TAHI levels. The results indicate that painting in crossdraft or semi-downdraft booths is significantly associated with increased TAHI levels in urine, stressing the importance of utilizing downdraft booths to reduce HDI isocyanurate exposures in the automotive refinishing industry. Additionally, wearing full-face air purifying, PAPR, or supplied-air respirators was significantly associated with decreased plasma TAHI levels indicating that half-face air purifying respirators may not adequately protect against HDI isocyanurate inhalation exposures. TAHI was detected in fewer urine and plasma samples than HDA despite higher exposures to HDI isocyanurate. The metabolism and excretion of HDI isocyanurate may be longer than HDI monomer. This study was designed to obtain optimal exposure and biomarker data for HDI monomer as no information existed for HDI isocyanurate,

and therefore, the optimal sample collection time for TAHI may have been somewhat missed in this study. Future exposure assessments in other exposed populations are warranted to better understand the relationships between short-term and/or cumulative HDI isocyanurate exposures and the associated biomarkers. In summary, the results obtained in this study confirm TAHI as a biomarker of HDI isocyanurate exposure and may eventually prove to be a stronger and more important biomarker than HDA for monitoring HDI exposures in the automotive refinishing industry.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

This dissertation includes three related manuscripts (Chapters 2 – 4) focused on the identification and quantification of hydrolyzed TAHI as a biomarker of HDI isocyanurate exposure. The sample treatment and analytical methods developed to quantify hydrolyzed TAHI in urine and plasma are described in Chapters 2 and 3. Chapter 2 details the synthesis and verification of analytical standards TAHI, TAAHI, TAHpI, and TAAHpI that were subsequently used for the development of a sensitive and specific sample treatment and analytical method. Hydrolyzed TAHI was quantified in urine of 15 spray-painters from North Carolina to validate the method. Chapter 3 details the adaptation of the sample treatment and analytical method to extract and analyze hydrolyzed TAHI in plasma of 46 spray-painters from North Carolina and Washington State. Because no changes were required to the sample treatment method, the work focused on the modification of the nano-UPLC-ESI-MS/MS method in order to improve sensitivity and specificity for quantification of hydrolyzed TAHI. Chapter 4 incorporates the levels of hydrolyzed TAHI measured in urine and plasma (as reported in Chapters 2 and 3) and previously published exposure and biomarker data for this worker population (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a) to investigate relationships between inhalation and skin exposures and biomarker levels in exposed workers.

The following sections outline the biomarker analysis (**Section 5.1**), the relationships between exposures, workplace factors, and biomarker levels (**Section 5.2**), urine levels compared to recommended limit values (**Section 5.3**), limitations of the biomarker analysis and study design (**Section 5.4**), and how these findings could translate for future research of HDI oligomer exposures and oligomeric isocyanate exposures in other occupations (**Section 5.5**).

5.1. Quantification of HDI isocyanurate biomarkers in urine and plasma

The significant and unique contribution of this research to the exposure science is the development of a sample treatment and analytical method to quantify TAHI in hydrolyzed urine and plasma samples as a biomarker of HDI isocyanurate exposures. The hazards associated with HDI monomer and oligomer exposures in occupational settings have been known for decades (NIOSH, 1978, Bernstein, 1996, NIOSH, 1996, Bello *et al.*, 2004), yet, exposure assessment and biomonitoring efforts have only focused on measurement of diisocyanate monomers and corresponding biomarkers.

HDI biomarker analysis has mostly been limited to metabolites of HDI monomer (free or conjugated) in urine or plasma (Brorson *et al.*, 1990a, Dalene *et al.*, 1990, Tinnerberg *et al.*, 1995, Maitre *et al.*, 1996, Williams *et al.*, 1999, Liu *et al.*, 2004, Creely *et al.*, 2006, Pronk *et al.*, 2006b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Jones *et al.*, 2013). In addition, HDI biuret- and HDI isocyanurate-specific IgE and IgG in human serum have been identified (Campo *et al.*, 2007, Pronk *et al.*, 2007), but attempts to relate HDI isocyanurate-specific IgE and IgG levels to HDI isocyanurate exposure levels in exposed individuals have been unsuccessful (Pronk *et al.*, 2007). This research demonstrates that biomarkers of HDI isocyanurate inhalation and skin exposures are measurable in hydrolyzed urine and plasma and is also the first study to identify biomarkers of oligomeric isocyanate exposures in urine or plasma.

Sample treatment methods from prior analysis of hydrolyzed HDA in urine and plasma (Flack *et al.*, 2010b, Gaines *et al.*, 2010a) were successfully adapted to extract and derivatize hydrolyzed TAHI using dichloromethane and acetic anhydride (Robbins *et al.*, 2018). The traditional methods of derivatizing HDA with polyfluorinated acid anhydrides proved difficult or non-applicable for analysis of TAHI derivatives by GC-MS. The reaction with HFBA creates semi-volatile and/or non-volatile products that are not suitable for GC-MS analysis. Although PFPA has been used in previous studies to derivatize HDA for GC-MS analysis (Flack *et al.*, 2010a), this reaction was not tested since it was presumed that the reaction would also create semi-volatile and/or non-volatile products.

Acetic anhydride was chosen as the derivatizing chemical, forming secondary amides by reaction with TAHI (TAAHI product) and TAHpI (TAAHpI product). The secondary amide products could not be volatilized for GC-MS analysis. On the other hand, since secondary amides are readily protonated, they are appropriate analytes for positive ESI for LC-MS analysis. The sample treatment and nano-UPLC-ESI-MS/MS analytical method that was developed to analyze the derivative TAAHI from extracted urine and plasma was as sensitive (urine MDL = 0.03 µg/L; plasma MDL = 0.02 µg/L) as recent analytical methods used to quantify HDA-HFBA in urine (MDL = 0.04 µg/L) and plasma (MDL = 0.02 µg/L) (Flack *et al.*, 2010b, Gaines *et al.*, 2010a, Robbins *et al.*, 2018). The combination of acetic anhydride derivatization and nano-UPLC-ESI-MS/MS analysis may have widespread applicability to monitor other potential biomarkers of oligomeric isocyanate exposures in urine and plasma. However, there are limitations and improvements to the sample treatment and analytical method that need to be addressed before it can be adapted for other toxicants of interest. These issues are discussed in **Section 5.4.1**.

5.2. HDI isocyanurate exposure and biomarker levels in exposed workers

5.2.1. The effects of exposures and workplace factors on biomarker levels

A major aim in this study was to investigate the relationships between HDI isocyanurate biomarker (*i.e.*, TAHI) levels and HDI exposure measures. As presented in Chapter 4, TAHI biomarker levels were significantly associated with HDI isocyanurate inhalation exposure levels and the duration of spray-painting task (*i.e.*, paint time) in both linear regression and linear mixed model analyses (**Tables 4.2, 4.5, and 4.6**). Significant correlations were also observed between HDI isocyanurate skin exposure and TAHI levels in creatinine-adjusted urine and plasma (**Table 4.2**), but interestingly, HDI isocyanurate skin exposure did not predict urine or plasma TAHI levels in the mixed model analyses (**Tables 4.5 and 4.6**).

HDI monomer skin exposure was significantly associated urine HDA levels in both linear regression (**Table 4.2**) and mixed model analyses (**Table 4.5**), corroborating previous findings in this study cohort (Gaines *et al.*, 2010a, Gaines *et al.*, 2011). We also observed that APF adjustment improved the significance of the associations between HDI monomer inhalation exposure and urine HDA levels in both linear regression (**Table 4.2**) and mixed model analyses (**Table 4.5**). Contrary to previous findings (Flack *et al.*, 2010b), we did not observe significant associations between HDI monomer inhalation or skin exposures and plasma HDA levels. Further, no exposure measures were significantly correlated with plasma HDA levels (**Table 4.2**), and the model fit statistics of the mixed models were poor compared to mixed models predicting plasma TAHI levels (**Table 4.6**). These differing results may be due to the calculation of the exposure variables. Flack *et al.* calculated daily exposure measures that included 20 inhalation exposure tasks and 18 skin exposure tasks after plasma samples were collected (Flack *et al.*, 2010b). Because linear correlations between HDI monomer exposures and plasma HDA

levels were close to the significance level of $\alpha = 0.05$ (HDI monomer inhalation $p = 0.026$, and HDI monomer skin $p = 0.040$) (Flack *et al.*, 2010b), it is likely that excluding exposures after plasma collection affected the statistical analyses presented in Chapter 4.

Multiple comparisons tests and linear mixed models showed that booth type was significantly associated with urine TAHI levels measured in the exposed workers (**Tables 4.3 and 4.5**). Workers who painted in downdraft booths had significantly lower creatinine-adjusted urine TAHI levels than workers who painted in crossdraft or semi-downdraft booths (**Table 4.3**), and booth type also significantly predicted urine TAHI levels in the mixed model analyses (**Table 4.5**). HDA biomarker levels were not significantly associated with booth type contrary to previous findings in this worker cohort (Flack *et al.*, 2010b, Gaines *et al.*, 2011). However, crossdraft and semi-downdraft booths were grouped together for multiple comparisons and mixed model analyses while previous studies considered booth types separately. Coverall use or glove type were not significantly associated with urine TAHI levels, however, workers who wore no gloves or wore latex gloves had significantly higher plasma TAHI levels when they wore coveralls or when they wore full-face air purifying, PAPR, or supplied-air respirators (**Table 4.4**). When painting in downdraft booths, workers who wore no respirator or wore half-face air purifying respirators had significantly higher plasma TAHI levels than workers who wore full-face air purifying, PAPR, or supplied-air respirators (**Table 4.4**). Respirator type was a borderline significant predictor of urine HDA levels (**Table 4.5**), but as shown in previous studies (Gaines *et al.*, 2010a, Gaines *et al.*, 2011), APF-adjusted HDI monomer inhalation exposure was more strongly associated with urine HDA levels than either unadjusted HDI monomer inhalation exposure or respirator type as independent variables.

HDA has been monitored in urine as a biomarker of short-term HDI monomer exposure in controlled exposure studies (Brorson *et al.*, 1990a, Dalene *et al.*, 1990, Tinnerberg *et al.*, 1995, Liu *et al.*, 2004, Budnik *et al.*, 2011) and in occupationally exposed populations (Maitre *et al.*, 1996, Pronk *et al.*, 2006b, Gaines *et al.*, 2010a, Gaines *et al.*, 2011). Additionally, HDA levels in plasma and hemoglobin were confirmed as biomarkers of short-term and cumulative exposures (Flack *et al.*, 2010b, Flack *et al.*, 2011). HDA levels in urine were not observed to be significantly associated with HDI biuret or total NCO inhalation exposures in a controlled inhalation challenge study (Liu *et al.*, 2004). However, associations between HDI oligomer exposures and HDA levels in urine or plasma in exposed workers have not been reported previously. Therefore, we sought to investigate whether biomarkers of HDI isocyanurate and HDI monomer exposures can be used interchangeably to estimate exposure to either compound. We observed that creatinine-adjusted urine TAHI levels were significantly correlated with HDI monomer inhalation exposures (INH $p = 0.0011$; **Table 4.2**), and plasma TAHI levels were significantly correlated with HDI monomer inhalation and skin exposures (INH $p = 0.0049$, and skin $p = 0.0285$). Creatinine-adjusted urine HDA levels were only significantly correlated with HDI isocyanurate skin exposure ($p = 0.0045$), and plasma HDA levels were not correlated with HDI isocyanurate inhalation or skin exposures (for all $p \geq 0.3407$; **Table 4.2**). These results show that TAHI biomarker levels are significantly associated with HDI monomer exposures but HDA levels in urine or plasma are not suitable biomarkers of HDI isocyanurate exposures.

5.2.2. Creatinine adjustment for urine biomarkers

Typically in the published scientific literature, urine HDA levels have been reported as creatinine-adjusted values (Maitre *et al.*, 1996, Williams *et al.*, 1999, Liu *et al.*, 2004, UK HSE, 2005b, Creely *et al.*, 2006, Pronk *et al.*, 2006b, Gaines *et al.*, 2010a, Budnik *et al.*, 2011, Gaines

et al., 2011, Jones *et al.*, 2013, ACGIH, 2015, Hu *et al.*, 2017, Jones *et al.*, 2017) because creatinine adjustment normalizes urine biomarker levels to account for variability in urine water content (Gaines *et al.*, 2010b). Also, the limit values for HDA in urine are published as creatinine-adjusted by ACGIH and UK HSE (UK HSE, 2005b, ACGIH, 2015). Therefore, the urine HDA and TAHI values reported in this thesis were also adjusted for creatinine level in the statistical analyses.

Consistent with previous studies (Gaines *et al.*, 2010a, Gaines *et al.*, 2011), we observed that creatinine level was a significant predictor of daily mean and post-shift urine HDA levels in mixed model analyses (**Tables 4.5 and C.3**). On the contrary, creatinine level was not a significant predictor for daily mean or post-shift urine TAHI levels in mixed model analyses (**Tables 4.5 and C.3**). The results indicate that creatinine adjustment is not necessary for HDI isocyanurate urine biomarker analyses, thus, it is likely that urine TAHI excretion is not related to the urine creatinine excretion rate in this worker population. However, this observation should be confirmed in future exposure assessment studies.

5.2.3. Daily mean urine concentration for exposure assessment

Many recommended limit values for urine biomarkers are determined for the concentration measured in the last urine sample collected during the work-shift (UK HSE, 2005b, ACGIH, 2019). Here, we report the measured urine biomarker levels as a geometric mean value of the samples collected during the workday for each worker. To evaluate the implications of averaging urine biomarker levels, we investigated the associations between daily HDI isocyanurate and HDI monomer exposures and the geometric means of urine TAHI and HDA levels measured in all urine samples collected during the work-shift in parallel with TAHI and HDA levels in spot urine samples collected post-shift. In order to minimize the impact of

exposure carryover from previous day exposure, a geometric mean of all individual urine samples collected after the first paint task of the day for each visit was calculated. The results of the statistical analyses using the spot urine samples collected post-shift are presented in Appendix C.

Mean urine TAHI levels were more strongly correlated with HDI isocyanurate inhalation and skin exposures than post-shift urine TAHI levels (**Tables C.1**). Painting in crossdraft or semi-downdraft booths was also significantly associated with higher post-shift TAHI levels after urine biomarker data was stratified by respirator type, coverall use, or glove type (for all $p \leq 0.0782$; **Table C.2**). Linear mixed model analyses in Chapter 4 (**Table 4.5**) for predicting mean urine levels were replicated with post-shift urine levels (**Table C.3**). Although paint time and booth type were significant predictors ($p = 0.0188$ and $p = 0.0024$, respectively) of post-shift urine TAHI levels in the final model (Model 3-C), HDI isocyanurate PBZ was not significant ($p = 0.1068$) and the AIC values for all three models were higher (Models 3A-C, AIC range 595.4 – 606.3) than the AIC values for mixed models predicting mean urine TAHI levels (Models 1A-C, AIC range 554.9 – 566.8). The linear regression and mixed model analyses indicate that the geometric mean of TAHI levels in spot urine samples collected during the work-shift is more strongly associated with HDI isocyanurate exposures than the TAHI levels in a single spot urine sample collected post-shift, emphasizing the need to collect urine throughout the work-shift.

5.3. Urine biomarker levels and recommended limit values

The ACGIH and UK HSE recommend maximum HDA levels in post-shift urine samples of 15 µg/g creatinine and 1 µmol/mol creatinine, respectively (UK HSE, 2005b, ACGIH, 2015). In this study, HDA was measured above the ACGIH BEI in one urine sample while 14 urine samples had HDA levels above the UK HSE BMGV (**Table 5.1**). In pre-shift urine, HDA was

measured above the BMGV in four samples indicating that HDA is systemically available and gradually released post-exposure excretion (*i.e.*, elimination kinetics is at least biphasic), as is also reported elsewhere (Tinnerberg *et al.*, 1995, Gaines *et al.*, 2010a, Budnik *et al.*, 2011).

No recommended biomarker limits exist for TAHI because an analytical method to detect it in biological media has not been available until now (Robbins *et al.*, 2018). However, hypothetical biomarker limits could be calculated by multiplying the HDA BEI and HDA BMGV with the TAHI:HDA molar ratio of 3.67. As a result of using the molar ratio, these hypothetical limit values assume that no differences in absorption, metabolism, and urinary excretion exist between HDI isocyanurate and HDI monomer in humans. The hypothetical TAHI BEI and TAHI BMGV were calculated to be 55.07 µg/g creatinine and 3.77 µg/g creatinine, respectively. It is noteworthy, that TAHI concentration was below the hypothetical TAHI BEI limit value in all urine samples in this study population. The HDA BEI originates from the estimated HDA level excreted in urine after full work-shift exposure (8 h) to TWA HDI monomer level of 34 µg/m^3 (ACGIH, 2015). Because the toxicokinetics and excretion of HDI isocyanurate are poorly understood, this hypothetical TAHI BEI concentration may not reflect a level of TAHI in urine when a worker is exposed day after day for a working lifetime to HDI isocyanurate at TWA level. On the other hand, TAHI was measured above the hypothetical TAHI BMGV in three urine samples. Interestingly, none of these samples were collected post-shift but instead after the first task. This may suggest that some absorbed HDI isocyanurate is rapidly metabolized and excreted in urine (*i.e.*, within an hour).

The UK HSE BMGV is not directly associated with an exposure limit. Instead it is determined as the 90th percentile of all UK biomonitoring data for exposures to HDI, IPDI, MDI, and TDI (Cocker, 2007). In this study cohort, the TAHI 90th percentile level from all the urine

samples was 0.46 µg/g creatinine. This 90th percentile level is markedly lower than the hypothetical TAHI BMGV calculated from the HDA BMGV using the molar ratio, but follows the same methodology used to calculate BMGV limits for urinary amine levels associated with diisocyanate monomer exposures. Additional studies monitoring TAHI concentrations in urine of workers exposed to HDI isocyanurate is warranted to further investigate these associations to determine if UK HSE methodology to calculate a recommended biomarker limit for urinary TAHI is appropriate.

Table 5.1. Urine concentrations above the MDL and above biomarker limits recommended by the ACGIH and UK HSE measured in samples collected from 47 automotive spray-painters in North Carolina (n = 15) and Washington State (n = 32).

			ACGIH	***UK HSE***
TAHI	*N*	*N* > MDL (%)	*N* > BEI[a] (%)	*N* > BMGV[b] (%)
Pre-shift	117	22 (19%)	0 (0%)	0 (0%)
After first task	283	105 (37%)	0 (0%)	3 (1%)
Post-shift	115	37 (32%)	0 (0%)	0 (0%)
All samples	400	127 (32%)	0 (0%)	3 (1%)
Daily mean	115	58 (50%)	0 (0%)	0 (0%)
Mean after first task	115	51 (44%)	0 (0%)	1 (1%)
HDA	*N*	*N* > MDL (%)	*N* > BEI[a] (%)	*N* > BMGV[b] (%)
Pre-shift	117	66 (56%)	0 (0%)	4 (3%)
After first task	283	186 (68%)	1 (0%)	10 (4%)
Post-shift	115	82 (71%)	1 (1%)	5 (4%)
All samples	400	252 (63%)	1 (0%)	14 (4%)
Daily mean	115	100 (87%)	0 (0%)	5 (4%)
Mean after first task	115	87 (76%)	0 (0%)	6 (5%)

N = number of samples; MDL = method detection limit; HDA = 1,6-diaminohexane; TAHI = trisaminohexyl isocyanurate; [a]ACGIH BEI = 15 µg/g creatinine for urinary HDA, and 55.07 µg/g creatinine for urinary TAHI calculated from HDA BEI; [b]UK HSE BMGV = 1.03 µg/g creatinine (1 µmol/mol creatinine) for urinary HDA, and 3.77 µg/g creatinine (1 µmol/mol creatinine) for urinary TAHI calculated from HDA BMGV.

5.4. Limitations

5.4.1. Limitations of the sample treatment and the analytical method

Some limitations for the quantification of TAHI in urine and plasma samples collected from this worker cohort may have affected the exposure-biomarker associations observed in this study. The sample treatment methods described in **Sections 2.2.4 and 3.2.4** are similar to sample treatment methods for extraction and derivatization of HDA from hydrolyzed urine and plasma samples (Flack *et al.*, 2010b, Gaines *et al.*, 2010a). However, the sample treatment method to extract TAHI is laborious and chemically intensive for processing large quantities of samples. Additionally, the sample treatment method was partly developed when free TAHI and TAHI-HFBA were analyzed by UPLC-ESI-MS/MS with poorer detection limits before the integration of acetic anhydride derivatization and the transition to the more sensitive nano-UPLC-ESI-MS/MS system. Therefore, the alternative extraction methods discussed in **Section 2.4** (SPE and HPLC) cannot be ruled out for extracting TAHI from hydrolyzed urine or plasma. The recovery of the sample treatment method has not been investigated since low quantities of standards were synthesized (Robbins *et al.*, 2018). Developing a reproducible sample treatment method that is sensitive and specific for quantitative TAHI analysis was prioritized over efficiency of the method. The hydrolysis and derivatization times of 16 h should be evaluated, and one of the drying steps (nitrogen gas or vacuum centrifugation) could be eliminated to reduce sample processing time and potential sample loss. Optimizing the sample treatment method may reduce total preparation time from 3 – 4 days to 2 – 3 days if the steps listed above can be modified.

The non-specific approach of sample treatment with acid hydrolysis left us unable to ascertain levels of macromolecule or acetylated TAHI conjugates in urine or plasma. Because the systemic availability of HDI isocyanurate in urine and plasma was unknown, acid hydrolysis was

chosen to maximize the quantifiable concentration in biological samples over identifying specific conjugates that may be present at low levels in the samples collected during the work-shift. Previous investigations of acetylated amines in urine indicate basic hydrolysis cleaves TAHI-macromolecule conjugates but would not revert amides to amines (Sepai *et al.*, 1995a, Sepai *et al.*, 1995b, Flack *et al.*, 2010a). Identifying TAHI-albumin conjugates in plasma and acetylated TAHI in urine would be relevant for investigating the favored metabolic processes of HDI isocyanurate after absorption.

Another limitation for TAHI analysis is the use of nano-UPLC for chromatographic separation. This is not readily available instrumentation and may limit the widespread applicability of the analytical method in its current form. Additionally, analysis time per sample was approximately 48 min with nano-UPLC-ESI-MS/MS, significantly longer than standard HPLC- and UPLC-MS/MS methods. It is possible the analytical method can be adapted for more accessible UPLC-ESI-MS/MS systems without significant loss in sensitivity or specificity. UPLC-ESI-MS/MS would also reduce analysis to an estimated time range of 15 – 20 min, more than doubling sample output.

Lastly, the SRM method for nano-UPLC-ESI-MS/MS analysis was modified for plasma TAAHI analysis. The original method was described in **Sections 2.2.5 and 2.4**, and the modification was described in **Sections 3.2.3 and 3.3**. Two additional mass transitions were added for plasma TAAHI analysis that were not included in the original urine TAAHI analysis. As a result, the calculated MDL for plasma TAHI analysis was lower (0.02 µg/L) than the calculated MDL for urine TAHI analysis (0.03 µg/L). The chromatograms of the mass transitions used for quantification in Chapters 2 and 3 are shown in **Figure 3.3**. Due to time and monetary constraints, urine samples with visible TAAHI mass transitions that either fell below the MDL or

below an s/n ratio = 3 were not reanalyzed. It is likely that some of these urine samples would be quantifiable with the addition of the two mass transitions described in **Section 3.2.3**. If more urine samples from this study cohort had TAHI levels above the MDL, this may affect the statistical associations and conclusions described in Chapter 4. It is estimated that 5 – 20 urine samples may have TAHI levels between 0.02 – 0.03 µg/L, which is the range between the MDLs calculated for plasma and urine TAHI analysis. Thus, the urine TAHI levels presented in this research should be considered a conservative estimate of measurable TAHI in these urine samples.

5.4.2. Limitations in study design and sample collection

HDI isocyanurate was measured in the breathing-zone and on the skin at considerably higher levels than HDI monomer (Fent *et al.*, 2009a, Fent *et al.*, 2009b), yet, TAHI was detected in 32% of urine samples and 22% of plasma samples (**Section 4.3.1**) while HDA was detected in 63% of urine samples and 74% of plasma samples (Flack *et al.*, 2010b, Gaines *et al.*, 2010a). Although, the limitations in the sample treatment and analytical method may have affected the detection of biomarkers in the biological samples, the low prevalence of TAHI may have been also affected by sample collection, and particularly timing of the sample collection.

Based on previous studies of HDI monomer exposures and calculated half-lives of urinary HDA excretion, it is probable that urinary TAHI excretion half-life is longer. In previous studies on human volunteers exposed to aerosols and vapors of HDI monomer and HDI biuret in exposure chambers or closed-circuit breathing apparatuses, half-life for urinary HDA excretion was observed to be 2.5 – 2.8 h (Tinnerberg *et al.*, 1995, Liu *et al.*, 2004, Budnik *et al.*, 2011). Gaines *et al.* corroborated these measured half-lives with an occupationally exposed population, calculating a urinary HDA excretion half-life of 2.9 h (Gaines *et al.*, 2010a). Budnik *et al.* did

observe a major, rapid excretion peak for HDA at 2 h. Major peaks were observed for 2,4- and 2,6-TDA at 4.1 h and 4.8 h, respectively, but no major peaks were observed for MDA or isophorone diamine (IPDA). Additionally, elevated excretion peaks with slower elimination kinetics were observed in groups with higher HDI, MDI, and IPDI exposures (Budnik *et al.*, 2011).

Exposures to IPDI, a cycloaliphatic isocyanate with a cyclohexane moiety, and urinary excretion of its metabolite IPDA may provide clues for the metabolism of HDI isocyanurate and urinary excretion of TAHI. The cycloaliphatic HDI isocyanurate with its isocyanurate moiety may have more similarities to the excretion kinetics of IPDI than to the excretion kinetics of HDI monomer. Budnik *et al.* exposed 9 volunteers to IPDI and estimated urinary IPDA excretion half-lives of 4 h after low IPDI exposure and 5.5 h after high IPDI exposure (Budnik *et al.*, 2011). IPDA excretion peaked at 5.6 h and was not fully eliminated after 24 h. By contrast, HDA peaked at 2 h with a small peak at 15 h and was fully eliminated after 24 h. The elongated excretion pattern was more pronounced in higher IPDI exposure groups and this pattern was not observed for low and high exposures to HDI monomer (Budnik *et al.*, 2011). The sample collection for this study was designed to obtain optimal exposure and biomarker data for HDI monomer and HDA (Fent *et al.*, 2009a, Fent *et al.*, 2009b, Flack *et al.*, 2010b, Gaines *et al.*, 2010a) as no information existed for HDI isocyanurate and associated biomarkers. If HDI isocyanurate excretion is similar to IPDI excretion, it is possible that TAHI was slowly excreted in urine hours after the sample collection ended and, thus, the optimal sample collection time was missed in this study. For future exposure assessments it is advisable to collect urine 24 h post-exposure, or longer, to monitor biomarkers of HDI isocyanurate.

5.5. Future research

The sample treatment and analytical methods presented in Chapters 2 and 3 combined with the exposure assessment analyses presented in Chapter 4 provides significant new information and knowledge to guide future research efforts to investigate isocyanate exposures and associated adverse health effects. The exposure assessment of automotive spray-painters utilized for this research was comprehensive with ample worker participation and robust sample size. However, future exposure assessments in other exposed populations are warranted to better understand the relationships between short-term and/or cumulative HDI isocyanurate exposures and the associated biomarkers. Because of the constraints of the study design in this study cohort (*i.e.*, the sample collection regimen was designed to capture biomarkers of HDI monomer exposure and not specifically biomarkers of HDI isocyanurate exposure), this dissertation research clearly indicates that these two compounds have different elimination characteristics in humans. The spray-painters who participated in this study worked full-time but samples were only collected during 1 – 3 full workday visits with a minimum of 3 weeks in between each visit. Thus, we were only able to investigate the relationship between the same-day exposure and biomarker levels and could not ascertain the possible contribution of past exposures to measured biomarker levels. We will not fully understand the metabolism and excretion of HDI isocyanurate, or the other HDI oligomers for that matter, in exposed populations unless post-exposure biological monitoring is extended beyond end of work-shift (*i.e.*, 24 h after shift, or even longer). Ideally, monitoring workers over a week and weekend to evaluate biomarker concentrations on days the workers are not exposed would be best practice. This data would provide valuable insight on excretion, variability, and factors that may influence TAHI and HDA biomarker levels long after the work-shift has ended. Most significantly this data would provide

information on setting a robust sampling scheme for exposure assessment and setting appropriate exposure limit values.

Future studies can build on this research to validate TAHI as a biomarker of HDI isocyanurate exposure in other exposed populations and to develop accurate exposure limit values to protect worker health. The results derived in this study can also be utilized investigate the toxicokinetics of HDI isocyanurate exposures and to identify additional biomarkers of exposure and effect. For example, TAHI macromolecule conjugates may be present in urine, blood, lung fluid, or skin in exposed workers and could potentially serve as biomarkers of exposure or early markers of adverse health effects. Because HDI and other isocyanates are known sensitizers and limit laboratory studies in human volunteers, murine studies to investigate biomarker levels after controlled inhalation and skin exposures would be useful for assessing metabolism and excretion in urine and feces. It would be advisable to use radiolabeled HDI monomer and HDI isocyanurate for controlled exposures because radioactivity can be measured to estimate isocyanate dose in lungs, skin, organs, urine, blood, and feces. Measuring levels of radioactivity would simplify the biomarker analysis, removing the laborious sample treatment and analytical methods for TAHI and HDA analysis. Our knowledge on HDI isocyanurate metabolism and excretion will remain limited unless comprehensive exposure assessment and biomonitoring can be performed for extended periods post-exposure; this is only achieved using appropriate murine models for isocyanates due to their strong sensitizing characteristics.

The current research in our laboratory continues to address issues with HDI exposure assessment and biomarker analyses in automotive and aircraft spray-painters. HDI exposure assessment and biomonitoring is far less common in the aircraft refinishing industry (Carlton and England, 2000, Wisnewski *et al.*, 2012, Ceballos *et al.*, 2017), thus, additional studies on other

worker cohorts, apart from automotive spray-painters, would provide further information on isocyanate exposure patterns and adverse health effects. The biological samples collected in these studies offer a unique opportunity to investigate associations between biomarkers measured in post-shift urine and end-of-week urine and plasma. In addition, the low HDI monomer and HDI oligomer exposures monitored by area and PBZ sampling in aircraft spray-painters will provide information on potential differences in biomarkers due to variability in exposure levels and products used. The data collected in these two studies presents a rare opportunity to evaluate and compare two skin exposure sampling methods and whether and how much the measured HDI isocyanurate and HDI monomer skin exposures contribute to systemic exposure (*i.e.*, urine and plasma TAHI and HDA levels) in these workers.

5.6. Conclusions

This dissertation research demonstrates that HDI isocyanurate, the oligomeric trimer of HDI monomer, is systemically available and circulated in blood for metabolism and excretion in exposed workers. Hydrolyzed trisaminohexyl isocyanurate (TAHI) in urine and plasma is a significant biomarker for HDI isocyanurate exposures in the automotive refinishing industry. Here, TAHI was measured in urine and plasma via acid hydrolysis, dichloromethane extraction, acetic anhydride derivatization, and quantitative analysis with nano-UPLC-ESI-MS/MS. The sample treatment and analytical method developed to quantify TAHI in hydrolyzed urine and plasma samples may also be adapted for biomonitoring of other oligomeric isocyanate exposures, although the method in its current form may require modifications to retain sensitivity and specificity.

Although TAHI was measured in fewer urine and plasma samples collected from occupational exposed workers, both HDI exposures and workplace factors were more strongly

associated with TAHI biomarker levels than with HDA biomarker levels. We observed that HDI isocyanurate inhalation exposure and paint time were significantly associated with TAHI levels in urine and plasma of exposed automotive spray-painters. HDI isocyanurate skin exposure was correlated with TAHI biomarker levels, however, skin exposure was not a significant predictor in mixed model analyses. We also observed that HDI monomer skin exposure was significantly associated with HDA levels in urine and plasma, corroborating previous findings in this study cohort (Gaines *et al.*, 2010a, Gaines *et al.*, 2011). Contrary to previously reported findings (Flack *et al.*, 2010b), HDI monomer inhalation or skin exposures were not significantly associated with plasma HDA levels. The disagreement between these observations is likely due to the altered approach for calculating both exposure and plasma variables.

Painting in downdraft booths significantly reduced exposure to HDI isocyanurate and urine TAHI levels, while more protective full-face air purifying, PAPR, and supplied-air respirators significantly reduced plasma TAHI and urine HDA levels. Additionally, workers who wore nitrile or neoprene gloves had significantly lower plasma TAHI levels. Based on the observations in this study, painting in downdraft booths, wearing nitrile or neoprene gloves, and wearing full-face air purifying, PAPR, or supplied-air respirators offers the greatest combination of respiratory and skin protection to reduce hazardous HDI exposures and, consequently, the amounts of the biomarkers of exposure in automotive spray-painters.

The associations between the exposure and biomarker levels presented here confirm TAHI as a biomarker of HDI isocyanurate exposure and may eventually prove to be a stronger indicator than HDA of HDI exposures in occupational settings. We observed that biomarkers of HDI isocyanurate are associated with HDI monomer exposures but HDA levels in urine or

plasma are not suitable biomarkers of HDI isocyanurate exposure. However, this observation needs to be confirmed in future studies.

In summary, HDI isocyanurate biomarker, TAHI, in urine and plasma can be used as a biomarker of HDI isocyanurate exposure in an occupational setting. The developed method for quantification of HDI isocyanurate biomarker, TAHI, in urine and plasma is a significant advancement for HDI exposure assessment and will advance future investigations to oligomeric isocyanate exposures and biomarkers as well as associated health effects.

APPENDIX A: SUPPLEMENTAL TABLE AND FIGURES FOR CHAPTER 2

A.1. Supplemental table for Chapter 2

Table A.1. Gradient program for the NanoAcquity ultra-performance liquid chromatography system (Waters Corp., Milford, MA) using a Symmetry C18 trapping column, 5 µm, 180 µm × 20 mm (Waters Corp.) and an Atlantis dC18 analytical column, 3 µm, 100 µm × 100 mm (Waters Corp.). Mobile phase A consisted of 0.1% formic acid in deionized water and mobile phase B consisted of 0.1% formic acid in acetonitrile.

Mobile Phase A	Mobile Phase B	Time (min)	Flow Rate (nL/min)
95	5	0	600
10	90	17	600
10	90	29	600
95	5	30	600
95	5	44	600

A.2. Supplemental figures for Chapter 2

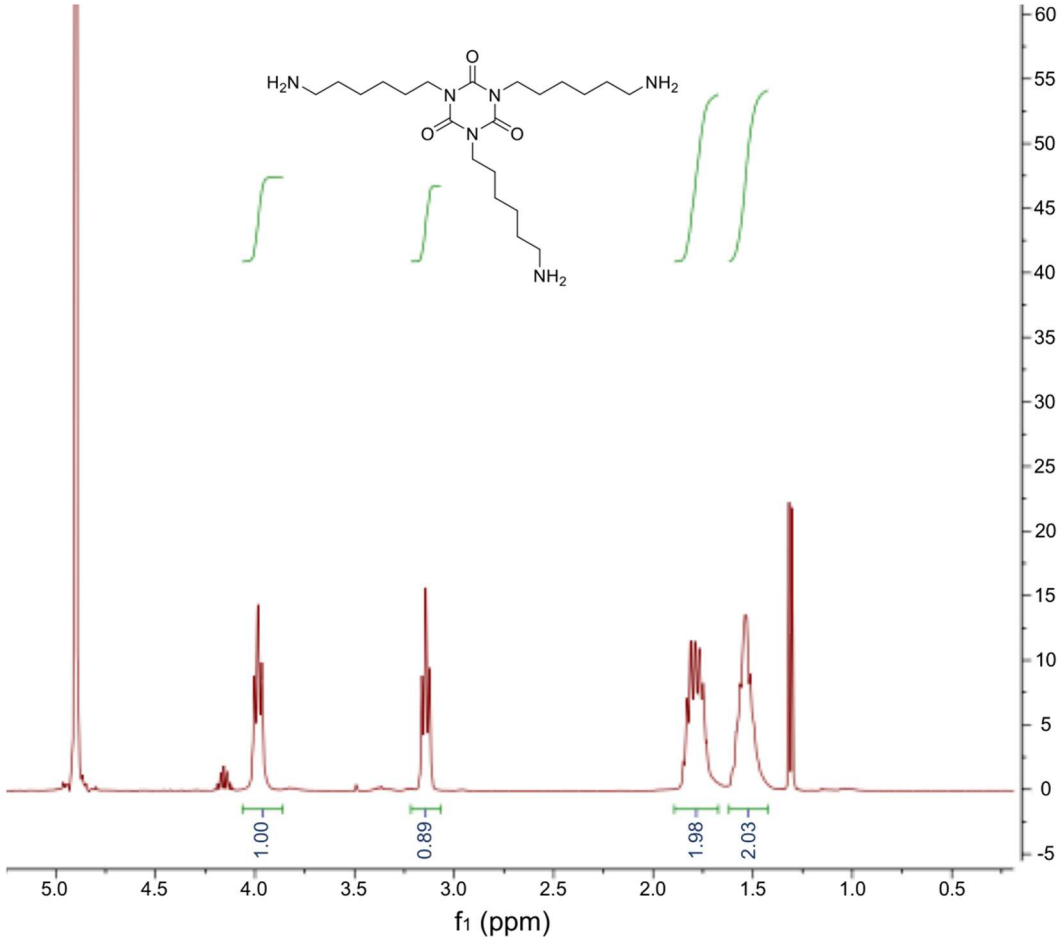

Figure A.1. Proton nuclear magnetic resonance spectrum for TAHI.

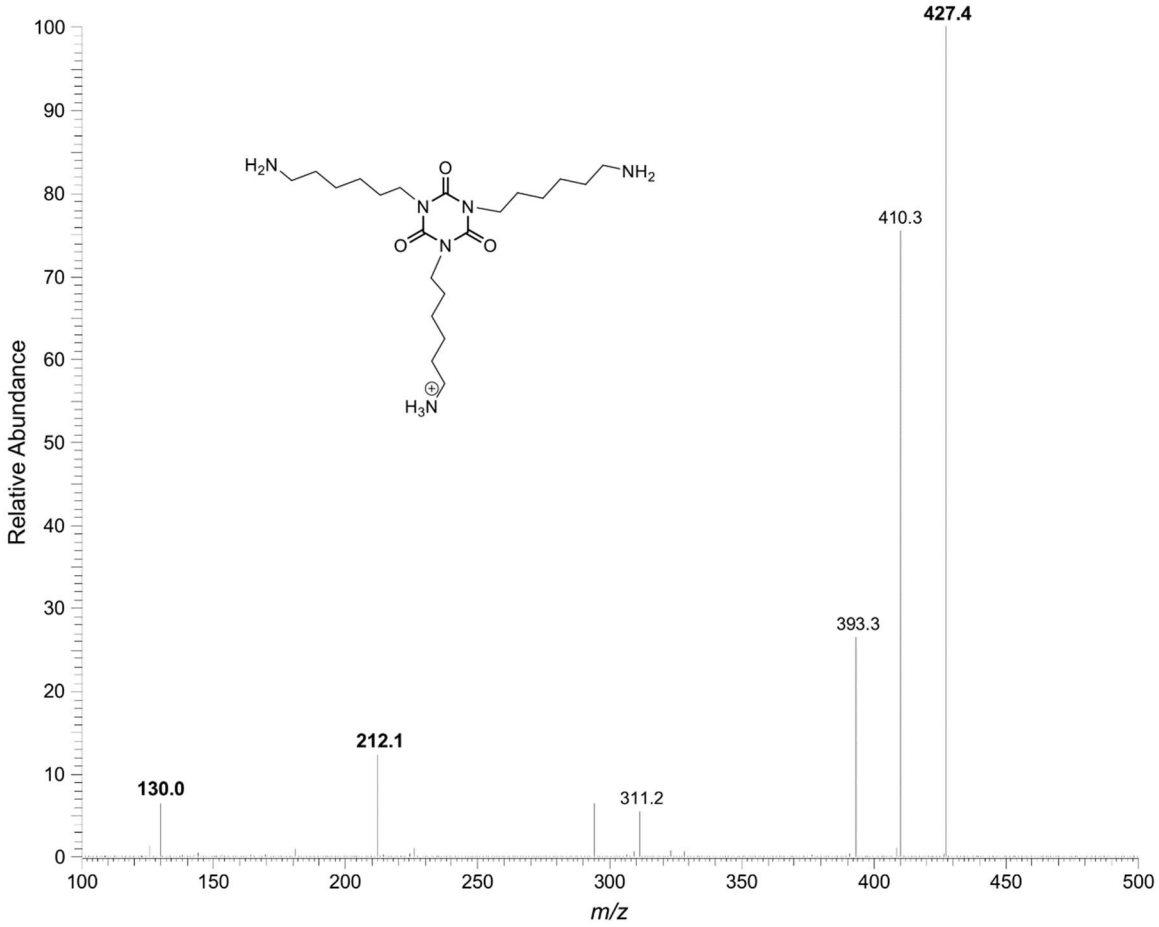

Figure A.2. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAHI (*m/z* 427.3) obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-500; collision energy, 25 eV).

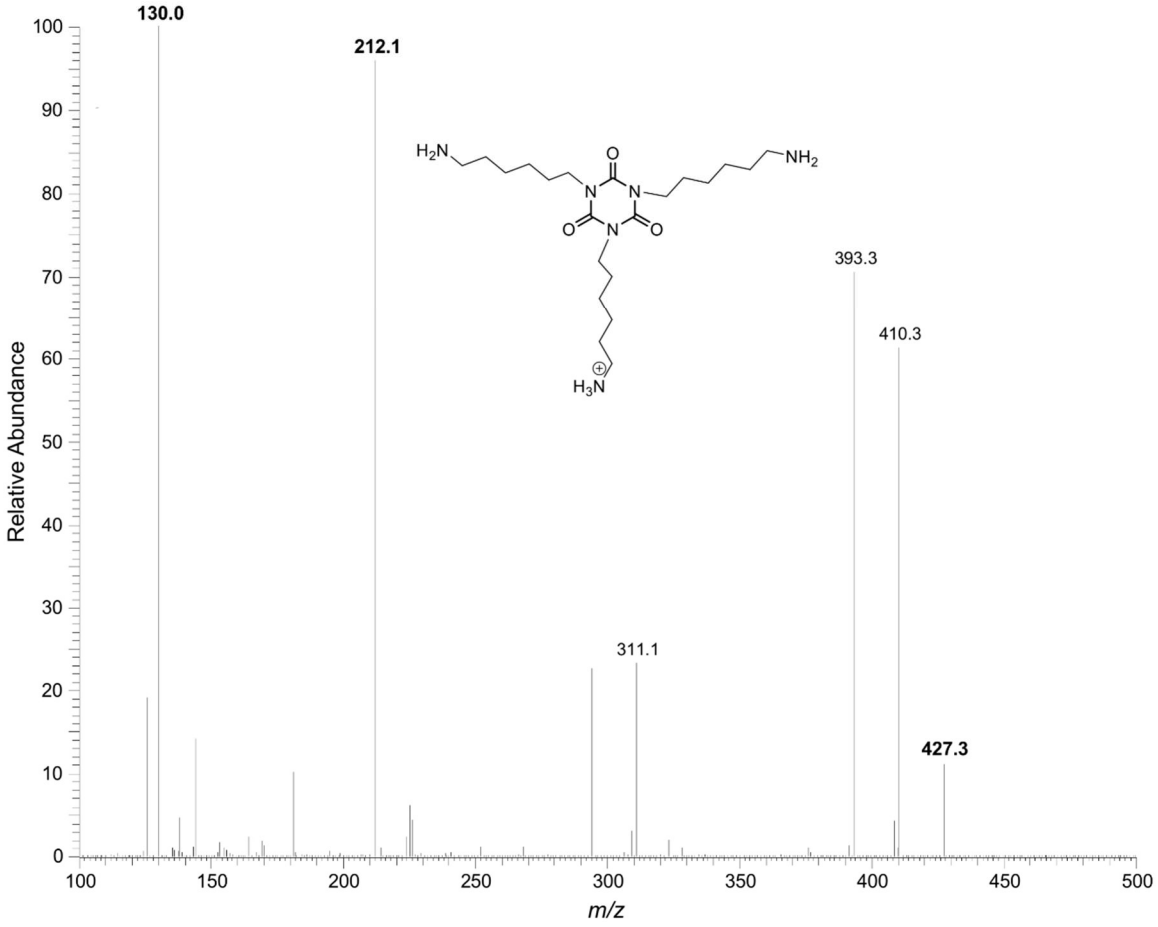

Figure A.3. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAHI (*m/z* 427.3) obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-500; collision energy, 35 eV).

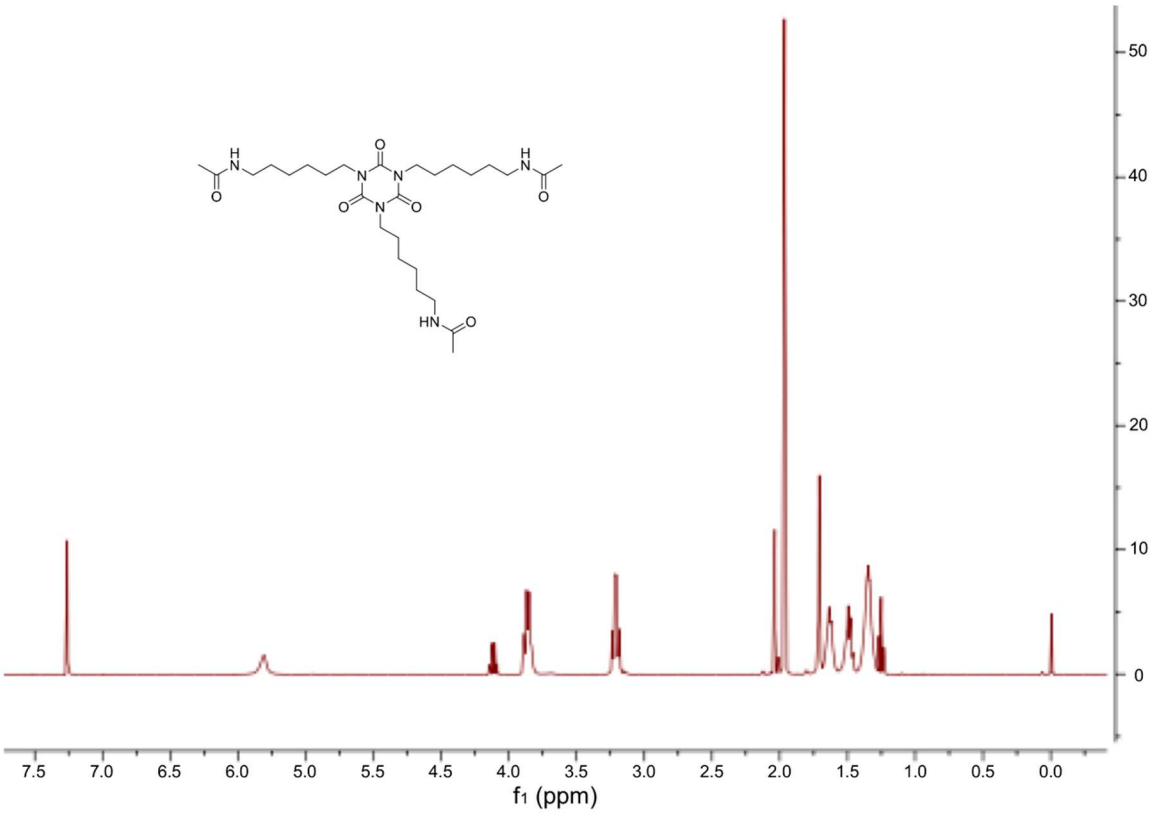

Figure A.4. Proton nuclear magnetic resonance spectrum for TAAHI.

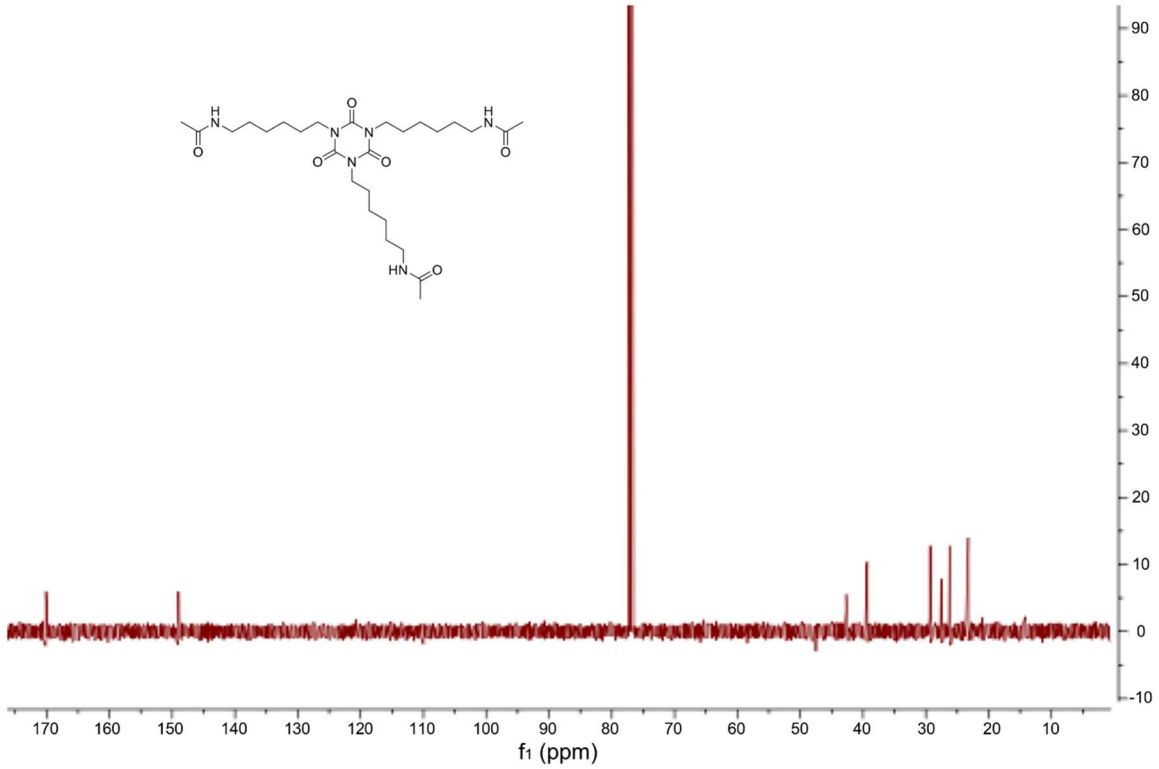

Figure A.5. Carbon-13 nuclear magnetic resonance spectrum for TAAHI.

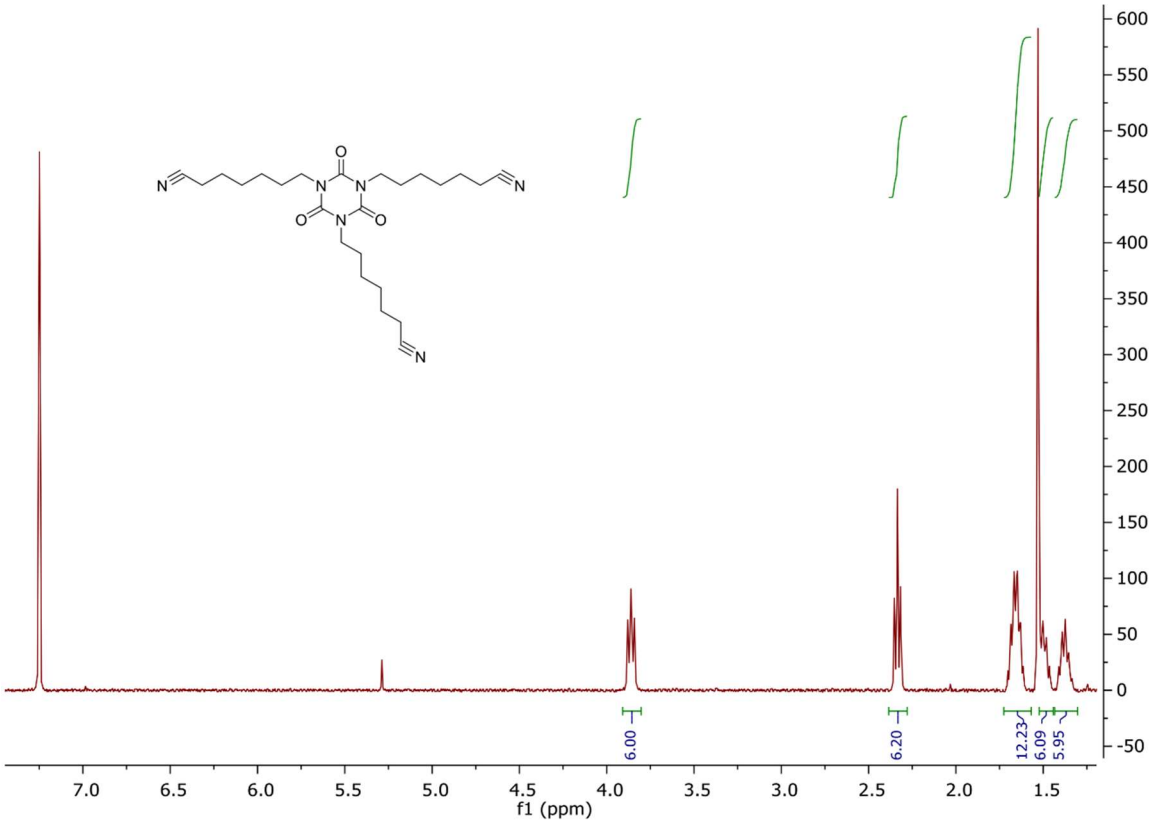

Figure A.6. Proton nuclear magnetic resonance spectrum for 7,7',7"-(2,4,6-trioxo-1,3,5-triazinane-1,3,5-triyl)triheptanenitrile.

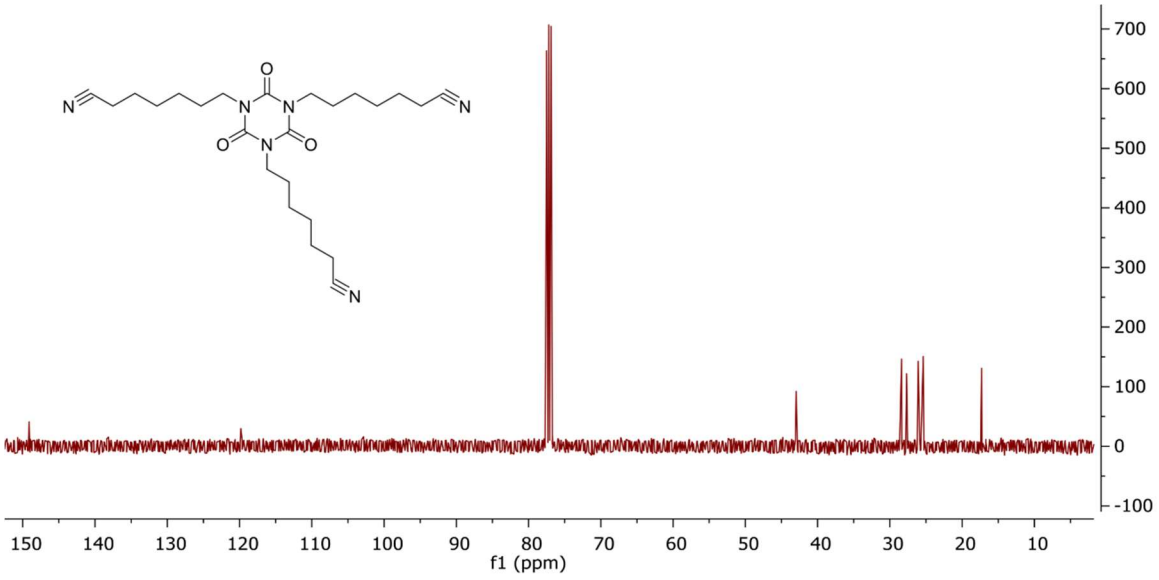

Figure A.7. Carbon-13 nuclear magnetic resonance spectrum for 7,7',7''-(2,4,6-trioxo-1,3,5-triazinane-1,3,5-triyl)triheptanenitrile.

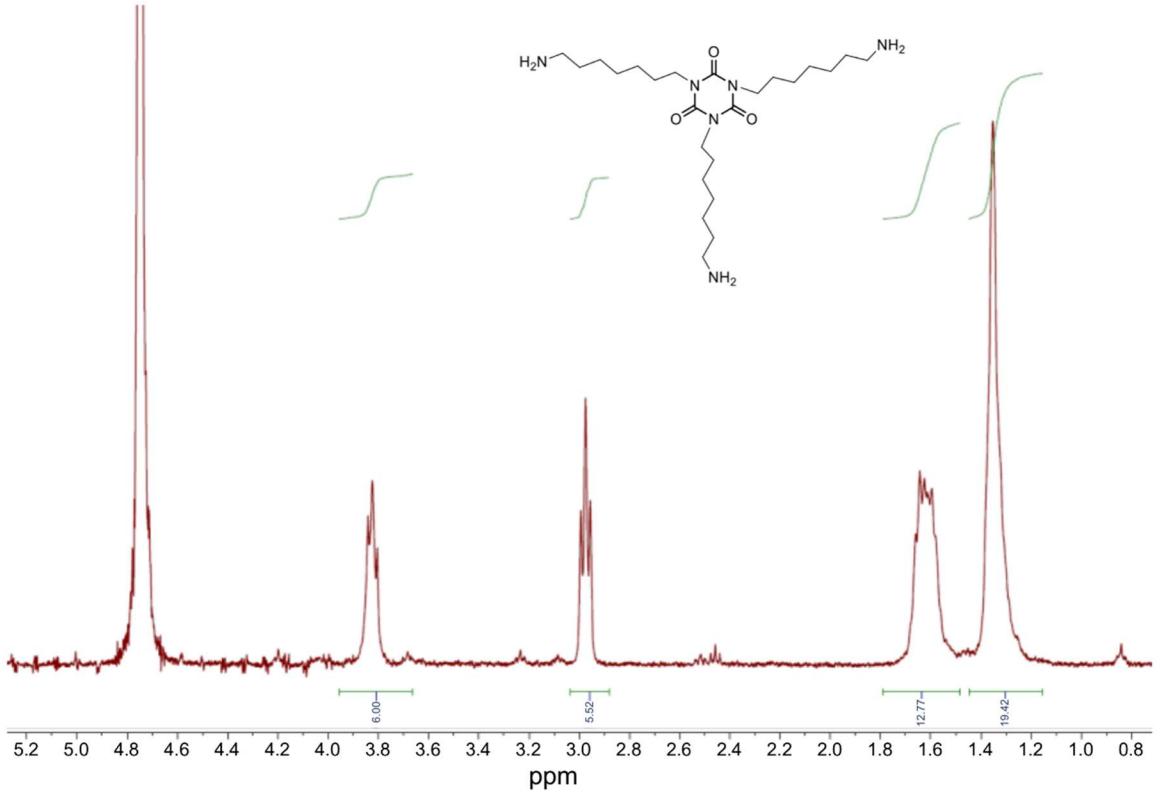

Figure A.8. Proton nuclear magnetic resonance spectrum for TAHpI.

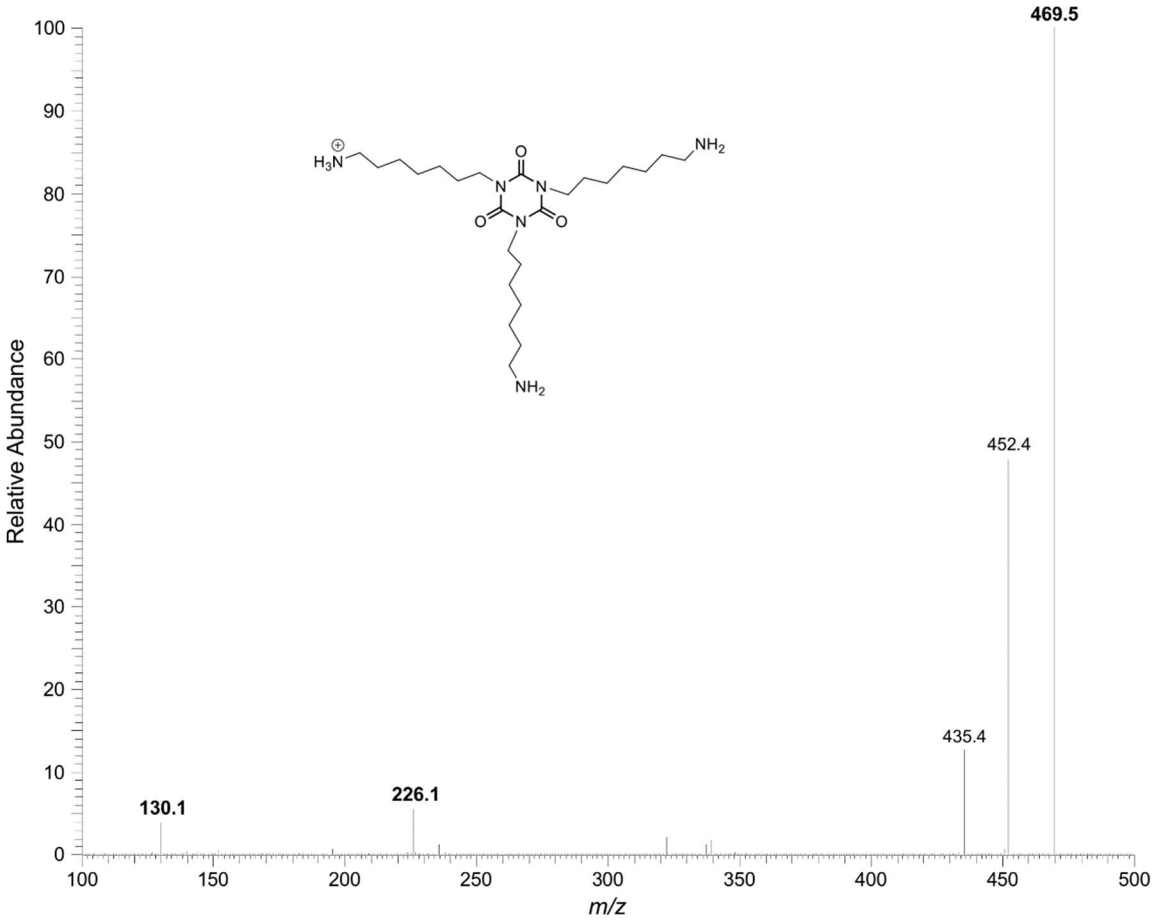

Figure A.9. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAHpI (*m/z* 469.3) obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-500; collision energy, 25 eV).

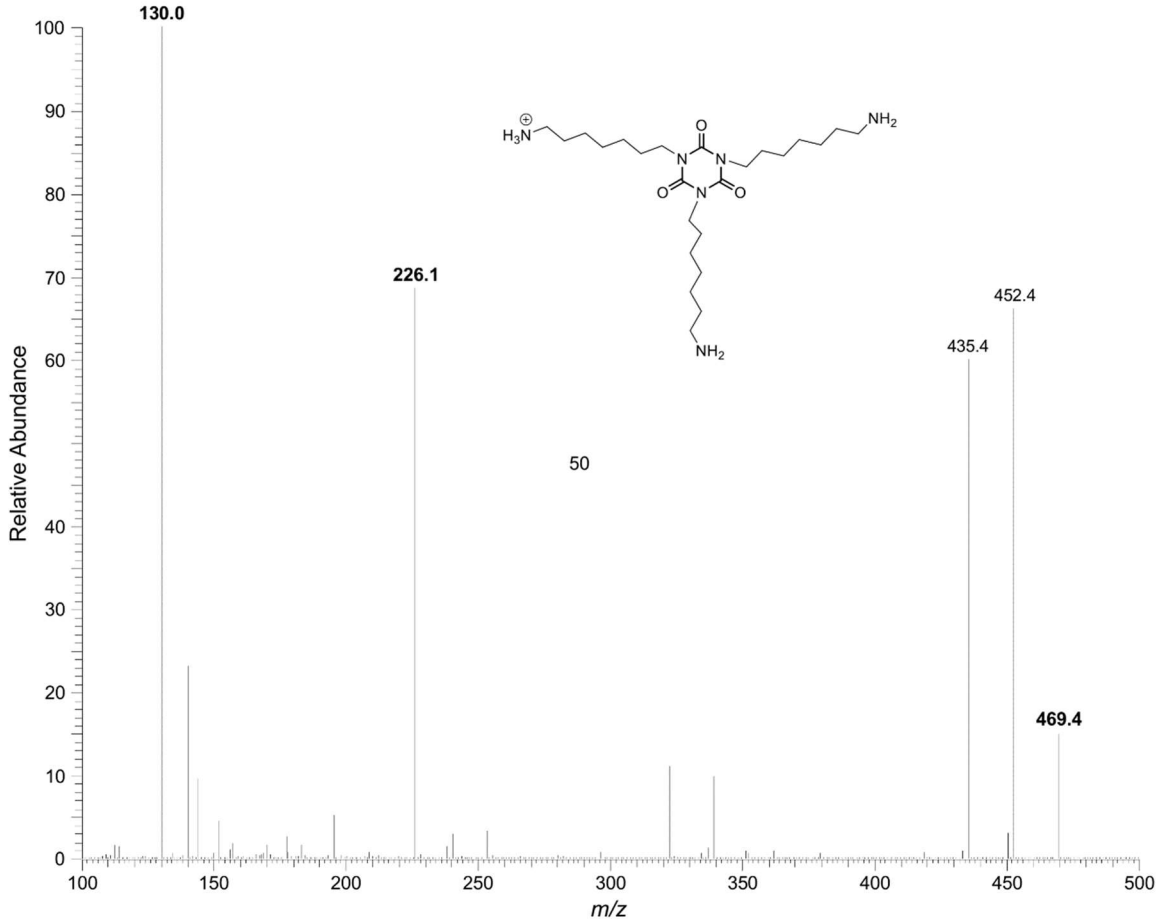

Figure A.10. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAHpI (*m/z* 469.3) obtained by nano-UPLC-ESI-MS/MS operated in positive ion-mode with nano-electrospray ionization (scan range, *m/z* 100-500; collision energy, 35 eV).

APPENDIX B: SAS CODES FOR CHAPTERS 4 AND 5

SAS code B1: PROC UNIVARIATE and PROC CORR procedures for evaluating the normality of each variable in the data set and the Pearson correlation coefficients. Non-transformed exposure and biomarker levels were calculated prior to the last urine sample collected.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExposureBeforeUrine.xlsx"
      OUT = test.S1_Day_ExposureBeforeUrine DBMS=xlsx REPLACE;
RUN;

*Shapiro-Wilk test for normality including distribution plot of the non-
transformed exposure and biomarker levels;

PROC UNIVARIATE DATA = test.S1_Day_ExposureBeforeUrine NORMAL PLOT;
VAR Day_Ptime TWAp_HDI TWAp_ISO TWAp_HDI_APF TWAp_ISO_APF DayHDI_IHug
DayISO_IHug DayHDI_IHugAPF DayISO_IHugAPF TotSkin_HDI_ug TotSkin_ISO_ug
AvgUR_HDA AvgUR_HDA_CR AvgUR_TAHI AvgUR_TAHI_CR EndUR_HDA EndUR_HDA_CR
EndUR_TAHI EndUR_TAHI_CR Pma_HDA Pma_TAHI PmaBSA_HDAug PmaBSA_TAHIug;
RUN;

*Shapiro-Wilk test for normality including distribution plot of the natural
log-transformed exposure and biomarker levels;

PROC UNIVARIATE DATA = test.S1_Day_ExposureBeforeUrine NORMAL PLOT;
VAR lnDay_Ptime lnTWAp_HDI lnTWAp_ISO lnTWAp_HDI_APF lnTWAp_ISO_APF
lnDayHDI_IHug lnDayISO_IHug lnDayHDI_IHugAPF lnDayISO_IHugAPF
lnTotSkin_HDI_ug lnTotSkin_ISO_ug AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI
AvgLnUR_TAHI_CR lnEndUR_HDA lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR
lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug;
RUN;

*Pearson correlation coefficient calculation for natural log-transformed
exposure and biomarker levels;

PROC CORR DATA = test.S1_Day_ExposureBeforeUrine PEARSON;
VAR lnDay_Ptime lnTWAp_HDI lnTWAp_ISO lnTWAp_HDI_APF lnTWAp_ISO_APF
lnDayHDI_IHug lnDayISO_IHug lnDayHDI_IHugAPF lnDayISO_IHugAPF
lnTotSkin_HDI_ug lnTotSkin_ISO_ug AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI
AvgLnUR_TAHI_CR lnEndUR_HDA lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR
lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug;
RUN;
```

SAS code B2: PROC UNIVARIATE and PROC CORR procedures for evaluating the normality of each variable in the data set and the Pearson correlation coefficients. Natural log-transformed exposure and biomarker levels were calculated prior to the plasma sample collection.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExposureBeforePL.xlsx"
      OUT = test.S1_Day_ExposureBeforePL DBMS=xlsx REPLACE;
RUN;

*Shapiro-Wilk test for normality including distribution plot of the non-
transformed exposure and biomarker levels;

PROC UNIVARIATE DATA = test.S1_Day_ExposureBeforePL NORMAL PLOT;
VAR Day_Ptime TWAp_HDI TWAp_ISO TWAp_HDI_APF TWAp_ISO_APF DayHDI_IHug
DayISO_IHug DayHDI_IHugAPF DayISO_IHugAPF TotSkin_HDI_ug TotSkin_ISO_ug
AvgUR_HDA AvgUR_HDA_CR AvgUR_TAHI AvgUR_TAHI_CR Pma_HDA Pma_TAHI PmaBSA_HDAug
PmaBSA_TAHIug;
RUN;

*Shapiro-Wilk test for normality including distribution plot of the natural
log-transformed exposure and biomarker levels;

PROC UNIVARIATE DATA = test.S1_Day_ExposureBeforePL NORMAL PLOT;
VAR lnDay_Ptime lnTWAp_HDI lnTWAp_ISO lnTWAp_HDI_APF lnTWAp_ISO_APF
lnDayHDI_IHug lnDayISO_IHug lnDayHDI_IHugAPF lnDayISO_IHugAPF
lnTotSkin_HDI_ug lnTotSkin_ISO_ug AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI
AvgLnUR_TAHI_CR lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug;
RUN;

*Pearson correlation coefficient calculation for natural log-transformed
exposure and biomarker levels;

PROC CORR DATA = test.S1_Day_ExposureBeforePL PEARSON;
VAR lnDay_Ptime lnTWAp_HDI lnTWAp_ISO lnTWAp_HDI_APF lnTWAp_ISO_APF
lnDayHDI_IHug lnDayISO_IHug lnDayHDI_IHugAPF lnDayISO_IHugAPF
lnTotSkin_HDI_ug lnTotSkin_ISO_ug AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI
AvgLnUR_TAHI_CR lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug;
RUN;
```

SAS code B3: PROC GLM procedure to perform Tukey-Kramer multiple comparisons tests for daily mean and post-shift urine TAHI and HDA levels. Urine samples were stratified by booth type with the BY statement and then by respirator type, coverall use, or glove type with the CLASS statement.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExpBefUR_CrossSemi.xlsx"
      OUT = test.S1_Day_ExpBefUR_CrossSemi DBMS=xlsx REPLACE;
RUN;

*Urine samples stratified by booth type and then by respirator type;

PROC GLM DATA = test.S1_Day_ExpBefUR_CrossSemi;
Class Resp_protC;
By CrossSemi;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = Resp_protC;
Means Resp_protC / Tukey;
RUN;

*Urine samples stratified by booth type and then by coverall use;

PROC GLM DATA = test.S1_Day_ExpBefUR_CrossSemi;
Class Cov;
By CrossSemi;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = Cov;
Means Cov / Tukey;
RUN;

*Urine samples stratified by booth type and then by glove type;

PROC GLM DATA = test.S1_Day_ExpBefUR_CrossSemi;
Class OrNitrile;
By CrossSemi;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = OrNitrile;
Means OrNitrile / Tukey;
RUN;
```

SAS code B4: PROC GLM procedure to perform Tukey-Kramer multiple comparisons tests for daily mean and post-shift urine TAHI and HDA levels. Urine samples were stratified by coverall use with the BY statement and then by respirator type, glove type, or booth type with the CLASS statement.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExpBefUR_Coverall.xlsx"
      OUT = test.S1_Day_ExpBefUR_Coverall DBMS=xlsx REPLACE;
RUN;

*Urine samples stratified by coverall use and then by respirator type;

PROC GLM DATA = test.S1_Day_ExpBefUR_Coverall;
Class Resp_protC;
By Cov;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = Resp_protC;
Means Resp_protC / Tukey;
RUN;

*Urine samples stratified by coverall use and then by glove type;

PROC GLM DATA = test.S1_Day_ExpBefUR_Coverall;
Class OrNitrile;
By Cov;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = OrNitrile;
Means OrNitrile / Tukey;
RUN;

*Urine samples stratified by coverall use and then by booth type;

PROC GLM DATA = test.S1_Day_ExpBefUR_Coverall;
Class CrossSemi;
By Cov;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = CrossSemi;
Means CrossSemi / Tukey;
RUN;
```

SAS code B5: PROC GLM procedure to perform Tukey-Kramer multiple comparisons tests for daily mean and post-shift urine TAHI and HDA levels. Urine samples were stratified by respirator type with the BY statement and then by coverall use, glove type, or booth type with the CLASS statement.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExpBefUR_RespProtC.xlsx"
      OUT = test.S1_Day_ExpBefUR_RespProtC DBMS=xlsx REPLACE;
RUN;

*Urine samples stratified by respirator type and then by coverall use;

PROC GLM DATA = test.S1_Day_ExpBefUR_RespProtC;
Class Cov;
By Resp_protC;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = Cov;
Means Cov / Tukey;
RUN;

*Urine samples stratified by respirator type and then by glove type;

PROC GLM DATA = test.S1_Day_ExpBefUR_RespProtC;
Class OrNitrile;
By Resp_protC;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = OrNitrile;
Means OrNitrile / Tukey;
RUN;

*Urine samples stratified by respirator type and then by booth type;

PROC GLM DATA = test.S1_Day_ExpBefUR_RespProtC;
Class CrossSemi;
By Resp_protC;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = CrossSemi;
Means CrossSemi / Tukey;
RUN;
```

SAS code B6: PROC GLM procedure to perform Tukey-Kramer multiple comparisons tests for daily mean and post-shift urine TAHI and HDA levels. Urine samples were stratified by glove type with the BY statement and then by respirator type, coverall use, or booth type with the CLASS statement.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExpBefUR_OrNitrile.xlsx"
      OUT = test.S1_Day_ExpBefUR_OrNitrile DBMS=xlsx REPLACE;
RUN;

*Urine samples stratified by glove type and then by respirator type;

PROC GLM DATA = test.S1_Day_ExpBefUR_OrNitrile;
Class Resp_protC;
By OrNitrile;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = Resp_protC;
Means Resp_protC / Tukey;
RUN;

*Urine samples stratified by glove type and then by coverall use;

PROC GLM DATA = test.S1_Day_ExpBefUR_OrNitrile;
Class Cov;
By OrNitrile;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = Cov;
Means Cov / Tukey;
RUN;

*Urine samples stratified by glove type and then by booth type;

PROC GLM DATA = test.S1_Day_ExpBefUR_OrNitrile;
Class CrossSemi;
By OrNitrile;
Model AvgLnUR_HDA AvgLnUR_HDA_CR AvgLnUR_TAHI AvgLnUR_TAHI_CR lnEndUR_HDA
lnEndUR_HDA_CR lnEndUR_TAHI lnEndUR_TAHI_CR = CrossSemi;
Means CrossSemi / Tukey;
RUN;
```

SAS code B7: PROC GLM procedure to perform Tukey-Kramer multiple comparisons tests for plasma TAHI and HDA levels. Plasma samples were stratified by booth type with the BY statement and then by respirator type, coverall use, or glove type with the CLASS statement.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExpBefPL_CrossSemi.xlsx"
      OUT = test.S1_Day_ExpBefPL_CrossSemi DBMS=xlsx REPLACE;
RUN;

*Plasma samples stratified by booth type and then by respirator type;

PROC GLM DATA = test.S1_Day_ExpBefPL_CrossSemi;
Class Resp_protC;
By CrossSemi;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = Resp_protC;
Means Resp_protC / Tukey;
RUN;

*Plasma samples stratified by booth type and then by coverall use;

PROC GLM DATA = test.S1_Day_ExpBefPL_CrossSemi;
Class Cov;
By CrossSemi;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = Cov;
Means Cov / Tukey;
RUN;

*Plasma samples stratified by booth type and then by glove type;

PROC GLM DATA = test.S1_Day_ExpBefPL_CrossSemi;
Class OrNitrile;
By CrossSemi;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = OrNitrile;
Means OrNitrile / Tukey;
RUN;
```

SAS code B8: PROC GLM procedure to perform Tukey-Kramer multiple comparisons tests for plasma TAHI and HDA levels. Plasma samples were stratified by coverall use with the BY statement and then by respirator type, glove type, or booth type with the CLASS statement.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExpBefPL_Coverall.xlsx"
      OUT = test.S1_Day_ExpBefPL_Coverall DBMS=xlsx REPLACE;
RUN;

*Plasma samples stratified by coverall use and then by respirator type;

PROC GLM DATA = test.S1_Day_ExpBefPL_Coverall;
Class Resp_protC;
By Cov;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = Resp_protC;
Means Resp_protC / Tukey;
RUN;

*Plasma samples stratified by coverall use and then by glove type;

PROC GLM DATA = test.S1_Day_ExpBefPL_Coverall;
Class OrNitrile;
By Cov;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = OrNitrile;
Means OrNitrile / Tukey;
RUN;

*Plasma samples stratified by coverall use and then by booth type;

PROC GLM DATA = test.S1_Day_ExpBefPL_Coverall;
Class CrossSemi;
By Cov;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = CrossSemi;
Means CrossSemi / Tukey;
RUN;
```

SAS code B9: PROC GLM procedure to perform Tukey-Kramer multiple comparisons tests for plasma TAHI and HDA levels. Plasma samples were stratified by respirator type with the BY statement and then by coverall use, glove type, or booth type with the CLASS statement.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExpBefPL_RespProtC.xlsx"
      OUT = test.S1_Day_ExpBefPL_RespProtC DBMS=xlsx REPLACE;
RUN;

*Plasma samples stratified by respirator type and then by coverall use;

PROC GLM DATA = test.S1_Day_ExpBefPL_RespProtC;
Class Cov;
By Resp_protC;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = Cov;
Means Cov / Tukey;
RUN;

*Plasma samples stratified by respirator type and then by glove type;

PROC GLM DATA = test.S1_Day_ExpBefPL_RespProtC;
Class OrNitrile;
By Resp_protC;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = OrNitrile;
Means OrNitrile / Tukey;
RUN;

*Plasma samples stratified by respirator type and then by booth type;

PROC GLM DATA = test.S1_Day_ExpBefPL_RespProtC;
Class CrossSemi;
By Resp_protC;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = CrossSemi;
Means CrossSemi / Tukey;
RUN;
```

SAS code B10: PROC GLM procedure to perform Tukey-Kramer multiple comparisons tests for plasma TAHI and HDA levels. Plasma samples were stratified by glove type with the BY statement and then by respirator type, coverall use, or booth type with the CLASS statement.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExpBefPL_OrNitrile.xlsx"
      OUT = test.S1_Day_ExpBefPL_OrNitrile DBMS=xlsx REPLACE;
RUN;

*Plasma samples stratified by glove type and then by respirator type;

PROC GLM DATA = test.S1_Day_ExpBefPL_OrNitrile;
Class Resp_protC;
By OrNitrile;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = Resp_protC;
Means Resp_protC / Tukey;
RUN;

*Plasma samples stratified by glove type and then by coverall use;

PROC GLM DATA = test.S1_Day_ExpBefPL_OrNitrile;
Class Cov;
By OrNitrile;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = Cov;
Means Cov / Tukey;
RUN;

*Plasma samples stratified by glove type and then by booth type;

PROC GLM DATA = test.S1_Day_ExpBefPL_OrNitrile;
Class CrossSemi;
By OrNitrile;
Model lnPma_HDA lnPma_TAHI lnPmaBSA_HDAug lnPmaBSA_TAHIug = CrossSemi;
Means CrossSemi / Tukey;
RUN;
```

SAS code B11: PROC MIXED procedure to build linear mixed models with restricted maximum likelihood estimation of repeated measures of each visit per worker. The TYPE statement sets the covariance structure as compound symmetry. The following PROC MIXED code was used to build mixed models predicting daily mean urine TAHI and HDA levels discussed in Chapter 4 (Models 1A-F; **Table 4.5**).

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExposureBeforeUrine.xlsx"
      OUT = test.S1_Day_ExposureBeforeUrine DBMS=xlsx REPLACE;
RUN;

*Compare base models with and without creatinine adjustment for urine TAHI
level;
*Compare base models with and without creatinine adjustment for urine TAHI
level;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug AvgLnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI_CR = lnTWAp_ISO lnTotSkin_ISO_ug / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug AvgLnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI_CR = lnTWAp_ISO_APF lnTotSkin_ISO_ug / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Higher AIC with creatinine-adjusted TAHI level, keep creatinine as
independent variable;
```

```
*Stepwise introduction of variables with urine TAHI level and TWAp_ISO;
*Stepwise introduction of variables with urine TAHI level and TWAp_ISO;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug AvgLnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add paint time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug AvgLnCRT lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Remove creatinine due to very low estimate and significance;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add booth type;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add respirator type, glove use, coverall use, shop location, weekday, and
smoking status one at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime CrossSemi Gloves
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime CrossSemi Cov /
CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime CrossSemi Smoker
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Retain paint time, booth type, and gloves, and remove skin;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnDay_Ptime CrossSemi Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Remove gloves;

*Final urine TAHI model with ISO air, paint time, and booth type;
*Final urine TAHI model with ISO air, paint time, and booth type;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnDay_Ptime CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Stepwise introduction of variables with urine TAHI level and TWAp_ISO_APF;
*Stepwise introduction of variables with urine TAHI level and TWAp_ISO_APF;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug AvgLnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
*Add paint time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug AvgLnCRT lnDay_Ptime /
CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Remove creatinine due to very low estimate and significance;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add booth type;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug AvgLnCRT lnDay_Ptime
CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add glove use, coverall use, shop location, weekday, and smoking status one
at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
Smoker / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Retain gloves and remove skin;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnDay_Ptime CrossSemi Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add back skin and remove gloves;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi /
CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Remove air;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTotSkin_ISO_ug lnDay_Ptime CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Remove skin;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnDay_Ptime CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Models are similar when air or skin are removed;

*Compare base models with and without creatinine adjustment for urine HDA
level;
*Compare base models with and without creatinine adjustment for urine HDA
level;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA_CR = lnTWAp_HDI lnTotSkin_HDI_ug / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA_CR = lnTWAp_HDI_APF lnTotSkin_HDI_ug / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Higher AIC with creatinine-adjusted HDA level, keep creatinine as
independent variable;

*Stepwise introduction of variables with urine HDA level and TWAp_HDI;
*Stepwise introduction of variables with urine HDA level and TWAp_HDI;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add paint time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Paint time does not help, remove, and add respirator type;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add booth type, glove use, coverall use, shop location, weekday, and smoking
status one at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT Resp_protC CrossSemi
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT Resp_protC Gloves /
CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT Resp_protC Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT Resp_protC Location
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT Resp_protC Day_week
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT Resp_protC Smoker /
CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Remove additional variables and retain respirator type;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Remove air or skin one at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTotSkin_HDI_ug AvgLnCRT Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI AvgLnCRT Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
*Retain skin and add other workplace factors back in one at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTotSkin_HDI_ug AvgLnCRT Resp_protC CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTotSkin_HDI_ug AvgLnCRT Resp_protC Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTotSkin_HDI_ug AvgLnCRT Resp_protC Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTotSkin_HDI_ug AvgLnCRT Resp_protC Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTotSkin_HDI_ug AvgLnCRT Resp_protC Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTotSkin_HDI_ug AvgLnCRT Resp_protC Smoker / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Workplace factors did not improve model with skin and respirator type;

*Final urine HDA model with HDI skin, creatinine, and respirator type;
*Final urine HDA model with HDI skin, creatinine, and respirator type;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTotSkin_HDI_ug AvgLnCRT Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Stepwise introduction of variables with urine HDA level and TWAp_HDI_APF;
*Stepwise introduction of variables with urine HDA level and TWAp_HDI_APF;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
*Add paint time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT lnDay_Ptime /
CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Paint time did not help, remove, add booth type, glove use, coverall use,
shop location, weekday, and smoking status one at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT Smoker / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Additional workplace factors did not improve base model with HDI_APF;
*Additional workplace factors did not improve base model with HDI_APF;
```

SAS code B12: PROC MIXED procedure to build linear mixed models with restricted maximum likelihood estimation of repeated measures of each visit per worker. The TYPE statement sets the covariance structure as compound symmetry. The following PROC MIXED code was used to build mixed models predicting plasma TAHI and HDA levels discussed in Chapter 4 (Models 2A-F; **Table 4.6**).

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExposureBeforePlasma.xlsx"
      OUT = test.S1_Day_ExposureBeforePlasma DBMS=xlsx REPLACE;
RUN;

*Stepwise introduction of variables with plasma TAHI level and TWAp_ISO;
*Stepwise introduction of variables with plasma TAHI level and TWAp_ISO;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add paint time;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Retain paint time, add respirator type;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime Resp_protC /
CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add booth type, glove use, coverall use, shop location, weekday, and smoking
status one at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime Resp_protC
CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime Resp_protB
Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime Resp_protB
Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime Resp_protB
Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime Resp_protB
Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug lnDay_Ptime Resp_protB
Smoker / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Retain respirator type and remove air or skin;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTotSkin_ISO_ug lnDay_Ptime Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnDay_Ptime Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Minimal difference between models with paint time and respirator type, and
either air or skin exposure;
```

```
*Stepwise introduction of variables with plasma TAHI level and TWAp_ISO_APF;
*Stepwise introduction of variables with plasma TAHI level and TWAp_ISO_APF;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add paint time;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Retain paint time, add booth type, glove use, coverall use, shop location,
weekday, and smoking status one at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime Gloves /
CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime Location
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime Day_week
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime Smoker /
CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Retain paint time and booth type;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add glove use;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Remove skin;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnDay_Ptime CrossSemi Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add skin back in, remove glove use;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnDay_Ptime CrossSemi
/ CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Retain paint time and booth type, remove air or skin;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTotSkin_ISO_ug lnDay_Ptime CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnDay_Ptime CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
*Retain paint time and booth type, remove booth type;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTotSkin_ISO_ug lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Final plasma TAHI model with TWAp_ISO and paint time;
*Final plasma TAHI model with TWAp_ISO and paint time;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Stepwise introduction of variables with plasma HDA level and TWAp_HDI;
*Stepwise introduction of variables with plasma HDA level and TWAp_HDI;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add paint time;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Paint time did not improve, remove, add respirator type, booth type, glove
use, coverall use, shop location, weekday, and smoking status one at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug Smoker / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Workplace factors did not improve base model with TWAp_HDI and skin;

*Stepwise introduction of variables with plasma HDA level and TWAp_HDI_APF;
*Stepwise introduction of variables with plasma HDA level and TWAp_HDI_APF;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Add paint time;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug lnDay_Ptime / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Paint time did not improve, remove, add respirator type, booth type, glove
use, coverall use, shop location, weekday, and smoking status one at a time;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug Smoker / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Workplace factors did not improve base model with TWAp_HDI_APF and skin;

*Repeat base models adding respirator type, booth type, glove use, coverall
use, shop location, weekday, and smoking status one at a time;
*This time remove TWAp_HDI, TWAp_HDI_APF, or skin for each added variable;

*Retain TWAp_HDI and remove skin;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI Smoker / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Retain TWAp_HDI_APF and remove skin;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF Smoker / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Retain skin and remove TWAp_HDI;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTotSkin_HDI_ug Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTotSkin_HDI_ug CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTotSkin_HDI_ug Gloves / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTotSkin_HDI_ug Cov / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTotSkin_HDI_ug Location / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTotSkin_HDI_ug Day_week / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

```
PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTotSkin_HDI_ug Smoker / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Try TWAp_HDI, TWAp_HDI_APF, and skin individually;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforePlasma METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnPmaBSA_HDAug = lnTotSkin_HDI_ug / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

*Skin has best model fit individually, but does not improve base models;
*Skin has best model fit individually, but does not improve base models;
```

SAS code B13: PROC GLM procedure to evaluate mixed model fit by calculating the marginal R^2 statistic for the goodness-of-fit of fixed effects. Because a REPEATED statement cannot be used within PROC GLM, geometric mean values for each worker were calculated with the daily exposure and biomarker levels. The following PROC GLM code was used to calculate the marginal R^2 statistics for mixed models predicting daily mean urine TAHI and HDA levels discussed in Chapter 4 (Models 1A-F; **Table 4.5**).

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Worker_ExposureBeforeUrine.xlsx"
      OUT = test.S1_Worker_ExposureBeforeUrine DBMS=xlsx REPLACE;
RUN;

*Calculating the marginal R2 statistic for goodness-of-fit of fixed effects
for Models 1A-C predicting daily urine TAHI levels;

*marginal R2 statistic for Model 1-A;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug AvgLnCRT;
RUN;

*marginal R2 statistic for Model 1-B;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL AvgLnUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug AvgLnCRT;
RUN;

*marginal R2 statistic for Model 1-C;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL AvgLnUR_TAHI = lnTWAp_ISO lnDay_Ptime CrossSemi;
RUN;

*Calculating the marginal R2 statistic for goodness-of-fit of fixed effects
for Models 1D-F predicting daily urine HDA levels;

*marginal R2 statistic for Model 1-D;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL AvgLnUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug AvgLnCRT;
RUN;
```

```
*marginal R2 statistic for Model 1-E;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL AvgLnUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug AvgLnCRT;
RUN;

*marginal R2 statistic for Model 1-F;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL AvgLnUR_HDA = lnTotSkin_HDI_ug AvgLnCRT Resp_protC;
RUN;
```

SAS code B14: PROC GLM procedure to evaluate mixed model fit by calculating the marginal R^2 statistic for the goodness-of-fit of fixed effects. Because a REPEATED statement cannot be used within PROC GLM, geometric mean values for each worker were calculated with the daily exposure and biomarker levels. The following PROC GLM code was used to calculate the marginal R^2 statistics for mixed models predicting plasma TAHI and HDA levels discussed in Chapter 4 (Models 2A-F; **Table 4.6**).

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Worker_ExposureBeforePlasma.xlsx"
      OUT = test.S1_Worker_ExposureBeforePlasma DBMS=xlsx REPLACE;
RUN;

*Calculating the marginal R2 statistic for goodness-of-fit of fixed effects
for Models 2A-C predicting plasma TAHI levels;

*marginal R2 statistic for Model 2-A;

PROC GLM DATA = test.S1_Worker_ExposureBeforePlasma;
CLASS WorkerNo;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO lnTotSkin_ISO_ug;
RUN;

*marginal R2 statistic for Model 2-B;

PROC GLM DATA = test.S1_Worker_ExposureBeforePlasma;
CLASS WorkerNo;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnTotSkin_ISO_ug;
RUN;

*marginal R2 statistic for Model 2-C;

PROC GLM DATA = test.S1_Worker_ExposureBeforePlasma;
CLASS WorkerNo;
MODEL lnPmaBSA_TAHIug = lnTWAp_ISO_APF lnDay_Ptime;
RUN;

*Calculating the marginal R2 statistic for goodness-of-fit of fixed effects
for Models 2D-F predicting plasma HDA levels;

*marginal R2 statistic for Model 2-D;

PROC GLM DATA = test.S1_Worker_ExposureBeforePlasma;
CLASS WorkerNo;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI lnTotSkin_HDI_ug;
RUN;
```

```
*marginal R2 statistic for Model 2-E;

PROC GLM DATA = test.S1_Worker_ExposureBeforePlasma;
CLASS WorkerNo;
MODEL lnPmaBSA_HDAug = lnTWAp_HDI_APF lnTotSkin_HDI_ug;
RUN;

*marginal R2 statistic for Model 2-F;

PROC GLM DATA = test.S1_Worker_ExposureBeforePlasma;
CLASS WorkerNo;
MODEL lnPmaBSA_HDAug = lnTotSkin_HDI_ug;
RUN;
```

SAS code B15: PROC MIXED procedure to build linear mixed-effects models with restricted maximum likelihood estimation of repeated measures. The TYPE statement sets the covariance structure as compound symmetry.

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Day_ExposureBeforeUrine.xlsx"
      OUT = test.S1_Day_ExposureBeforeUrine DBMS=xlsx REPLACE;
RUN;


PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnEndUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnEndUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnEndUR_TAHI = lnTWAp_ISO lnDay_Ptime CrossSemi / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;



PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnEndUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug lnEndCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnEndUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug lnCRT / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;

PROC MIXED DATA = test.S1_Day_ExposureBeforeUrine METHOD = reml Covtest;
CLASS WorkerNo Visit;
MODEL lnEndUR_HDA = lnTotSkin_HDI_ug lnCRT Resp_protC / CL;
REPEATED Visit / Type=CS Subject=WorkerNo;
RUN;
```

SAS code B16: PROC GLM procedure to evaluate mixed model fit by calculating the marginal R^2 statistic for the goodness-of-fit of fixed effects. Because a REPEATED statement cannot be used within PROC GLM, geometric mean values for each worker were calculated with the daily exposure and biomarker levels. The following PROC GLM code was used to calculate the marginal R^2 statistics for mixed models predicting post-shift urine TAHI and HDA levels discussed in Chapter 5 (Models 3A-F; **Table C.3**).

```
*Make a new library;

LIBNAME test "C:\Users\Zachary Robbins\Desktop\SAS_Input";

*Import exposure and biomarker data from Excel;

PROC IMPORT DATAFILE = "C:\Users\Zachary
Robbins\Desktop\SAS_Input\S1_Worker_ExposureBeforeUrine.xlsx"
      OUT = test.S1_Worker_ExposureBeforeUrine DBMS=xlsx REPLACE;
RUN;

*Calculating the marginal R2 statistic for goodness-of-fit of fixed effects
for Models 3A-C predicting post-shift urine TAHI levels;

*marginal R2 statistic for Model 3-A;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL lnEndUR_TAHI = lnTWAp_ISO lnTotSkin_ISO_ug lnCRT;
RUN;

*marginal R2 statistic for Model 3-B;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL lnEndUR_TAHI = lnTWAp_ISO_APF lnTotSkin_ISO_ug lnCRT;
RUN;

*marginal R2 statistic for Model 3-C;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL lnEndUR_TAHI = lnTWAp_ISO lnDay_Ptime CrossSemi;
RUN;

*Calculating the marginal R2 statistic for goodness-of-fit of fixed effects
for Models 3D-F predicting post-shift urine HDA levels;

*marginal R2 statistic for Model 3-D;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL lnEndUR_HDA = lnTWAp_HDI lnTotSkin_HDI_ug lnCRT;
RUN;
```

```
*marginal R2 statistic for Model 3-E;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL lnEndUR_HDA = lnTWAp_HDI_APF lnTotSkin_HDI_ug lnCRT;
RUN;

*marginal R2 statistic for Model 3-F;

PROC GLM DATA = test.S1_Worker_ExposureBeforeUrine;
CLASS WorkerNo;
MODEL lnEndUR_HDA = lnTotSkin_HDI_ug lnCRT Resp_protC;
RUN;
```

APPENDIX C: SUPPLEMENTAL TABLES FOR CHAPTER 5

Table C.1. Pearson correlations between HDI isocyanurate and HDI monomer exposure measures and creatinine-adjusted daily mean and post-shift urine TAHI and HDA levels (μg/g creatinine).

Table C.2. Associations between workplace factors and the post-shift creatinine-adjusted urine TAHI and HDA levels (μg/g creatinine) by stratification. General linear modeling was used to evaluate the significance of the compared variable in predicting urine biomarker levels given the indicated workplace condition.

Table C.3. Summary of linear mixed models for predicting post-shift urine TAHI and HDA levels (μg/L).

Table C.1. Pearson correlations between HDI isocyanurate and HDI monomer exposure measures and creatinine-adjusted daily mean and post-shift urine TAHI and HDA levels.

HDI isocyanurate			**Daily Mean Urine**				**Post-shift Urine**			
	Exposure level		TAHI (μg/g)		HDA (μg/g)		TAHI (μg/g)		HDA (μg/g)	
Explanatory	GM	GSD	*r*	*p* value	*r*	*p* value[a]	*r*	*p* value	*r*	*p* value[a]
PBZ (μg/m^3)	1969.2	2.9	0.27	**0.0038**	-0.07	0.4775	0.18	**0.0485**	-0.08	0.4006
PBZ-APF (μg/m^3)	65.3	9.4	0.05	0.5622	0.12	0.2210	-0.03	0.7763	0.04	0.6607
INH (μg)	635.0	3.9	0.34	**0.0002**	0.02	0.8382	0.29	**0.0014**	0.02	0.8249
INH-APF (μg)	21.1	9.9	0.13	0.1536	0.15	0.0990	0.06	0.5081	0.09	0.3434
Skin (μg)	170.2	15.4	0.22	**0.0162**	0.26	**0.0045**	0.18	0.0550	0.20	**0.0371**
HDI monomer			**Daily Mean Urine**				**Post-shift Urine**			
	Exposure level		TAHI (μg/g)		HDA (μg/g)		TAHI (μg/g)		HDA (μg/g)	
Explanatory	GM	GSD	*r*	*p* value	*r*	*p* value[a]	*r*	*p* value	*r*	*p* value[a]
PBZ (μg/m^3)	5.7	5.1	0.22	**0.0182**	0.14	0.1229	0.17	0.0686	0.18	0.0591
PBZ-APF (μg/m^3)	0.2	11.5	0.08	0.3876	0.23	**0.0132**	0.01	0.9185	0.19	**0.0425**
INH (μg)	1.9	6.1	0.30	**0.0011**	0.18	**0.0485**	0.27	**0.0040**	0.22	**0.0173**
INH-APF (μg)	0.1	11.8	0.16	0.0973	0.27	**0.0038**	0.09	0.3296	0.23	**0.0122**
Skin (μg)	0.005	331.7	0.18	0.0535	0.29	**0.0016**	0.19	**0.0488**	0.27	**0.0032**
Paint time (min)	13.9	2.4	0.22	**0.0194**	0.12	0.2191	0.24	**0.0101**	0.13	0.1519

μg/g = μg/g creatinine; GM = geometric mean; GSD = geometric standard deviation; *r* = Pearson correlation coefficient ([a]significance was determined at α-level 0.05); PBZ = personal breathing-zone (μg/m^3); PBZ-APF = APF-adjusted personal breathing-zone (μg/m^3); INH = inhalation exposure (μg); INH-APF = APF-adjusted inhalation exposure (μg).

Table C.2. Associations between workplace factors and the post-shift creatinine-adjusted urine TAHI and HDA levels (µg/g creatinine) by stratification. General linear modeling was used to evaluate the significance of the compared variable in predicting urine biomarker levels given the indicated workplace condition.

Workplace Condition		Compared Variable	Compared Categories	*N*	TAHI (µg/g creatinine)		HDA (µg/g creatinine)	
					N > MDL (%)	*p* value[a]	*N* > MDL (%)	*p* value[a]
Booth type	Cross/Semi	Respirator	None/Half-face	24	12 (50%)	0.6691	18 (75%)	0.9588
			Full-face[b]	13	8 (62%)		10 (77%)	
Booth type	Downdraft	Respirator	None/Half-face	61	12 (20%)	0.4963	43 (70%)	0.3773
			Full-face[b]	17	5 (29%)		11 (65%)	
Respirator	None/Half-face	Glove type	None/Latex	50	17 (34%)	0.2832	38 (76%)	0.9015
			Nitrile/Neoprene	30	6 (20%)		20 (67%)	
Respirator	Full-face[b]	Glove type	None/Latex	14	7 (50%)	0.5030	9 (64%)	0.6596
			Nitrile/Neoprene	16	6 (38%)		12 (75%)	
Coverall	No	Glove type	None/Latex	28	9 (32%)	0.7949	23 (82%)	0.7830
			Nitrile/Neoprene	5	1 (20%)		3 (60%)	
Coverall	Yes	Glove type	None/Latex	36	15 (42%)	0.2086	24 (67%)	0.5265
			Nitrile/Neoprene	41	11 (27%)		29 (71%)	
Respirator	None/Half-face	Booth type	Cross/Semi	24	12 (50%)	**0.0030**	18 (75%)	0.5052
			Downdraft	61	12 (20%)		43 (70%)	
Respirator	Full-face[b]	Booth type	Cross/Semi	13	8 (62%)	0.0782	10 (77%)	0.2968
			Downdraft	17	5 (29%)		11 (65%)	
Coverall	No	Booth type	Cross/Semi	17	8 (47%)	**0.0348**	13 (76%)	0.9113
			Downdraft	21	3 (14%)		16 (76%)	
Coverall	Yes	Booth type	Cross/Semi	20	12 (60%)	**0.0022**	15 (75%)	0.3267
			Downdraft	57	14 (25%)		38 (67%)	
Glove type	None/Latex	Booth type	Cross/Semi	22	13 (59%)	**0.0061**	17 (77%)	0.3791
			Downdraft	42	11 (26%)		30 (71%)	
Glove type	Nitrile/Neoprene	Booth type	Cross/Semi	15	7 (47%)	**0.0227**	11 (73%)	0.5494
			Downdraft	31	5 (16%)		21 (68%)	

N = number of samples; MDL = method detection limit; [a]Significance was determined by Tukey-Kramer multiple comparisons tests at α-level 0.05; [b]Full-face variable includes full-face air purifying, PAPR, or supplied-air respirators.

Table C.3. Summary of linear mixed models for predicting post-shift urine TAHI and HDA levels (µg/L).

TAHI level	Explanatory	Estimate	Standard Error	*p* value	AIC	R^2	HDA level	Explanatory	Estimate	Standard Error	*p* value	AIC	R^2
Base	Intercept	-5.99	3.93	0.1347	604.8	0.03	Base	Intercept	-15.30	2.34	<0.0001	569.4	0.29
Model 3-A	ISO PBZ	0.33	0.34	0.3330			Model 3-D	HDI PBZ	0.21	0.19	0.2615		
	ISO Skin	0.18	0.13	0.1826				HDI Skin	0.09	0.05	0.0923		
	Creatinine	-0.65	0.55	0.2360				Creatinine	2.48	0.44	**<0.0001**		
	Worker var	5.14	1.95	0.0085				Worker var	1.78	1.09	0.1038		
	Residual var	7.91	1.39	<0.0001				Residual var	6.70	1.16	<0.0001		
Base	Intercept	-2.33	3.07	0.4522	606.3	0.05	Base	Intercept	-15.31	2.25	<0.0001	566.9	0.30
Model 3-B	ISO PBZ-APF	-0.16	0.18	0.3928			Model 3-E	HDI PBZ-APF	0.28	0.13	**0.0355**		
	ISO Skin	0.25	0.13	0.0546				HDI Skin	0.08	0.05	0.1400		
	Creatinine	-0.83	0.55	0.1407				Creatinine	2.63	0.45	**<0.0001**		
	Worker var	4.98	1.95	0.0107				Worker var	1.83	1.08	0.0909		
	Residual var	8.02	1.41	<0.0001				Residual var	6.44	1.12	<0.0001		
Final	Intercept	-10.39	2.64	0.0003	595.4	0.25	Final	Intercept	-14.92	2.27	<0.0001	566.3	0.29
Model 3-C	ISO PBZ	0.49	0.30	0.1068			Model 3-F	HDI Skin	0.11	0.05	**0.0273**		
	Paint time	0.96	0.40	**0.0188**				Creatinine	2.54	0.45	**<0.0001**		
	Booth type	-2.49	0.77	**0.0024**				Respirator type	-0.95	0.74	0.2012		
	Worker var	2.60	1.48	0.0802				Worker var	1.94	1.13	0.0870		
	Residual var	8.30	1.45	<0.0001				Residual var	6.57	1.14	<0.0001		

AIC = Akaike's Information Criterion; R^2 = marginal R^2 statistic calculated with the PROC GLM procedure in SAS; ISO = HDI isocyanurate; HDI = HDI monomer; var = variance.

REFERENCES

Aalto-Korte, K., Pesonen, M., Kuuliala, O., Alanko, K. & Jolanki, R., 2010. Contact allergy to aliphatic polyisocyanates based on hexamethylene-1,6-diisocyanate (HDI). *Contact Dermatitis,* 63**,** 357-63.

Aalto-Korte, K., Suuronen, K., Kuuliala, O., Henriks-Eckerman, M.L. & Jolanki, R., 2012. Occupational contact allergy to monomeric isocyanates. *Contact Dermatitis,* 67**,** 78-88.

ACGIH, 2015. 1,6-Hexamethylene Diisocyanate: BEI® 7th Edition Documentation. Cincinnati, OH: American Conference of Governmental Industrial Hygienists.

ACGIH, 2019. *TLVs® and BEIs®. Guide to Occupational Exposure Values* Cincinnati, OH: American Conference of Governmental Industrial Hygienists.

Adams, W.C., 1993. *Measurement of breathing rate and volume in routinely performed daily activities.* California Air Resources Board A033-205.

Alexandersson, R., Hedenstierna, G., Plato, N. & Kolmodin-Hedman, B., 1987. Exposure, lung function, and symptoms in car painters exposed to hexamethylendiisocyanate and biuret modified hexamethylendiisocyanate. *Arch Environ Health,* 42**,** 367-73.

Almeida, A.M., Castel-Branco, M.M. & Falcao, A.C., 2002. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. *J Chromatogr B Analyt Technol Biomed Life Sci,* 774**,** 215-22.

Austin, S., 2007. Biological monitoring of TDI-derived amines in polyurethane foam production. *Occup Med (Lond),* 57**,** 444-8.

Bailey, E., Brooks, A.G., Bird, I., Farmer, P.B. & Street, B., 1990. Monitoring exposure to 4,4'-methylenedianiline by the gas chromatography-mass spectrometry determination of adducts to hemoglobin. *Anal Biochem,* 190**,** 175-81.

Bayer MaterialScience, 2005. *The Chemistry of Polyurethane Coatings* [online]. https://www.pharosproject.net/uploads/files/cml/1383145151.pdf [Accessed 31 January 2019].

Bello, A., Xue, Y., Gore, R., Woskie, S. & Bello, D., 2019. Assessment and control of exposures to polymeric methylene diphenyl diisocyanate (pMDI) in spray polyurethane foam applicators. *Int J Hyg Environ Health,* 222**,** 804-815.

Bello, D., Herrick, C.A., Smith, T.J., Woskie, S.R., Streicher, R.P., Cullen, M.R., Liu, Y. & Redlich, C.A., 2007a. Skin exposure to isocyanates: reasons for concern. *Environ Health Perspect,* 115**,** 328-35.

Bello, D., Redlich, C.A., Stowe, M.H., Sparer, J., Woskie, S.R., Streicher, R.P., Hosgood, H.D. & Liu, Y., 2008. Skin exposure to aliphatic polyisocyanates in the auto body repair and refinishing industry: II. A quantitative assessment. *Ann Occup Hyg,* 52**,** 117-24.

Bello, D., Smith, T.J., Woskie, S.R., Streicher, R.P., Boeniger, M.F., Redlich, C.A. & Liu, Y., 2006. An FTIR investigation of isocyanate skin absorption using in vitro guinea pig skin. *J Environ Monit,* 8**,** 523-9.

Bello, D., Sparer, J., Redlich, C.A., Ibrahim, K., Stowe, M.H. & Liu, Y., 2007b. Slow curing of aliphatic polyisocyanate paints in automotive refinishing: a potential source for skin exposure. *J Occup Environ Hyg,* 4**,** 406-11.

Bello, D., Streicher, R.P., Liu, Y.C., Sparer, J., Young, F. & Woskie, S.R., 2002. Field comparison of impingers and treated filters for sampling of total aliphatic isocyanates with the MAP reagent. *AIHA J (Fairfax, Va),* 63**,** 790-6.

Bello, D., Woskie, S.R., Streicher, R.P., Liu, Y., Stowe, M.H., Eisen, E.A., Ellenbecker, M.J., Sparer, J., Youngs, F., Cullen, M.R. & Redlich, C.A., 2004. Polyisocyanates in occupational environments: a critical review of exposure limits and metrics. *Am J Ind Med,* 46**,** 480-91.

Bennett, J., Marlow, D., Nourian, F., Breay, J., Feng, A. & Methner, M., 2018. Effect of ventilation velocity on hexavalent chromium and isocyanate exposures in aircraft paint spraying. *J Occup Environ Hyg,* 15**,** 167-181.

Bernstein, J.A., 1996. Overview of diisocyanate occupational asthma. *Toxicology,* 111**,** 181-9.

Blake, C.L., Johnson, G.T., Abritis, A.J., Lieckfield, R., Jr. & Harbison, R.D., 2012. Isocyanate and VOC exposure analysis using Flexane(R). *Regul Toxicol Pharmacol,* 63**,** 363-70.

Booth, K., Cummings, B., Karoly, W.J., Mullins, S., Robert, W.P., Spence, M., Lichtenberg, F.W. & Banta, J., 2009. Measurements of airborne methylene diphenyl diisocyanate (MDI) concentration in the U.S. workplace. *J Occup Environ Hyg,* 6**,** 228-38.

Boutin, M., Dufresne, A., Ostiguy, C. & Lesage, J., 2006. Determination of airborne isocyanates generated during the thermal degradation of car paint in body repair shops. *Ann Occup Hyg,* 50**,** 385-93.

Brorson, T., Skarping, G. & Nielsen, J., 1990a. Biological monitoring of isocyanates and related amines. II. Test chamber exposure of humans to 1,6-hexamethylene diisocyanate (HDI). *Int Arch Occup Environ Health,* 62**,** 385-9.

Brorson, T., Skarping, G., Sandstrom, J.F. & Stenberg, M., 1990b. Biological monitoring of isocyanates and related amines. I. Determination of 1,6-hexamethylene diamine (HDA) in hydrolysed human urine after oral administration of HDA. *Int Arch Occup Environ Health,* 62**,** 79-84.

Brzeznicki, S. & Bonczarowska, M., 2015. Occupational exposure to selected isocyanates in Polish industry. *Med Pr,* 66**,** 291-301.

Budnik, L.T., Nowak, D., Merget, R., Lemiere, C. & Baur, X., 2011. Elimination kinetics of diisocyanates after specific inhalative challenges in humans: mass spectrometry analysis, as a basis for biomonitoring strategies. *J Occup Med Toxicol,* 6**,** 9.

California OEHHA, 2019. *Proposed Reference Exposure Levels for Hexamethylene Diisocyanate (Monomer and Polyisocyanates): Scientific Review Panel Draft, OEHHA*. Officue of Environmental Health Hazard Assessment, California Environmental Protection Agency, Sacramento, CA. Available from: https://oehha.ca.gov/media/downloads/crnr/hdirelssrpreviewdraft020419.pdf [Accessed 12 June 2019]

Campo, P., Wisnewski, A.V., Lummus, Z., Cartier, A., Malo, J.L., Boulet, L.P. & Bernstein, D.I., 2007. Diisocyanate conjugate and immunoassay characteristics influence detection of specific antibodies in HDI-exposed workers. *Clin Exp Allergy,* 37**,** 1095-102.

Carbonnelle, P., Boukortt, S., Lison, D. & Buchet, J.P., 1996. Determination of toluenediamines in urine of workers occupationally exposed to isocyanates by high-performance liquid chromatography. *Analyst,* 121**,** 663-9.

Carlton, G.N. & England, E.C., 2000. Exposures to 1,6-hexamethylene diisocyanate during polyurethane spray painting in the U.S. Air Force. *Appl Occup Environ Hyg,* 15**,** 705-12.

Ceballos, D., Reeb-Whitaker, C., Glazer, P., Murphy-Robinson, H. & Yost, M., 2014a. Understanding factors that influence protective glove use among automotive spray painters. *J Occup Environ Hyg,* 11**,** 306-13.

Ceballos, D.M., Fent, K.W., Whittaker, S.G., Gaines, L.G., Thomasen, J.M., Flack, S.L., Nylander-French, L.A., Yost, M.G. & Reeb-Whitaker, C.K., 2011. Survey of dermal protection in Washington State collision repair industry. *J Occup Environ Hyg,* 8**,** 551-60.

Ceballos, D.M., Sasakura, M., Reeb-Whitaker, C., Broadwater, K., Milbauer, M., Crampton, R., Dills, R. & Yost, M.G., 2014b. Testing of glove efficacy against sprayed isocyanate coatings utilizing a reciprocating permeation panel. *Ann Occup Hyg,* 58**,** 50-9.

Ceballos, D.M., West, C., Methner, M. & Gong, W., 2017. Evaluation of chromium, hexavalent chromium, cadmium, and isocyanate exposures in an aircraft refinishing plant. *In* H.H. Evaluations (ed.) Cincinnati, OH: Centers for Disease Control and Prevention.

Chan-Yeung, M. & Malo, J.L., 1995. Occupational asthma. *N Engl J Med,* 333**,** 107-12.

Cocker, J., 2007. Biological monitoring for isocyanates. *Occup Med (Lond),* 57**,** 391-3.

Cocker, J., Jones, K., Morton, J. & Mason, H.J., 2007. Biomonitoring at the UK Health and Safety Laboratory. *Int J Hyg Environ Health,* 210**,** 383-6.

Covestro, 2017. *Aliphatic Diisocyanate Monomers: Safe Handling Guidance* [online]. https://www.productsafetyfirst.covestro.com/en/resources/resources/detail-pages/aliphatic-diisocyanate-monomers-safe-handling-guidance [Accessed 14 June 2019].

Covestro, 2018a. *Product Safety Summary for HDI* [online]. https://www.productsafetyfirst.covestro.com/en/resources/resources/detail-pages/product-safety-summary-hdi [Accessed 14 June 2019].

Covestro, 2018b. *Product Safety Summary for HDI Based Polyisocyanates* [online]. https://www.productsafetyfirst.covestro.com/en/resources/resources/detail-pages/product-safety-summary-hdi-based-polyisocyanates [Accessed 21 July 2019].

Creely, K.S., Hughson, G.W., Cocker, J. & Jones, K., 2006. Assessing isocyanate exposures in polyurethane industry sectors using biological and air monitoring methods. *Ann Occup Hyg,* 50**,** 609-21.

Crespo, J. & Galan, J., 1999. Exposure to MDI during the process of insulating buildings with sprayed polyurethane foam. *Ann Occup Hyg,* 43**,** 415-9.

Dahlqvist, M., Tornling, G., Plato, N. & Ulfvarson, U., 1995. Effects within the week on forced vital capacity are correlated with long term changes in pulmonary function: reanalysis of studies on car painters exposed to isocyanate. *Occup Environ Med,* 52**,** 192-5.

Dalene, M., Skarping, G. & Brorson, T., 1990. Chromatographic determination of amines in biological fluids with special reference to the biological monitoring of isocyanates and amines. IV. Determination of 1,6-hexamethylenediamine in human urine using capillary gas chromatography and selective ion monitoring. *J Chromatogr,* 516**,** 405-13.

Dalene, M., Skarping, G. & Brunmark, P., 1995. Assessment of occupational exposure to 4,4'-methylenedianiline by the analysis of urine and blood samples. *Int Arch Occup Environ Health,* 67**,** 67-72.

Dalene, M., Skarping, G. & Tinnerberg, H., 1994. Biological monitoring of hexamethylene diisocyanate by determination of 1,6-hexamethylene diamine as the trifluoroethyl chloroformate derivative using capillary gas chromatography with thermoionic and selective-ion monitoring. *J Chromatogr B Biomed Appl,* 656**,** 319-28.

Darcey, D., Lipscomb, H.J., Epling, C., Pate, W., Cherry, L.P. & Bernstein, J., 2002. Clinical findings for residents near a polyurethane foam manufacturing plant. *Arch Environ Health,* 57**,** 239-46.

De Palma, G., Cortesi, I., Ghitti, R., Festa, D., Bergonzi, R. & Apostoli, P., 2012. Biological monitoring as a valid tool to assess occupational exposure to mixtures of 2,4-:2,6-toluene diisocyanate. *Med Lav,* 103**,** 361-71.

De Vries, T.T., Bello, D., Stowe, M.H., Harari, H., Slade, M.D. & Redlich, C.A., 2012. Transferability of aliphatic isocyanates from recently applied paints to the skin of auto body shop workers. *J Occup Environ Hyg,* 9**,** 699-711.

Dow Chemical Company, 2010. *Product Safety Assessment DOW Hexamethylene Diisocyanate (HDI)-Based Polymer Products* [online]. http://msdssearch.dow.com/PublishedLiteratureDOWCOM/dh_07ed/0901b803807edd97.pdf?filepath=productsafety/pdfs/noreg/233-00638.pd.&fromPage=GetDoc [Accessed 07 February 2019].

Fent, K.W., Gaines, L.G., Thomasen, J.M., Flack, S.L., Ding, K., Herring, A.H., Whittaker, S.G. & Nylander-French, L.A., 2009a. Quantification and statistical modeling--part I: breathing-zone concentrations of monomeric and polymeric 1,6-hexamethylene diisocyanate. *Ann Occup Hyg,* 53**,** 677-89.

Fent, K.W., Jayaraj, K., Ball, L.M. & Nylander-French, L.A., 2008. Quantitative monitoring of dermal and inhalation exposure to 1,6-hexamethylene diisocyanate monomer and oligomers. *J Environ Monit,* 10**,** 500-7.

Fent, K.W., Jayaraj, K., Gold, A., Ball, L.M. & Nylander-French, L.A., 2006. Tape-strip sampling for measuring dermal exposure to 1,6-hexamethylene diisocyanate. *Scand J Work Environ Health,* 32**,** 225-40.

Fent, K.W., Trelles Gaines, L.G., Thomasen, J.M., Flack, S.L., Ding, K., Herring, A.H., Whittaker, S.G. & Nylander-French, L.A., 2009b. Quantification and statistical modeling--part II: dermal concentrations of monomeric and polymeric 1,6-hexamethylene diisocyanate. *Ann Occup Hyg,* 53**,** 691-702.

Ferguson, J.S., Schaper, M. & Alarie, Y., 1987. Pulmonary effects of a polyisocyanate aerosol: hexamethylene diisocyanate trimer (HDIt) or Desmodur-N (DES-N). *Toxicol Appl Pharmacol,* 89**,** 332-46.

Flack, S.L., Ball, L.M. & Nylander-French, L.A., 2010a. Occupational exposure to HDI: progress and challenges in biomarker analysis. *J Chromatogr B Analyt Technol Biomed Life Sci,* 878**,** 2635-42.

Flack, S.L., Fent, K.W., Gaines, L.G., Thomasen, J.M., Whittaker, S.G., Ball, L.M. & Nylander-French, L.A., 2011. Hemoglobin adducts in workers exposed to 1,6-hexamethylene diisocyanate. *Biomarkers,* 16**,** 261-70.

Flack, S.L., Fent, K.W., Trelles Gaines, L.G., Thomasen, J.M., Whittaker, S., Ball, L.M. & Nylander-French, L.A., 2010b. Quantitative plasma biomarker analysis in HDI exposure assessment. *Ann Occup Hyg,* 54**,** 41-54.

Fletcher, M., 2015. Spray Painters' Skin Exposure to 1,6-Hexamethylene Diisocyanate Monomer and Its Oligomers. Masters Paper. University of North Carolina at Chapel Hill.

Floyd, E.L., Henry, J.B. & Johnson, D.L., 2018. Influence of facial hair length, coarseness, and areal density on seal leakage of a tight-fitting half-face respirator. *J Occup Environ Hyg,* 15**,** 334-340.

Frick, M., Bjorkner, B., Hamnerius, N. & Zimerson, E., 2003a. Allergic contact dermatitis from dicyclohexylmethane-4,4'-diisocyanate. *Contact Dermatitis,* 48**,** 305-9.

Frick, M., Isaksson, M., Bjorkner, B., Hindsen, M., Ponten, A. & Bruze, M., 2003b. Occupational allergic contact dermatitis in a company manufacturing boards coated with isocyanate lacquer. *Contact Dermatitis,* 48**,** 255-60.

Gaines, L.G., Fent, K.W., Flack, S.L., Thomasen, J.M., Ball, L.M., Richardson, D.B., Ding, K., Whittaker, S.G. & Nylander-French, L.A., 2010a. Urine 1,6-hexamethylene diamine (HDA) levels among workers exposed to 1,6-hexamethylene diisocyanate (HDI). *Ann Occup Hyg,* 54**,** 678-91.

Gaines, L.G., Fent, K.W., Flack, S.L., Thomasen, J.M., Ball, L.M., Zhou, H., Whittaker, S.G. & Nylander-French, L.A., 2010b. Effect of creatinine and specific gravity normalization on urinary biomarker 1,6-hexamethylene diamine. *J Environ Monit,* 12**,** 591-9.

Gaines, L.G., Fent, K.W., Flack, S.L., Thomasen, J.M., Whittaker, S.G. & Nylander-French, L.A., 2011. Factors affecting variability in the urinary biomarker 1,6-hexamethylene diamine in workers exposed to 1,6-hexamethylene diisocyanate. *J Environ Monit,* 13**,** 119-27.

Geddie, J.E., North, C., Occupational, S. & Health, D., 2011. *A guide to occupational exposure to isocyanates* Raleigh, N.C.: Occupational Safety and Health Division, N.C. Dept. of Labor.

Geens, T., Dugardin, S., Schockaert, A., De Cooman, G. & Van Sprundel, M., 2012. Air exposure assessment of TDI and biological monitoring of TDA in urine in workers in polyurethane foam industry. *Occup Environ Med,* 69**,** 93-8.

Glindmeyer, H.W., Lefante, J.J., Jr., Rando, R.J., Freyder, L., Hnizdo, E. & Jones, R.N., 2004. Spray-painting and chronic airways obstruction. *Am J Ind Med,* 46**,** 104-11.

Goossens, A., Detienne, T. & Bruze, M., 2002. Occupational allergic contact dermatitis caused by isocyanates. *Contact Dermatitis,* 47**,** 304-8.

Gui, W., Wisnewski, A.V., Neamtiu, I., Gurzau, E., Sparer, J.A., Stowe, M.H., Liu, J., Slade, M.D., Rusu, O.A. & Redlich, C.A., 2014. Inception cohort study of workers exposed to toluene diisocyanate at a polyurethane foam factory: initial one-year follow-up. *Am J Ind Med,* 57**,** 1207-15.

Harari, H., Bello, D., Woskie, S. & Redlich, C., 2016. Development of an Interception Glove Sampler for Skin Exposures to Aromatic Isocyanates. *Ann Occup Hyg,* 60**,** 1092-1103.

Haycock, G.B., Schwartz, G.J. & Wisotsky, D.H., 1978. Geometric method for measuring body surface area: a height-weight formula validated in infants, children, and adults. *J Pediatr,* 93**,** 62-6.

Henneken, H., Vogel, M. & Karst, U., 2007. Determination of airborne isocyanates. *Anal Bioanal Chem,* 387**,** 219-36.

Henriks-Eckerman, M.L., Makela, E.A., Laitinen, J., Ylinen, K., Suuronen, K., Vuokko, A. & Sauni, R., 2015. Role of dermal exposure in systemic intake of methylenediphenyl diisocyanate (MDI) among construction and boat building workers. *Toxicol Lett,* 232**,** 595-600.

Herrick, C.A., Xu, L., Wisnewski, A.V., Das, J., Redlich, C.A. & Bottomly, K., 2002. A novel mouse model of diisocyanate-induced asthma showing allergic-type inflammation in the lung after inhaled antigen challenge. *J Allergy Clin Immunol,* 109**,** 873-8.

Hu, J., Cantrell, P. & Nand, A., 2017. Comprehensive Biological Monitoring to Assess Isocyanates and Solvents Exposure in the NSW Australia Motor Vehicle Repair Industry. *Ann Work Expo Health,* 61**,** 1015-1023.

Hurley, P.J., 1975. Red cell and plasma volumes in normal adults. *J Nucl Med,* 16**,** 46-52.

Janko, M., Mccarthy, K., Fajer, M. & Van Raalte, J., 1992. Occupational exposure to 1,6-hexamethylene diisocyanate-based polyisocyanates in the state of Oregon, 1980-1990. *Am Ind Hyg Assoc J,* 53**,** 331-8.

Jarand, C.W., Akapo, S.O., Swenson, L.J. & Kelman, B.J., 2002. Diisocyanate emission from a paint product: a preliminary analysis. *Appl Occup Environ Hyg,* 17**,** 491-4.

Jones, K., Cocker, J. & Piney, M., 2013. Isocyanate exposure control in motor vehicle paint spraying: evidence from biological monitoring. *Ann Occup Hyg,* 57**,** 200-9.

Jones, K., Johnson, P.D., Baldwin, P.E.J., Coldwell, M., Cooke, J., Keen, C., Harding, A.H., Smith, D. & Cocker, J., 2017. Exposure to Diisocyanates and Their Corresponding Diamines in Seven Different Workplaces. *Ann Work Expo Health,* 61**,** 383-393.

Kaaria, K., Hirvonen, A., Norppa, H., Piirila, P., Vainio, H. & Rosenberg, C., 2001. Exposure to 2,4- and 2,6-toluene diisocyanate (TDI) during production of flexible foam: determination of airborne TDI and urinary 2,4- and 2,6-toluenediamine (TDA). *Analyst,* 126**,** 1025-31.

Karol, M.H., Hauth, B.A., Riley, E.J. & Magreni, C.M., 1981. Dermal contact with toluene diisocyanate (TDI) produces respiratory tract hypersensitivity in guinea pigs. *Toxicol Appl Pharmacol,* 58**,** 221-30.

Karoly, W.J., Flatley, J.J., Stevenson, R.D. & Bowers, J.D., 2004. Airborne concentrations of methylene diphenyl diisocyanate (MDI) in North American wood mills during the manufacturing of oriented strand board (OSB). *J Occup Environ Hyg,* 1**,** 789-98.

Kelly, T.J., Myers, J.D. & Holdren, M.W., 1999. Testing of household products and materials for emission of toluene diisocyanate. *Indoor Air,* 9**,** 117-24.

Kiec-Swierczynska, M., Swierczynska-Machura, D., Chomiczewska-Skora, D., Nowakowska-Swirta, E. & Krecisz, B., 2014. Occupational allergic and irritant contact dermatitis in workers exposed to polyurethane foam. *Int J Occup Med Environ Health,* 27**,** 196-205.

Lesage, J., Stanley, J., Karoly, W.J. & Lichtenberg, F.W., 2007. Airborne methylene diphenyl diisocyanate (MDI) concentrations associated with the application of polyurethane spray foam in residential construction. *J Occup Environ Hyg,* 4**,** 145-55.

Liippo, J. & Lammintausta, K., 2008. Contact sensitization to 4,4'-diaminodiphenylmethane and to isocyanates among general dermatology patients. *Contact Dermatitis,* 59**,** 109-14.

Liljelind, I., Norberg, C., Egelrud, L., Westberg, H., Eriksson, K. & Nylander-French, L.A., 2010. Dermal and inhalation exposure to methylene bisphenyl isocyanate (MDI) in iron foundry workers. *Ann Occup Hyg,* 54**,** 31-40.

Lind, P., Dalene, M., Skarping, G. & Hagmar, L., 1996. Toxicokinetics of 2,4- and 2,6-toluenediamine in hydrolysed urine and plasma after occupational exposure to 2,4- and 2,6- toluene diisocyanate. *Occup Environ Med,* 53**,** 94-9.

Lind, P., Dalene, M., Tinnerberg, H. & Skarping, G., 1997. Biomarkers in hydrolysed urine, plasma and erythrocytes among workers exposed to thermal degradation products from toluene diisocyanate foam. *Analyst,* 122**,** 51-6.

Littorin, M., Rylander, L., Skarping, G., Dalene, M., Welinder, H., Stromberg, U. & Skerfving, S., 2000. Exposure biomarkers and risk from gluing and heating of polyurethane: a cross sectional study of respiratory symptoms. *Occup Environ Med,* 57**,** 396-405.

Liu, Q. & Wisnewski, A.V., 2003. Recent developments in diisocyanate asthma. *Ann Allergy Asthma Immunol,* 90**,** 35-41.

Liu, Y., Bello, D., Sparer, J.A., Stowe, M.H., Gore, R.J., Woskie, S.R., Cullen, M.R. & Redlich, C.A., 2007. Skin exposure to aliphatic polyisocyanates in the auto body repair and refinishing industry: a qualitative assessment. *Ann Occup Hyg,* 51**,** 429-39.

Liu, Y., Berode, M., Stowe, M.H., Holm, C.T., Walsh, F.X., Slade, M.D., Boeniger, M.F. & Redlich, C.A., 2004. Urinary hexane diamine to assess respiratory exposure to hexamethylene diisocyanate aerosol: a human inhalation study. *Int J Occup Environ Health,* 10**,** 262-71.

Liu, Y., Sparer, J., Woskie, S.R., Cullen, M.R., Chung, J.S., Holm, C.T. & Redlich, C.A., 2000. Qualitative assessment of isocyanate skin exposure in auto body shops: a pilot study. *Am J Ind Med,* 37**,** 265-74.

Liu, Y., Stowe, M.H., Bello, D., Woskie, S.R., Sparer, J., Gore, R., Youngs, F., Cullen, M.R. & Redlich, C.A., 2006. Respiratory protection from isocyanate exposure in the autobody repair and refinishing industry. *J Occup Environ Hyg,* 3**,** 234-49.

Lockey, J.E., Redlich, C.A., Streicher, R., Pfahles-Hutchens, A., Hakkinen, P.B., Ellison, G.L., Harber, P., Utell, M., Holland, J., Comai, A. & White, M., 2015. Isocyanates and human health: multistakeholder information needs and research priorities. *J Occup Environ Med,* 57**,** 44-51.

Ma-Hock, L., Gamer, A.O., Deckardt, K., Leibold, E. & Van Ravenzwaay, B., 2007. Determination of pulmonary irritant threshold concentrations of hexamethylene-1,6-diisocyanate (HDI) prepolymers by bronchoalveolar lavage in acute rat inhalation studies according to TRGS 430. *Food Chem Toxicol,* 45**,** 237-43.

Maitre, A., Berode, M., Perdrix, A., Romazini, S. & Savolainen, H., 1993. Biological monitoring of occupational exposure to toluene diisocyanate. *Int Arch Occup Environ Health,* 65**,** 97-100.

Maitre, A., Berode, M., Perdrix, A., Stoklov, M., Mallion, J.M. & Savolainen, H., 1996. Urinary hexane diamine as an indicator of occupational exposure to hexamethylene diisocyanate. *Int Arch Occup Environ Health,* 69**,** 65-8.

Malo, J.L., Ouimet, G., Cartier, A., Levitz, D. & Zeiss, C.R., 1983. Combined alveolitis and asthma due to hexamethylene diisocyanate (HDI), with demonstration of crossed respiratory and immunologic reactivities to diphenylmethane diisocyanate (MDI). *J Allergy Clin Immunol,* 72**,** 413-9.

Marand, A., Karlsson, D., Dalene, M. & Skarping, G., 2004a. Determination of amines as pentafluoropropionic acid anhydride derivatives in biological samples using liquid chromatography and tandem mass spectrometry. *Analyst,* 129**,** 522-8.

Marand, A., Karlsson, D., Dalene, M. & Skarping, G., 2004b. Extractable organic compounds in polyurethane foam with special reference to aromatic amines and derivatives thereof. *Analytica Chimica Acta,* 510**,** 109-119.

Mellette, M.P., Bello, D., Xue, Y., Yost, M., Bello, A. & Woskie, S., 2019. Evaluation of Disposable Protective Garments against Isocyanate Permeation and Penetration from Polyurethane Anticorrosion Coatings. *Ann Work Expo Health,* 63**,** 592-603.

NIOSH, 1978. *Criteria Documents - Criteria for a Recommended Standard: Occupational Exposure to Diisocyanates.* National Institute for Occupational Safety and Health, Cincinnati, OH, 78-215.

NIOSH, 1996. *Preventing Asthma and Death from Diisocyanate Exposure*. National Institute for Occupational Safety and Health, Cincinnati, OH, 96-111.

NIOSH, 2004. *A Summary of Health Hazard Evaluations: Issues Related to Occupational Exposure to Isocyanates, 1989 to 2002*. National Institute for Occupational Safety and Health, Cincinnati, OH, 2004-116.

Oregon OSHA, 2017. *Oregon Rules for Air Contaminants*. Oregon Occupational Safety and Health Division, Salem, OR, AO 3-2017.

Orelien, J.G. & Edwards, L.J., 2008. Fixed-effect variable selection in linear mixed models using R2 statistics. *Computational Statistics & Data Analysis,* 52**,** 1896-1907.

OSHA, 2009. *Assigned protection factors for the revised respiratory protection standard*. Occupational Safety and Health Administration, U.S. Department of Labor, Washington, DC, 3352-02.

OSHA, 2018. *Hexamethylene Diisocyanate: OSHA Occupational Chemical Database: Occupational Safety and Health Administration, U.S. Department of Labor* [online]. https://www.osha.gov/chemicaldata/chemResult.html?recNo=715 [Accessed 09 June 2019].

OSHA, 2019a. *1,6-Hexamethylene Diisocyanate Homopolymer: OSHA Occupational Chemical Database: Occupational Safety and Health Administration, U.S. Department of Labor* [online]. https://www.osha.gov/chemicaldata/chemResult.html?recNo=808 [Accessed 09 June 2019].

OSHA, 2019b. *Hexamethylene Diisocyanate Biuret: OSHA Occupational Chemical Database: Occupational Safety and Health Administration, U.S. Department of Labor* [online]. https://www.osha.gov/chemicaldata/chemResult.html?recNo=788 [Accessed 09 June 2019].

Pauluhn, J., 2000. Inhalation toxicity of 1,6-hexamethylene diisocyanate homopolymer (HDI-IC) aerosol: results of single inhalation exposure studies. *Toxicol Sci,* 58**,** 173-81.

Pauluhn, J., 2002a. Critical analysis of biomonitoring endpoints for measuring exposure to polymeric diphenyl-methane-4,4'-diisocyanate (MDI) in rats: a comparison of markers of exposure and markers of effect. *Arch Toxicol,* 76**,** 13-22.

Pauluhn, J., 2002b. Short-term inhalation toxicity of polyisocyanate aerosols in rats: comparative assessment of irritant-threshold concentrations by bronchoalveolar lavage. *Inhal Toxicol,* 14**,** 287-301.

Pauluhn, J., 2004. Pulmonary irritant potency of polyisocyanate aerosols in rats: comparative assessment of irritant threshold concentrations by bronchoalveolar lavage. *J Appl Toxicol,* 24**,** 231-47.

Pauluhn, J., 2015. Analysis of the interrelationship of the pulmonary irritation and elicitation thresholds in rats sensitized with 1,6-hexamethylene diisocyanate (HDI). *Inhal Toxicol,* 27**,** 191-206.

Pauluhn, J., Eidmann, P. & Mohr, U., 2002. Respiratory hypersensitivity in guinea pigs sensitized to 1,6-hexamethylene diisocyanate (HDI): comparison of results obtained with the monomer and homopolymers of HDI. *Toxicology,* 171**,** 147-60.

Pauluhn, J. & Lewalter, J., 2002. Analysis of markers of exposure to polymeric methylene-diphenyl diisocyanate (pMDI) in rats: a comparison of dermal and inhalation routes of exposure. *Exp Toxicol Pathol,* 54**,** 135-46.

Pauluhn, J. & Mohr, U., 2001. Inhalation toxicity of 1,6-hexamethylene diisocyanate homopolymers (HDI-IC and HDI-BT): results of subacute and subchronic repeated inhalation exposure studies. *Inhal Toxicol,* 13**,** 513-32.

Petsonk, E.L., Wang, M.L., Lewis, D.M., Siegel, P.D. & Husberg, B.J., 2000. Asthma-like symptoms in wood product plant workers exposed to methylene diphenyl diisocyanate. *Chest,* 118**,** 1183-93.

Piirila, P.L., Nordman, H., Keskinen, H.M., Luukkonen, R., Salo, S.P., Tuomi, T.O. & Tuppurainen, M., 2000. Long-term follow-up of hexamethylene diisocyanate-, diphenylmethane diisocyanate-, and toluene diisocyanate-induced asthma. *Am J Respir Crit Care Med,* 162**,** 516-22.

Pourabedian, S., Barkhordari, A., Habibi, E., Rismanchiyan, M. & Zare, M., 2010. Effect of 1,6-hexamethylene diisocyanate exposure on peak flowmetry in automobile paint shop workers in Iran. *Arh Hig Rada Toksikol,* 61**,** 183-9.

Pronk, A., Preller, L., Raulf-Heimsoth, M., Jonkers, I.C., Lammers, J.W., Wouters, I.M., Doekes, G., Wisnewski, A.V. & Heederik, D., 2007. Respiratory symptoms, sensitization, and exposure response relationships in spray painters exposed to isocyanates. *Am J Respir Crit Care Med,* 176**,** 1090-7.

Pronk, A., Tielemans, E., Skarping, G., Bobeldijk, I., J, V.a.N.H., Heederik, D. & Preller, L., 2006a. Inhalation exposure to isocyanates of car body repair shop workers and industrial spray painters. *Ann Occup Hyg,* 50**,** 1-14.

Pronk, A., Yu, F., Vlaanderen, J., Tielemans, E., Preller, L., Bobeldijk, I., Deddens, J.A., Latza, U., Baur, X. & Heederik, D., 2006b. Dermal, inhalation, and internal exposure to 1,6-HDI and its oligomers in car body repair shop workers and industrial spray painters. *Occup Environ Med,* 63**,** 624-31.

Randall, D. & Lee, S., 2002. *The Polyurethanes Book*, New York, NY: Wiley.

Randolph, B.W., Lalloo, U.G., Gouws, E. & Colvin, M.S., 1997. An evaluation of the respiratory health status of automotive spray-painters exposed to paints containing hexamethylene di-isocyanates in the greater Durban area. *S Afr Med J,* 87**,** 318-23.

Rattray, N.J., Botham, P.A., Hext, P.M., Woodcock, D.R., Fielding, I., Dearman, R.J. & Kimber, I., 1994. Induction of respiratory hypersensitivity to diphenylmethane-4,4'-diisocyanate (MDI) in guinea pigs. Influence of route of exposure. *Toxicology,* 88**,** 15-30.

Redlich, C.A., 2010. Skin exposure and asthma: is there a connection? *Proc Am Thorac Soc,* 7**,** 134-7.

Redlich, C.A. & Karol, M.H., 2002. Diisocyanate asthma: clinical aspects and immunopathogenesis. *Int Immunopharmacol,* 2**,** 213-24.

Reeb-Whitaker, C., Whittaker, S.G., Ceballos, D.M., Weiland, E.C., Flack, S.L., Fent, K.W., Thomasen, J.M., Trelles Gaines, L.G. & Nylander-French, L.A., 2012. Airborne isocyanate exposures in the collision repair industry and a comparison to occupational exposure limits. *J Occup Environ Hyg,* 9**,** 329-39.

Reeb-Whitaker, C.K. & Schoonover, T.M., 2016. Isocyanate Exposure Below Analytical Detection When a Paint Brush and Roller Are Used to Apply Moisture-Cure Polyurethane Paint. *Ann Occup Hyg,* 60**,** 513-8.

Robbins, Z., Bodnar, W., Zhang, Z., Gold, A. & Nylander-French, L.A., 2018. Trisaminohexyl isocyanurate, a urinary biomarker of HDI isocyanurate exposure. *J Chromatogr B Analyt Technol Biomed Life Sci,* 1076**,** 117-129.

Robert, A., Ducos, P., Francin, J.M. & Marsan, P., 2007. Biological monitoring of workers exposed to 4,4'-methylenediphenyl diisocyanate (MDI) in 19 French polyurethane industries. *Int Arch Occup Environ Health,* 80**,** 412-22.

Rosenberg, C., Nikkila, K., Henriks-Eckerman, M.L., Peltonen, K. & Engstrorm, K., 2002. Biological monitoring of aromatic diisocyanates in workers exposed to thermal degradation products of polyurethanes. *J Environ Monit,* 4**,** 711-6.

Rosenberg, C. & Savolainen, H., 1986. Determination in urine of diisocyanate-derived amines from occupational exposure by gas chromatography-mass fragmentography. *Analyst,* 111**,** 1069-71.

Rudzinski, W.E., Dahlquist, B., Svejda, S.A., Richardson, A. & Thomas, T., 1995. Sampling and Analysis of Isocyanates in Spray-Painting Operations. *American Industrial Hygiene Association Journal,* 56**,** 284-289.

Sabbioni, G., Wesp, H., Lewalter, J. & Rumler, R., 2007. Determination of isocyanate biomarkers in construction site workers. *Biomarkers,* 12**,** 468-83.

Sakai, T., Morita, Y., Kim, Y. & Tao, Y.X., 2002. LC-MS determination of urinary toluenediamine in workers exposed to toluenediisocyanate. *Toxicol Lett,* 134**,** 259-64.

Sakai, T., Morita, Y., Roh, J., Kim, H. & Kim, Y., 2005. Improvement in the GC-MS method for determining urinary toluene-diamine and its application to the biological monitoring of workers exposed to toluene-diisocyanate. *Int Arch Occup Environ Health,* 78**,** 459-66.

Sennbro, C.J., Lindh, C.H., Tinnerberg, H., Gustavsson, C., Littorin, M., Welinder, H. & Jonsson, B.A., 2003. Development, validation and characterization of an analytical method for the quantification of hydrolysable urinary metabolites and plasma protein adducts of 2,4- and 2,6-toluene diisocyanate, 1,5-naphthalene diisocyanate and 4,4'-methylenediphenyl diisocyanate. *Biomarkers,* 8**,** 204-17.

Sepai, O., Henschler, D. & Sabbioni, G., 1995a. Albumin adducts, hemoglobin adducts and urinary metabolites in workers exposed to 4,4'-methylenediphenyl diisocyanate. *Carcinogenesis,* 16**,** 2583-7.

Sepai, O., Schutze, D., Heinrich, U., Hoymann, H.G., Henschler, D. & Sabbioni, G., 1995b. Hemoglobin adducts and urine metabolites of 4,4'-methylenedianiline after 4,4'-methylenediphenyl diisocyanate exposure of rats. *Chem Biol Interact,* 97**,** 185-98.

Skarping, G., Dalene, M. & Brunmark, P., 1994a. Liquid-Chromatography and Mass-Spectrometry Determination of Aromatic-Amines in Hydrolyzed Urine from Workers Exposed to Thermal-Degradation Products of Polyurethane. *Chromatographia,* 39**,** 619-623.

Skarping, G., Dalene, M., Svensson, B.G., Littorin, M., Akesson, B., Welinder, H. & Skerfving, S., 1996. Biomarkers of exposure, antibodies, and respiratory symptoms in workers heating polyurethane glue. *Occup Environ Med,* 53**,** 180-7.

Skarping, G., Dalene, M.D. & Tinnerberg, H., 1994b. Biological monitoring of hexamethylene- and isophorone-diisocyanate by the determination of hexamethylene- and isophorone-diamine in hydrolysed urine using liquid chromatography and mass spectrometry. *Analyst,* 119**,** 2051-5.

Skretvedt, O.T. & Loschiavo, J.G., 1984. Effect of facial hair on the face seal of negative-pressure respirators. *Am Ind Hyg Assoc J,* 45**,** 63-6.

Sparer, J., Stowe, M.H., Bello, D., Liu, Y., Gore, R.J., Youngs, F., Cullen, M.R., Redlich, C.A. & Woskie, S.R., 2004. Isocyanate exposures in autobody shop work: the SPRAY study. *J Occup Environ Hyg,* 1**,** 570-81.

Stobbe, T.J., Daroza, R.A. & Watkins, M.A., 1988. Facial hair and respirator fit: a review of the literature. *Am Ind Hyg Assoc J,* 49**,** 199-204.

Swierczynska-Machura, D., Brzeznicki, S., Nowakowska-Swirta, E., Walusiak-Skorupa, J., Wittczak, T., Dudek, W., Bonczarowska, M., Wesolowski, W., Czerczak, S. & Palczynski, C., 2015. Occupational exposure to diisocyanates in polyurethane foam factory workers. *Int J Occup Med Environ Health,* 28**,** 985-98.

Thomas, A., 2015. *Improving polyurethane surface coatings Part 1: Polyurethane and polyisocyanate chemistry - a review*.

Thomasen, J.M., Fent, K.W. & Nylander-French, L.A., 2011. Development of a sampling patch to measure dermal exposures to monomeric and polymeric 1,6-hexamethylene diisocyanate: a pilot study. *J Occup Environ Hyg,* 8**,** 709-17.

Thomasen, J.M. & Nylander-French, L.A., 2012. Penetration patterns of monomeric and polymeric 1,6-hexamethylene diisocyanate monomer in human skin. *J Environ Monit,* 14**,** 951-60.

Tinnerberg, H., Broberg, K., Lindh, C.H. & Jonsson, B.A., 2014. Biomarkers of exposure in Monday morning urine samples as a long-term measure of exposure to aromatic diisocyanates. *Int Arch Occup Environ Health,* 87**,** 365-72.

Tinnerberg, H., Dalene, M. & Skarping, G., 1997. Air and biological monitoring of toluene diisocyanate in a flexible foam plant. *Am Ind Hyg Assoc J,* 58**,** 229-35.

Tinnerberg, H., Skarping, G., Dalene, M. & Hagmar, L., 1995. Test chamber exposure of humans to 1,6-hexamethylene diisocyanate and isophorone diisocyanate. *Int Arch Occup Environ Health,* 67**,** 367-74.

U.S. Bureau of Labor Statistics, 2019a. *Painters, Construction and Maintenance: Occupational Outlook Handbook: U.S. Bureau of Labor Statistics* [online]. https://www.bls.gov/ooh/construction-and-extraction/painters-construction-and-maintenance.htm [Accessed 09 June 2019].

U.S. Bureau of Labor Statistics, 2019b. *Painting and Coating Workers: Occupational Outlook Handbook: U.S. Bureau of Labor Statistics* [online]. https://www.bls.gov/ooh/production/painting-and-coating-workers.htm [Accessed 09 June 2019].

U.S. Census Bureau, 2018. *2016 SUSB Annual Data Tables by Establishment Industry* [online]. https://www.census.gov/data/tables/2016/econ/susb/2016-susb-annual.html [Accessed 28 January 2019].

U.S. EPA, 1990. *The Clean Air Act Amendments of 1990 List of Hazardous Air Pollutants* [online]. United States Environmental Protection Agency. Available from: https://www3.epa.gov/airtoxics/orig189.html [Accessed 21 July 2019].

U.S. EPA, 2016. *Definition and Procedure for the Determination of the Method Detection Limint, Revision 2*. U.S. Environmental Protection Agency, Washington, DC, 821-R-16-006.

UK HSE, 2005a. *EH40/2005 Workplace Exposure Limits* [online]. http://www.hse.gov.uk/pubns/priced/eh40.pdf [Accessed 25 July 2019].

UK HSE, 2005b. *Isocyanates: Biological Monitoring Guidance Values, Health and Safety Laboratory* [online]. https://www.hsl.gov.uk/media/1196200/isocyanates%20in%20urine.pdf [Accessed 25 July 2019].

Vandenplas, O., Cartier, A., Lesage, J., Cloutier, Y., Perreault, G., Grammer, L.C., Shaughnessy, M.A. & Malo, J.L., 1993a. Prepolymers of hexamethylene diisocyanate as a cause of occupational asthma. *J Allergy Clin Immunol,* 91**,** 850-61.

Vandenplas, O., Cartier, A., Lesage, J., Perrault, G., Grammer, L.C. & Malo, J.L., 1992. Occupational asthma caused by a prepolymer but not the monomer of toluene diisocyanate (TDI). *J Allergy Clin Immunol,* 89**,** 1183-8.

Vandenplas, O., Malo, J.L., Dugas, M., Cartier, A., Desjardins, A., Levesque, J., Shaughnessy, M.A. & Grammer, L.C., 1993b. Hypersensitivity pneumonitis-like reaction among workers exposed to diphenylmethane [correction to piphenylmethane] diisocyanate (MDI). *Am Rev Respir Dis,* 147**,** 338-46.

Vandenplas, O., Malo, J.L., Saetta, M., Mapp, C.E. & Fabbri, L.M., 1993c. Occupational asthma and extrinsic alveolitis due to isocyanates: current status and perspectives. *Br J Ind Med,* 50**,** 213-28.

Vonesh, E.F., 1997. Linear and nonlinear models for the analysis of repeated measurements. *Statistics, Textbooks and Monographs,* 154**,** 293-294.

Weyel, D.A., Rodney, B.S. & Alarie, Y., 1982. Sensory irritation, pulmonary irritation, and acute lethality of a polymeric isocyanate and sensory irritation of 2,6-toleune diisocyanate. *Toxicol Appl Pharmacol,* 64**,** 423-30.

Whittaker, S.G. & Reeb-Whitaker, C., 2009. Characterizing the health and safety needs of the collision repair industry. *J Occup Environ Hyg,* 6**,** 273-82.

Wikman, H., Piirila, P., Rosenberg, C., Luukkonen, R., Kaaria, K., Nordman, H., Norppa, H., Vainio, H. & Hirvonen, A., 2002. N-Acetyltransferase genotypes as modifiers of diisocyanate exposure-associated asthma risk. *Pharmacogenetics,* 12**,** 227-33.

Wilder, L.C., Langley, R.L., Middleton, D.C., Ernst, K., Lummus, Z.L., Streicher, R.P., Campbell, D.S., Wattigney, W.A., Bernstein, J.A., Bernstein, D.I. & Dearwent, S.M., 2011. Communities near toluene diisocyanate sources: an investigation of exposure and health. *J Expo Sci Environ Epidemiol,* 21**,** 587-94.

Wilkinson, S.M., Cartwright, P.H., Armitage, J. & English, J.S., 1991. Allergic contact dermatitis from 1,6-diisocyanatohexane in an anti-pill finish. *Contact Dermatitis,* 25**,** 94-6.

Williams, N.R., Jones, K. & Cocker, J., 1999. Biological monitoring to assess exposure from use of isocyanates in motor vehicle repair. *Occup Environ Med,* 56**,** 598-601.

Wisnewski, A.V., 2007. Developments in laboratory diagnostics for isocyanate asthma. *Curr Opin Allergy Clin Immunol,* 7**,** 138-45.

Wisnewski, A.V., Mhike, M., Hettick, J.M., Liu, J. & Siegel, P.D., 2013. Hexamethylene diisocyanate (HDI) vapor reactivity with glutathione and subsequent transfer to human albumin. *Toxicol In Vitro,* 27**,** 662-71.

Wisnewski, A.V., Srivastava, R., Herick, C., Xu, L., Lemus, R., Cain, H., Magoski, N.M., Karol, M.H., Bottomly, K. & Redlich, C.A., 2000. Identification of human lung and skin proteins conjugated with hexamethylene diisocyanate in vitro and in vivo. *Am J Respir Crit Care Med,* 162**,** 2330-6.

Wisnewski, A.V., Stowe, M.H., Cartier, A., Liu, Q., Liu, J., Chen, L. & Redlich, C.A., 2004. Isocyanate vapor-induced antigenicity of human albumin. *J Allergy Clin Immunol,* 113**,** 1178-84.

Wisnewski, A.V., Stowe, M.H., Nerlinger, A., Opare-Addo, P., Decamp, D., Kleinsmith, C.R. & Redlich, C.A., 2012. Biomonitoring Hexamethylene diisocyanate (HDI) exposure based on serum levels of HDI-specific IgG. *Ann Occup Hyg,* 56**,** 901-10.

Wisnewski, A.V., Xu, L., Robinson, E., Liu, J., Redlich, C.A. & Herrick, C.A., 2011. Immune sensitization to methylene diphenyl diisocyanate (MDI) resulting from skin exposure: albumin as a carrier protein connecting skin exposure to subsequent respiratory responses. *J Occup Med Toxicol,* 6**,** 6.

Woskie, S.R., Sparer, J., Gore, R.J., Stowe, M., Bello, D., Liu, Y., Youngs, F., Redlich, C., Eisen, E. & Cullen, M., 2004. Determinants of isocyanate exposures in auto body repair and refinishing shops. *Ann Occup Hyg,* 48**,** 393-403.

Yeh, H.J., Shih, T.S., Tsai, P.J. & Chang, H.Y., 2002. Toluene diisocyanate concentration investigation among TDI-related factories in Taiwan and their relations to the type of industry. *J Expo Anal Environ Epidemiol,* 12**,** 157-64.

Yucesoy, B., Kissling, G.E., Johnson, V.J., Lummus, Z.L., Gautrin, D., Cartier, A., Boulet, L.P., Sastre, J., Quirce, S., Tarlo, S.M., Cruz, M.J., Munoz, X., Luster, M.I. & Bernstein, D.I., 2015. N-Acetyltransferase 2 Genotypes Are Associated With Diisocyanate-Induced Asthma. *J Occup Environ Med,* 57**,** 1331-6.

Zissu, D., Binet, S. & Limasset, J.C., 1998. Cutaneous sensitization to some polyisocyanate prepolymers in guinea pigs. *Contact Dermatitis,* 39**,** 248-51.