Final Progress Report

Quantifying Determinants of Spray Painters' Isocyanurate Exposure

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List of Terms and Abbreviations

^{13}C NMR	carbon-13 nuclear magnetic resonance spectroscopy
ESI	electrospray ionization
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
HDA	1,6-hexamethylene diamine
HDI	1,6-hexamethylene diisocyanate
HFBA	heptafluorobutyric acid
HPLC	high-performance liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LLE	liquid-liquid extraction
LMM	linear mixed-effects model
MAPE	mean absolute percentage error
MDI	methylene diphenyl diisocyanate
MDL	method detection limit
NIOSH	National Institute for Occupational Safety and Health
NORA	National Occupational Research Agenda
r2p	research to practice
SPE	solid-phase extraction
SRM	selected reaction monitoring
TAAHI	trisacetamidohexyl isocyanurate
TAAHpI	trisacetamidoheptyl isocyanurate
TAHI	trisaminohexyl isocyanurate
TAHpI	trisaminoheptyl isocyanurate
TDA	diaminotoluene
TDI	toluene diisocyanate
UPLC	ultra-performance liquid chromatography

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Abstract

Biological monitoring of occupational exposure to 1,6-hexamethylene diisocyanate (HDI)-containing spray-paints is limited to the analysis of a hydrolysis metabolite of HDI monomer although polymeric HDI isocyanurate constitutes the predominant inhalation and skin exposures for workers in the automotive paint industry. We developed a novel method to quantify trisaminohexyl isocyanurate (TAHI), as a biomarker of HDI isocyanurate exposure in urine and blood samples collected from occupationally exposed spray-painters. We utilized the quantitative measures of inhalation and skin exposure and the biomarker levels as well as sophisticated exposure modeling to link the observed biomarker levels to other predictors of systemic exposure such as personal and workplace factors. To identify specific metabolites as predictive biomarkers following exposure to HDI isocyanurate, we developed a novel nano-UPLC-ESI-MS/MS method for small molecule quantification. Urine samples collected from 72 workers (*N* = 607) and plasma samples from 69 workers (*N* = 173) with exposure to HDI-containing spray-paints were processed using a novel sample extraction and treatment method for TAHI. The protocol was sensitive and specific for analysis of a derivatized product of TAHI, trisacetamidohexyl isocyanurate (TAAHI) in workers' urine and plasma with method detection limits at 0.03 µg/L and at 0.02 µg/L, respectively. TAHI was detected in 195 of 607 urine samples (55 of 72 workers) and in 26 of 173 plasma samples (15 of 69 workers). A positive linear correlation was observed between the measured total daily breathing-zone HDI isocyanurate concentration and the daily average urine TAHI concentration (r = 0.21), while the respective correlation for HDI monomer and urine 1,6-hexamethylene diamine (HDA, a biomarker of HDI monomer exposure) was r = -0.03. A stronger linear correlation was observed between the measured total daily breathing-zone HDI isocyanurate and the daily plasma TAHI (r = 0.61) while no correlation was observed between HDI monomer and plasma HDA (r = -0.01). Our results confirm that TAHI is a suitable biomarker for HDI isocyanurate exposure and will allows us to distinguish between HDI isocyanurate and HDI monomer exposure and, thus, provide a major advance in characterizing both exposures through multiple exposure routes. The identification and quantification of TAHI as a biomarker of HDI isocyanurate exposure is critical for improvement of diisocyanate exposure assessment through characterization of exposure-dose relationships for both HDI monomer and HDI isocyanurate in occupationally exposed populations.

Section 1

Significant (Key) Findings

Our study was designed to provide cutting-edge knowledge and a path forward to identify specific metabolites as predictive biomarkers following exposure to 1,6-hexamethylene diisocyanate (HDI) monomer and its oligomer, HDI isocyanurate. Development of a biomarker for HDI isocyanurate, a polymeric HDI isocyanate, is pertinent for exposure monitoring of diisocyanate exposed workers because (1) the greatest exposure mass to spray painters using HDI-containing paints is HDI isocyanurate, (2) individual differences in sensitization is greater for HDI isocyanurate than HDI monomer exposure (Aalto-Korte et al., 2010; Vandenplas et al., 1993a; Zissu et al., 1998), (3) development of this biomarker will increase our ability to explain reported intra- and inter-individual variability between HDI isocyanurate and HDI exposure (Fent et al., 2009a; Fent et al., 2009b; Flack et al., 2011; Flack et al., 2010b; Gaines et al., 2010a; Gaines et al., 2011; Thomasen and Nylander-French, 2012), and (4) development of validated biomarkers for HDI isocyanurate exposure has been recognized as one of the most critical needs by the stakeholders (Lockey et al., 2015).

We successfully developed a novel method using nanoflow ultra-performance liquid chromatography coupled to nano-electrospray ionization tandem mass spectrometry (nano-UPLC-ESI-MS/MS) to quantify trisaminohexyl isocyanurate (TAHI), a hydrolysis product of HDI isocyanurate, in the urine and blood of workers exposed to automotive spray-paints. Urine from 72 workers (N = 607) and plasma from 69 workers (N = 173) were analyzed using nano-UPLC-ESI-MS/MS. TAHI was detected in 32% of urine samples and in 15% of plasma samples. A positive linear correlation was observed between the measured total daily breathing-zone HDI isocyanurate concentration and the daily average urine TAHI concentration (r = 0.21 without creatinine adjustment; r = 0.10 with creatinine adjustment). A stronger linear correlation was observed between measured total daily breathing-zone HDI isocyanurate concentration and the daily average plasma TAHI concentration (r = 0.61).

As is the case for the urine HDI monomer biomarker 1,6-hexamethylene diamine (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. 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 (Aalto-Korte et al., 2010; Zissu et al., 1998), 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 and developing regulatory limits for HDI isocyanurate to ensure worker safety.

Translation of Findings

The developed monitoring method for urine and blood biomarkers of HDI isocyanurate exposure will help reduce uncertainty in biomarker of exposure/early biological effect classification in diisocyanate exposure and risk assessment. Analysis of HDI isocyanurate biomarker along with HDI monomer biomarker will increase our ability to explain reported intra- and inter-individual variability between HDI isocyanurate and HDI exposure (Fent et al., 2009a; Fent et al., 2009b; Flack et al., 2011; Flack et al., 2010b; Gaines et al., 2010a; Gaines et al., 2011; Thomasen and Nylander-French, 2012). These tools have the potential to provide an effective approach to identify the basis for individual exposure and the biomarker of exposure outcome and significantly contribute to our understanding of exposure dose-effects relationships. Through the methodology developed in this project, we can also account for individual differences in exposure-dose relationships that may be related to individual's threshold for toxicity (i.e., individual susceptibility governed by gene- and epigenetic-environment interactions) (Sun, 2016; Taylor, 2017) and, potentially in future studies, to disease (e.g., sensitization and occupational asthma). Thus, we are able to develop more predictive exposure assessment models and more

effective worker protection strategies based upon individual genetic and exposure variation (Sun, 2016; Taylor, 2017). These results benefit NIOSH NORA and Research to Practice (r2p) initiatives by providing critical new information on factors affecting individual differences in HDI biomarker levels and to assess strategies to implement future interventions.

Research Outcomes/Impact

This project benefits two NIOSH NORA sectors (1) Services (Automotive Body, Paint, and Interior Repair and Maintenance) and (2) Manufacturing (Paint, Coating, and Adhesive Manufacturing) by providing critical new information on HDI monomer and its oligomer HDI isocyanurate exposures as well as on factors affecting individual differences in exposure and biomarker levels. To summarize the significance and impact:

- This work addressed a significant area of exposure science by validating specific metabolites as predictive biomarkers following exposure to HDI monomer and its oligomer, HDI isocyanurate and providing the foundation for monitoring diisocyanate monomer and oligomer exposures.
- The biomarker trisaminohexyl isocyanurate is the first biomarker established for HDI isocyanurate exposure and significant levels were measured in the urine and blood of automotive spray painters.
- The quantification of the HDI isocyanurate biomarker, trisaminohexyl isocyanurate, will provide a more realistic measure of the received internal dose and a better understanding of the overall range of exposure profiles in diisocyanate exposed workers.
- This biomarker assay is critical for investigation of dose-response relationships, to understand of how exposure pathways (*i.e.*, inhalation and skin) as well as personal and workplace factors contribute to both HDI isocyanurate and HDI monomer exposure, to establish causality for associated health effects from diisocyanate exposure, and to improve exposure and risk assessment for diisocyanate exposures.
- The primary strength of this research is the potential to characterize the exposure-dose relationships for both HDI isocyanurate and HDI monomer that will significantly contribute towards realistic exposure assessment in spray painters who have an increased risk of developing allergic contact dermatitis and/or occupational asthma.
- Knowledge developed from this research will increase our understanding of biomarker modifiers for HDI isocyanurate and HDI monomer exposures that should be particularly useful to identify workers whose exposure must be controlled to minimize exposure and who may be at the greatest risk for developing isocyanate-related adverse health effects.
- This research will also allow us to provide useful input in setting exposure limits, which are currently lacking for HDI monomer and its oligomers, by taking into account the individual variation in exposure and biomarker levels to both HDI monomer and HDI isocyanurate.

Section 2: Scientific Report

Background

Aromatic and aliphatic diisocyanates are highly reactive, low-molecular-weight compounds included in the 187 hazardous air pollutants of the Clean Air Act Amendments of 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; Weber, 2004). Diisocyanate exposure is associated with a multitude of adverse health outcomes, most notably occupationally-induced asthma and allergic contact dermatitis (Aalto-Korte et al., 2010; Bernstein, 1996; Chan-Yeung and Malo, 1995; Piirila et al., 2000; Vandenplas et al., 1993b). Acute exposure can cause shortness of breath, rhinitis, irritation of the skin, eyes, and mucous membranes, and pulmonary edema (Bello et al., 2007a; Bello et al., 2007b; Bernstein, 1996; Goossens et al., 2002). Despite the widespread use of diisocyanate-containing products and the current knowledge on their exposure related serious adverse health effects, the causal link between external exposure pathways (*i.e.*, inhalation and skin), systemic and target-tissue dose (*i.e.*, biomarkers of exposure), and an adverse health effect is poorly understood. One of the most commonly used isocyanates is 1,6-hexamethylene diisocyanate (HDI), comprised of its monomer and oligomers (NIOSH, 1978). Occupational exposure to HDI occurs during industrial production or during spray-painting operations such as auto-body refinishing or application of marine coatings (NIOSH, 1996).

The predominant inhalation and skin exposure in automotive spray-painting is to HDI isocyanurate (Fent et al., 2009a; Fent et al., 2009b; Fletcher, 2015; Kim, 2015; Reeb-Whitaker et al., 2012), but the relative contributions of exposure to the HDI monomer and HDI 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 (Aalto-Korte et al., 2010; Zissu et al., 1998), and occupational asthma has been linked to HDI oligomer exposure without an immune response to the monomer (Vandenplas et al., 1993a). We have demonstrated that the most abundant HDI oligomer in the automotive spray-painting environment is HDI isocyanurate and that spray painters' inhalation and skin exposure to HDI isocyanurate is ≈300-fold greater than HDI monomer (Fent et al., 2009a; Fent et al., 2009b). Furthermore, we have shown that HDI isocyanurate also penetrates skin at much faster rates (approximately 350 to 500 times) than HDI monomer (Thomasen and Nylander-French, 2012). However, it has been shown that measured biomarker levels of HDI monomer exposure do not correlate with HDI oligomer exposure (Liu et al., 2004). Biological monitoring to estimate the systemic doses of HDI monomer and oligomers through exposure has been limited primarily to 1,6-hexamethylene diamine (HDA), the hydrolysis metabolite of HDI monomer, in urine and blood (Flack et al., 2010a; Flack et al., 2011; Flack et al., 2010b; Gaines et al., 2010a; Maitre et al., 1996; Pronk et al., 2006; Rosenberg et al., 2002; Tinnerberg et al., 1995). Therefore, identification and quantitation of a biomarker of isocyanurate exposure represents a critical element in the improvement of exposure and risk assessment for diisocyanate exposures. At present, knowledge of metabolites of any diisocyanate oligomers that would reflect the internal dose received by oligomer exposure is completely lacking. Furthermore, no methods exist to measure metabolites of HDI isocyanurate (*e.g.*, amines or protein adducts) in urine or plasma. The critical question remains, which form(s) of HDI (monomer, oligomers, or both) contribute to biologically relevant systemic exposure? The results obtained from this project represent the first comprehensive evaluation of exposure and biological uptake and elimination of HDI isocyanurate in comparison to HDI monomer and its metabolite HDA in exposed workers. Establishing the relationship between exposure and biomarkers of HDI isocyanurate and HDI monomer exposures in urine and plasma provides critical information on the uptake and elimination of these compounds and can elucidate the relative difference between HDI isocyanurate and HDI monomer exposures. Ultimately, we will be able to characterize the exposure-dose relationship for HDI isocyanurate and HDI monomer exposures and, thus, significantly contribute towards realistic exposure assessment in spray painters who have an increased risk of developing allergic contact dermatitis and/or occupational asthma.

Specific Aims

Our goal in this study was to develop a sensitive and specific analytical method to quantify systemic levels of the HDI isocyanurate biomarker, trisaminohexyl isocyanurate (TAHI), in a well-characterized exposed spray-painter population and to refine the exposure analysis to include both TAHI and HDA, as biomarkers of oligomer and monomer exposure, respectively, in both urine and blood. This research project was designed to test the following overall hypothesis "*Biomarkers of HDI isocyanurate and HDI monomer exposure are unique*

and affected by an individual's inhalation and skin exposure as well as factors related to work practices and work environment" with the following specific aims:

Aim 1. *Quantify trisaminohexyl isocyanurate (TAHI), a biomarker of HDI isocyanurate exposure, in urine and blood samples collected from occupationally exposed spray painters during multiple independent sampling visits.*

Aim 2: *Investigate the exposure-dose relationship between inhalation and skin exposure to HDI isocyanurate and its biomarker TAHI in urine and blood using linear mixed effects models in order to identify sources of intra- and inter-individual variability to elucidate the relative difference between HDI isocyanurate and HDI monomer exposure and their respective biomarker levels.*

Methodology

Study population

We utilized our previously collected samples and data (NIOSH R01-0H007598 "Dermal Exposure to 1,6-Hexamethylene Diisocyanate" and NIOSH R01-OH009364 "Evaluation of Protective Clothing to Prevent Diisocyanate Exposures in the Collision Repair Industry") (Ceballos et al., 2011; Fent et al., 2009a; Fent et al., 2009b; Flack et al., 2011; Flack et al., 2010b; Fletcher, 2015; Gaines et al., 2010a; Gaines et al., 2010b; Gaines et al., 2011; Kim, 2015; Reeb-Whitaker et al., 2012) (referred to as Study I & II, respectively). These studies were approved by the Institutional Review Board in the Office of Human Research Ethics at the University of North Carolina at Chapel Hill and by the Washington State Institutional Review Board at the Washington State Department of Social and Health Services. Workers' consents to conduct all the analyses proposed were obtained.

In the two studies, environmental (inhalation and skin) and biological samples (blood and urine) were collected for expanded analysis of exposure and biomarker levels. Respiratory and skin exposure to HDI monomer and the associated urine and blood biomarker, HDA, levels were quantified for 72 spray painters during 1 – 4 repeated independent exposure assessment surveys in North Carolina and Washington State (Ceballos et al., 2011; Fent et al., 2009a; Fent et al., 2009b; Flack et al., 2011; Flack et al., 2010b; Fletcher, 2015; Gaines et al., 2010a; Gaines et al., 2010b; Gaines et al., 2011; Kim, 2015; Reeb-Whitaker et al., 2012). In addition, skin and respiratory exposure to HDI isocyanurate (HDI oligomer) were quantified for these spray painters during these repeated independent exposure assessment surveys (Ceballos et al., 2011; Fent et al., 2009a; Fent et al., 2009b; Flack et al., 2011; Flack et al., 2010b; Fletcher, 2015; Gaines et al., 2010a; Gaines et al., 2010b; Gaines et al., 2011; Kim, 2015; Reeb-Whitaker et al., 2012). Collections of urine and blood samples were accomplished as described in our publications (Flack et al., 2011; Flack et al., 2010b; Gaines et al., 2010a). Briefly, one spot urine sample was obtained before the start of work and spot urine samples were obtained from the worker each time he urinated during the workday. Venous blood (30 mL) was obtained from each worker after the work shift while multiple urine samples per worker were collected throughout the workday. Immediately after collection, the samples were packed in ice and shipped to Dr. Nylander-French's laboratory for processing and analyses. Within 24 h of collection, urine samples were transferred into individual sterile 50 mL polypropylene tubes for storage at −80°C until analysis. Plasma and red blood cells were separated by centrifugation at 2,000×g and samples stored at –80˚C until analysis. The following exposure and biomarker levels from the 72 healthy workers were available for this study: (A) breathing-zone and skin tape-strip samples analyzed for HDI monomer and HDI isocyanurate; (B) urine samples analyzed for total HDA, creatinine levels, and specific gravity; and (C) blood samples analyzed for total HDA in plasma.

Development of Protocol for Trisaminohexyl Isocyanurate Analysis in Urine

We developed a sample treatment and extraction protocol to isolate trisaminohexyl isocyanurate (TAHI) from urine of exposed workers. TAHI (1,3,5-Tris(6-aminohexyl)-1,3,5-triazinane-2,4,6-trione) is not commercially available, consequently, we synthesized TAHI in-house. Additionally, we synthesized an internal standard 1,3,5-tris(7-aminoheptyl)-1,3,5-triazinane-2,4,6-trione (trisaminoheptyl isocyanurate, TAHpI) for the sample treatment protocol. For mass spectral characterization of acetylated standards after sample treatment, we synthesized *N,N',N"*-((2,4,6-trioxo-1,3,5-triazinane-1,3,5-triyl)tris(hexane-6,1-diyl))triacetamide (trisacetamido-hexyl isocyanurate; TAAHI) and *N,N',N"*-((2,4,6-trioxo-1,3,5-triazinane-1,3,5-triyl)tris(heptane-7,1-diyl))triacet-amide (trisacetamidoheptyl isocyanurate; TAAHpI). Nuclear magnetic resonance spectra for all four synthesized compounds were characterized with proton nuclear magnetic resonance (^{1}H NMR) at 400 MHz and carbon-13 nuclear magnetic resonance (^{13}C NMR) at 100 MHz on a Varian INOVA 400 (Palo Alto, CA). Mass spectra were acquired on 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.). The structures of all four synthesized standards are displayed in Figure 1.

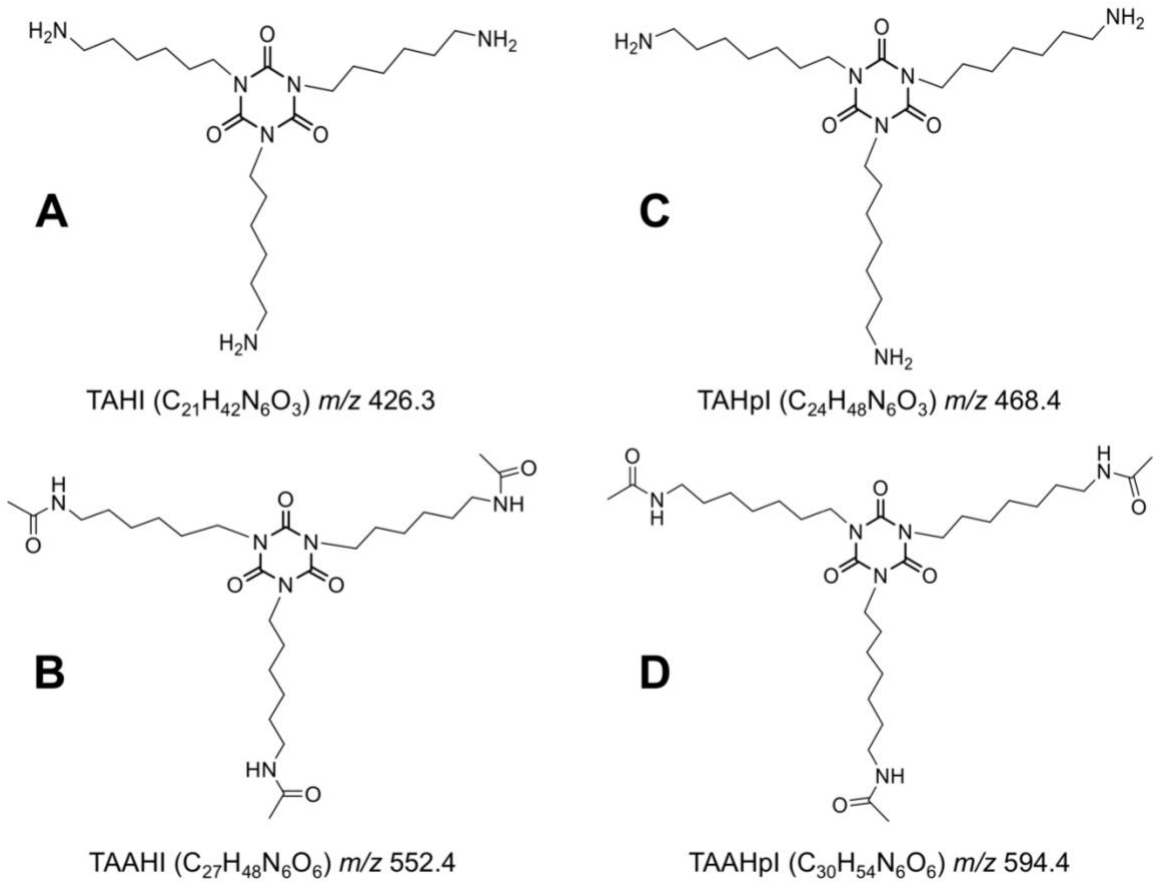

Figure 1. Chemical structures of [A] trisaminohexyl isocyanurate (TAHI), [B] trisacetamidohexyl isocyanurate (TAAHI), [C] trisaminoheptyl isocyanurate (TAHpI), and [D] trisacetamidoheptyl isocyanurate (TAAHpI).

During the first year of the project, we investigated three extractions methods for control urine spiked with TAHI: liquid-liquid extraction (LLE), solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) fractionation. The LLE protocol was adopted from previous methods for HDA analysis in urine and blood (Flack et al., 2010b; Gaines et al., 2010a), while the SPE and HPLC-fractionation protocols were developed in the laboratory. TAHI was analyzed with nano-UPLC-ESI-MS/MS in its amine form and its derivatized form using heptafluorobutyric acid (HFBA). The LLE, SPE, and HPLC-fractionation methods were successfully used to isolate TAHI and TAHpI. However, we were unable to detect TAHI and TAHI-HFBA below 1.0 µg/L with LLE and HPLC-fractionation. With SPE we could not detect TAHI below 0.75 µg/L.

We developed an alternative derivatization method using acetic anhydride, and proceeded to use the LLE method with dichloromethane as the extraction solvent. We chose LLE over SPE and HPLC-fractionation because for the extracted TAHI free amine, LLE had limited confounding matrix effects. Additionally, LLE has the advantages of lower cost and shorter procedural time. The experimental protocol was based on previous studies for HDI, methylene diphenyl diisocyanate (MDI), and toluene diisocyanate (TDI) biomarkers in urine and plasma (Brorson et al., 1990a; Brorson et al., 1990b; Dalene et al., 1995; Flack et al., 2010b; Gaines et al., 2010a; Liu et al., 2004; Maitre et al., 1996; Pronk et al., 2006; Rosenberg et al., 2002; Sabbioni et al., 2007; Sennbro et al., 2003; Skarping et al., 1996; Tinnerberg et al., 1995). 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; Flack et al., 2010a; Pauluhn, 2002; Sepai et al., 1995a; Sepai et al., 1995b). Dichloromethane, an extraction solvent reported in the analytical literature (Flack et al., 2010a; Flack et al., 2011; Kaaria et al., 2001; Sabbioni et al., 2007; Sakai et al., 2005; Sepai et al., 1995a; Sepai et al., 1995b), 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 (Almeida et al., 2002; Brorson et al., 1990a; Flack et al., 2010b; Gaines et al., 2010a; Liu et al., 2004; Maitre et al., 1996; Marand et al., 2004; Pronk et al., 2006; Rosenberg et al., 2002; Sennbro et al., 2003; Skarping et al., 1996; Tinnerberg et al., 1995), hexane, and ethyl acetate (Bailey et al., 1990; Sakai et al., 2002; Sepai et al., 1995b). No analyte could be detected by extraction with hexane or toluene, and confounding matrix effects persisted with ethyl acetate.

Analysis of TAHI in Urine of Exposed Workers

Our liquid-liquid extraction method is similar to methods used for analysis of HDA in urine and plasma of spray painters (Flack et al., 2010b; Gaines et al., 2010a). The work-up procedure involved acid hydrolysis, dichloromethane extraction, and derivatization with acetic anhydride prior to analysis by nano-UPLC-ESI-MS/MS. Acetylated amines (acetamides) protonate well with positive electrospray ionization under acidic conditions and are highly sensitive with liquid chromatography-mass spectrometry (LC-MS) analysis. Briefly, 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 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.

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. The method detection limit (MDL) was calculated using the procedure established by the US EPA (EPA, 2016b). 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 = 3.7 ng/L, N = 10, and t = 2.821 at α = 0.1), the MDL was calculated to be 0.03 µg/L.

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. 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 2A), 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 2B). Fragmentation spectra acquired for TAAHI with nano-UPLC-ESI-MS/MS to use for quantification are displayed in Figure 3A and 3B.

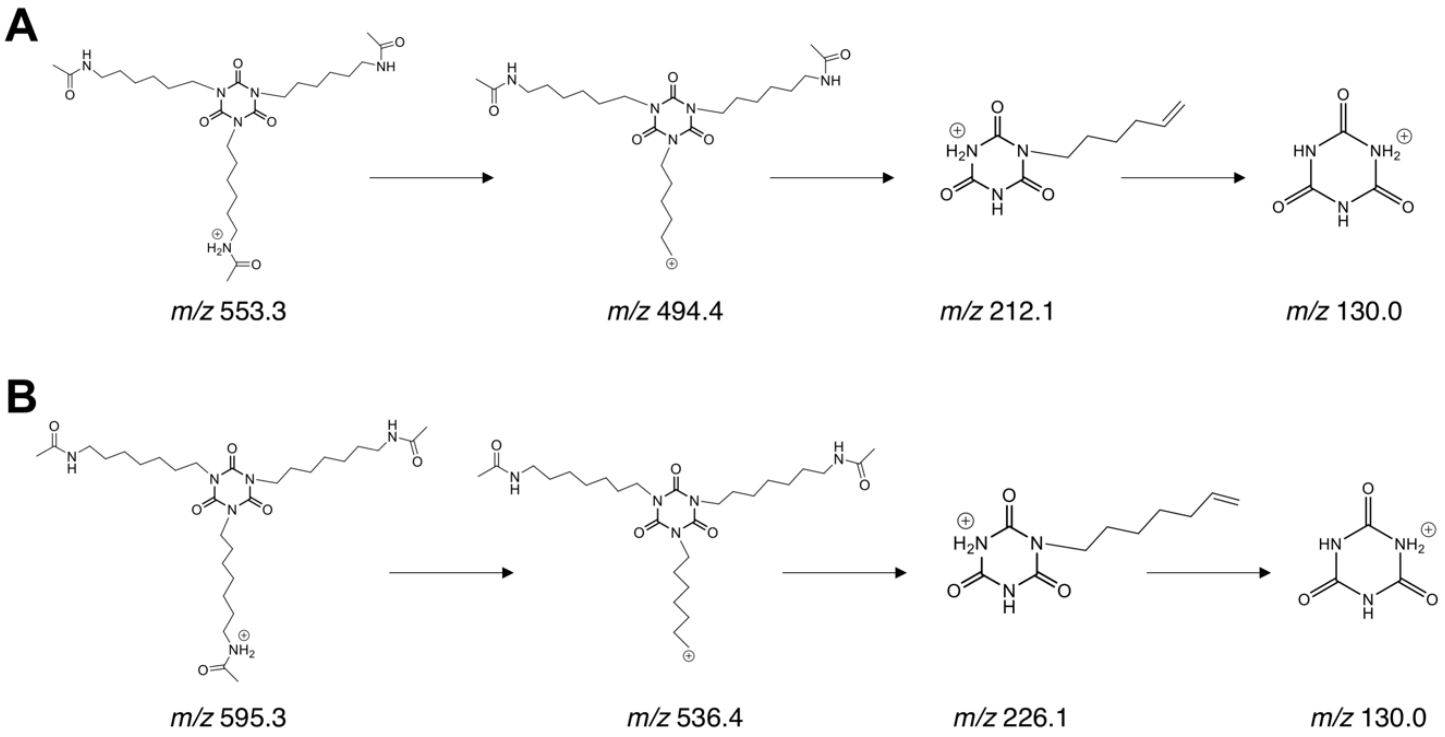

Figure 2. [A] TAAHI mass spectral fragments and [B] TAAHpI mass spectral fragments.

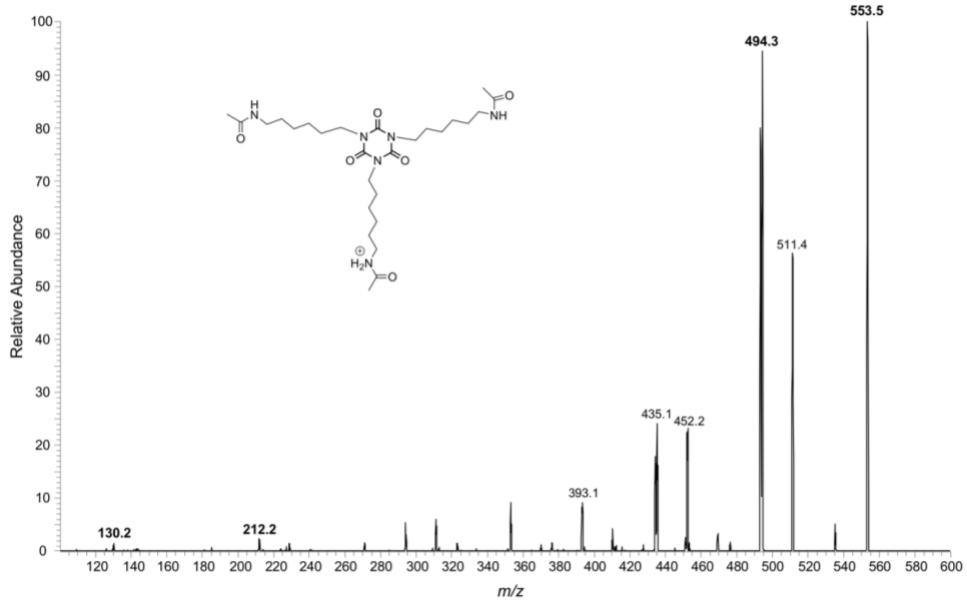

Figure 3A. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) obtained by direct injection on ESI-MS/MS operated in positive ion-mode with electrospray ionization (scan range, *m/z* 100-600; collision energy, 25 eV).

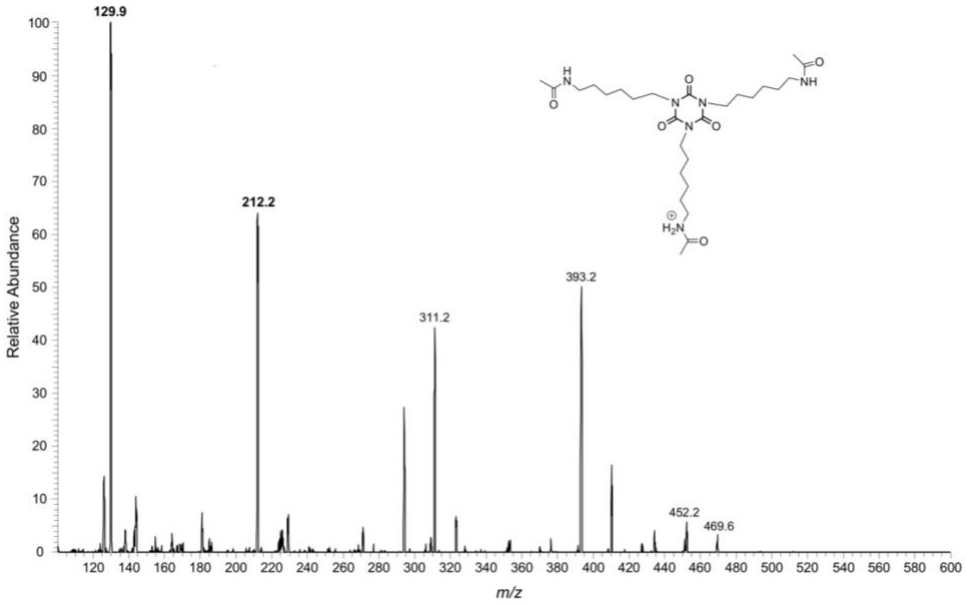

Figure 3B. Fragmentation spectrum of precursor ion $[M + H]^+$ for TAAHI (*m/z* 553.3) obtained by direct injection on ESI-MS/MS operated in positive ion-mode with electrospray ionization (scan range, *m/z* 100-600; collision energy, 50 eV).

The total ion chromatograms used for quantification are displayed in Figure 4 for control urine from a non-exposed volunteer spiked with 0.25 µg/L TAHI and 2.0 µg/L TAHpI (Figure 4A) and urine sample 8 from worker #7 spiked with 2.0 µg/L TAHpI (Figure 4B). 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.

Analysis of TAHI in Blood of Exposed Workers

The sample treatment protocol was replicable in control plasma from non-exposed persons. No changes were made in the work-up of spiked plasma. Due to higher confounding matrix effects for the three fragments of TAAHI during nano-UPLC-ESI-MS/MS analysis, we added two additional fragments. Five fragments were monitored for TAAHI: *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). There were no changes in the mass spectrometer settings for TAAHpI. We observed an improvement in the calibration curve (0.03 – 3.99 µg/L; $N = 15$; $w = y^{-2}$; $r = 0.999$) and MDL (0.02 µg/L) for TAHI in spiked control plasma. Unlike urine analysis, TAAHI fragments *m/z* 393.3 and *m/z* 452.3 were included in the instrument response ratio (*m/z* 494.4, 212.1, and 130.0 for analyte confirmation only).

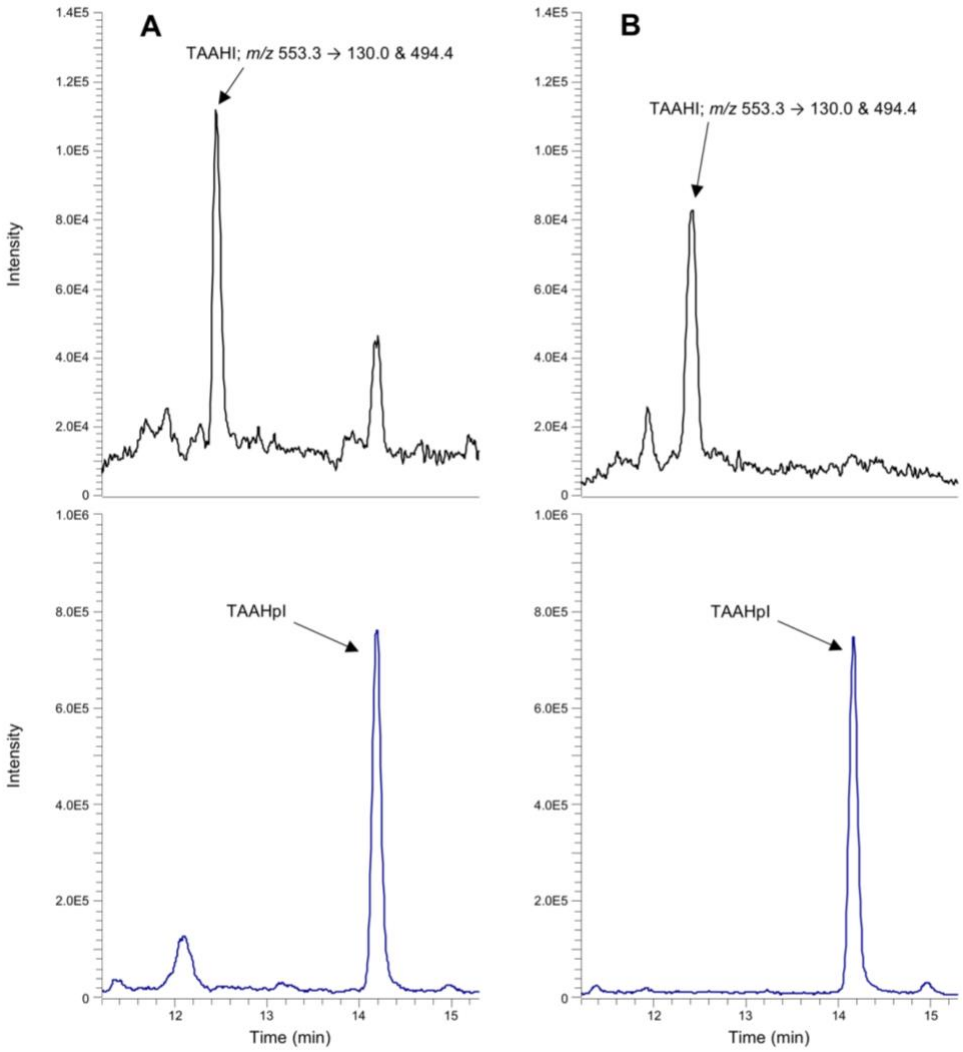

Figure 4. [A] Total ion chromatograms acquired by selected reaction monitoring for TAAHI (combined *m/z* 553.3 → 130.0 and 494.4) and TAAHpI (combined *m/z* 595.3 → 130.0, 226.1, and 536.4); obtained for control urine spiked with TAHI (0.25 µg/L) and TAHpI (2.0 µg/L). [B] Total ion chromatograms acquired by selected reaction monitoring for TAAHI (combined *m/z* 553.3 → 130.0 and 494.4), and TAAHpI (combined *m/z* 595.3 → 130.0, 226.1, and 536.4); obtained for urine sample 8 from worker #7 spiked with TAHpI (2.0 µg/L) and a calculated concentration of 0.36 µg/L for TAHI.

Statistical Analyses and Linear Mixed-Effects Model

To accomplish Aim 2, we have begun to use the HDI isocyanurate urine and blood biomarker data derived in Aim 1 and our unique set of samples and data from simultaneous monitoring of breathing-zone and skin exposure to HDI isocyanurate and HDI monomer and HDA levels in urine and blood from the 72 spray painters. The relationship between the biomarkers (*i.e.*, TAHI and HDA in blood and urine) and inhalation and skin exposure to HDI isocyanurate and HDI monomer as well as personal and work environment factors are investigated using linear mixed-effects models (LMM). The measurements of the biomarkers in urine and blood provide internal biomarker levels among workers, which are used as outcomes in modeling the contribution of exposure routes and personal and workplace factors. Model analyses include investigation of how the physical and structural parameters of the work environment (*e.g.*, personal protective equipment use, paint time) modify the exposure-dose relationship. The detailed information on demographics as well as work practice and workplace determinants collected from our worker cohort are also utilized in statistical analyses. The LMM accounts for (1) personal inhalation exposure to HDI isocyanurate and HDI monomer, (2) personal skin exposure to HDI isocyanurate and HDI monomer, and (3) individual and environmental determinants (*e.g.*, glove use, coverall use, booth type), which are available from our detailed exposure surveys. Construction of the LMM is accomplished using Proc Mixed in SAS. Co-linearity between covariates is investigated using the criterion of Pearson's r <0.7 for model inclusion.

The LMM has been developed to model biomarker level for the i^{th} worker at the j^{th} visit (Y_{ij}) as a function of the measured and/or observed fixed effects (*i.e.*, skin and inhalation exposure levels and other personal and work environment factors), and random effects associated with group (job), worker, and error. Specifically, we used the following Model 1:

$$Y_{ij} = b_0 + \sum_{p=1}^{P} b_p \ln(X_{ijp}) + \sum_{q=1}^{Q} g_g C_{ijq} + a_i + e_{ij}$$

where Y_{ij} represents the natural logarithm of the biomarker level in urine or blood on j^{th} visit for i^{th} worker; X_{ijp} represents the p^{th} exposure level (inhalation or skin) on j^{th} visit for the i^{th} worker; C_{ijq} represents the q^{th} covariate value (*i.e.*, personal and workplace factors) on j^{th} visit for the i^{th} worker; β_p and γ_q, represent the regression coefficient for p^{th} exposure level and q^{th} covariate, respectively; β_0 is the intercept, α_i is random effect of the i^{th} worker, and ε_{ij} is the random error on j^{th} visit for the i^{th} worker. It is assumed that under LMM α_i and ε_{ij} are mutually independent and normally distributed, with mean of zero and variances σ_B^2 (between-person variability) and σ_W^2 (within-person variability), respectively. The total variability $\sigma_y^2 = \sigma_B^2 + \sigma_W^2$, thus, under LMM Y_{ij} is normally distributed with mean μ_y and variance σ_y^2.

Results and Discussion

We successfully developed an extraction and derivatization protocol and liquid chromatography-mass spectrometry method for analysis of trisaminohexyl isocyanurate (TAHI), a hydrolysis product and novel urine and plasma biomarker of HDI isocyanurate, and applied this method first to quantify TAHI in urine of 15 workers exposed to HDI isocyanurate during automotive spray-painting operations (Robbins et al., submitted). 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. Table 1 summarizes the mean breathing-zone and skin concentrations of HDI isocyanurate and urine levels of TAHI measured in 15 spray painters during 1 – 3 exposure monitoring visits (Robbins et al., submitted). The mean and standard deviation for the breathing-zone exposure ranged from 65.8 ± 44.0 to 34304.1 ± 27191.4 µg/m^3 and for the skin exposure from 4.6 ± 3.5 to 3984.7 ± 3874.1 µg/mm^3. A positive linear correlation was observed between the measured daily total breathing-zone HDI isocyanurate concentration and the daily average urine TAHI concentration (r = 0.21 without creatinine adjustment; r = 0.14 with creatinine adjustment), while the respective correlation for HDI and creatinine adjusted HDA in urine was 0.06.

Table 1. Mean ± standard deviation for breathing-zone and skin HDI isocyanurate levels and urine 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 TAHI (µg/L)
1	3	10	5.5 ± 3.4	10622.6 ± 11430.5	897.0 ± 1197.4	10	<MDL[a]
2	3	5	7.9 ± 3.5	3655.6 ± 1819.8	204.1 ± 181.1	7	<MDL
3	3	3	3.8 ± 1.0	10231.6 ± 6569.5	316.1 ± 273.6	12	0.321 ± 0.243
4	1	2	8.0 ± 1.4	10752.0 ± 12539.1	1387.5 ± 1814.6	3	<MDL
5	1	1	19.5	21930.9	637.3	3	0.039 ± 0.067
6	2	6	6.2 ± 2.5	34304.1 ± 27191.4	1181.0 ± 569.8	8	0.141 ± 0.185
7	3	12	5.2 ± 3.3	17101.3 ± 14805.1	734.9 ± 499.2	19	0.137 ± 0.231
8	2	3	6.5 ± 3.8	18126.8 ± 7693.7	675.4 ± 519.6	5	0.059 ± 0.131
9	2	4	7.0 ± 5.0	9602.6 ± 17965.8	635.0 ± 835.9	6	<MDL
10	2	5	4.1 ± 0.6	21868.1 ± 29490.3	208.4 ± 425.1	6	0.024[b] ± 0.059
11	1	2	4.8 ± 1.8	65.8 ± 44.0	4.6 ± 3.5	3	0.107 ± 0.097
12	3	3	4.5 ± 2.5	25738.5 ± 34422.9	3984.7 ± 3874.1	6	0.345 ± 0.550
13	1	1	1.5	20926.6	366.0	2	1.989 ± 2.812
14	3	10	7.6 ± 3.1	12486.3 ± 11750.2	263.4 ± 232.0	12	0.866 ± 2.843
15	3	8	5.6 ± 2.1	6410.9 ± 5749.5	10.9 ± 7.9	9	0.080 ± 0.240

[a] <MDL = all samples below method detection limit

[b] 5 of 6 urine samples were below MDL

Next, TAHI was quantified in urine and plasma samples collected from all workers in Study I & II (Table 2). TAHI was detected in 195 of 607 urine samples (55 of 72 workers) and in 26 of 173 plasma samples (15 of 69 workers). The percentage of urine samples with TAHI greater than the MDL was consistent across both studies. However, only 3 plasma samples from Study II contained TAHI, while 23 plasma samples in Study I contained TAHI. Individual plasma TAHI values were significantly different between Study I and Study II ($p < 0.01$). The likely reason for this is that the TAHI values were affected by the lower HDI isocyanurate air and skin concentrations measured in Study II, considering that urine TAHI population mean values were not statistically different between the two study populations.

Table 2. The summary of HDA and TAHI levels measured in individual urine (2A) and individual plasma (2B) samples collected from workers in Study I & II.

Table 2A Urine			**1,6-Diaminohexane (HDA)**		**Trisaminohexyl Isocyanurate (TAHI)**	
	Number of Workers	Number of Samples	>MDL (%)	Mean (µg/L)	>MDL (%)	Mean (µg/L)
Study I	48	417	259 (62.1)	0.53 ± 3.32	130 (31.2)	0.20 ± 0.71
Study II	24	190	183 (96.3)	0.40 ± 0.68	65 (34.2)	0.14 ± 0.44
Study I & II	72	607	442 (72.8)	0.49 ± 2.78	195 (32.1)	0.18 ± 0.63

Table 2B Plasma			**1,6-Diaminohexane (HDA)**		**Trisaminohexyl Isocyanurate (TAHI)**	
	Number of Workers	Number of Samples	>MDL (%)	Mean (µg/L)	>MDL (%)	Mean (µg/L)
Study I	46	112	83 (74.1)	0.10 ± 0.14	23 (20.5)	0.01 ± 0.04**
Study II	23	61	9 (14.8)	0.14 ± 0.78	3 (4.9)	0.00 ± 0.01**
Study I & II	69	173	92 (53.2)	0.11 ± 0.47	26 (15.0)	0.01 ± 0.03

>MDL (%) – Number of samples above the method detection limit (percentage)
** two-tail *p*-value <0.01 using Student's *t*-test: two-sample assuming unequal variances between Study I & II

The means of daily total paint time, breathing-zone and skin HDI monomer and HDI isocyanurate levels, and daily average urine HDA and TAHI levels (non-adjusted and adjusted for creatinine) are shown in Table 3. We analyzed Study I and Study II separately and together due to disparities in the number of workers and samples available from each study, and the observed differences in exposure and biomarker levels. The statistically significant differences in exposure and biomarker measurements were determine using ANOVA and Student's *t*-test two-sample (assuming unequal variances) analyses. Significant differences were observed in the daily total paint time ($p < 0.05$), breathing-zone HDI monomer ($p < 0.05$), breathing-zone HDI isocyanurate ($p < 0.01$), and skin HDI isocyanurate levels ($p < 0.001$).

Table 3. Mean and standard deviation for the total paint time, total breathing-zone and skin HDI monomer and HDI isocyanurate levels, and average urine and HDA and TAHI levels (non-adjusted and adjusted for creatinine level) collected during each visit.

	Paint Time (min)	**Air HDI (µg/m^3)**	**Air Isocyanurate (µg/m^3)**	**Skin HDI (µg/mm^3)**	**Skin Isocyanurate (µg/mm^3)**	**HDA (µg/L)**	**HDA (µg/g creatinine)**	**TAHI (µg/L)**	**TAHI (µg/g creatinine)**
Study I	20.3 ± 20.0*	25.5 ± 29.9*	7056.3 ± 6715.4**	1.8 ± 11.8	540.0 ± 1175.1***	1.85 ± 6.68	1.01 ± 2.57	0.71 ± 1.55	0.74 ± 1.91
Study II	14.7 ± 12.3*	14.2 ± 31.6*	4246.9 ± 6853.0**	0.03 ± 0.08	68.0 ± 126.0***	1.03 ± 1.70	0.86 ± 1.37	0.37 ± 0.88	0.30 ± 0.67
Study I & II	18.0 ± 17.4	20.8 ± 31.0	5895.3 ± 6896.0	1.3 ± 10.1	407.2 ± 1019.7	1.54 ± 5.38	0.95 ± 2.19	0.58 ± 1.35	0.57 ± 1.57

* two-tail *p*-value <0.05 using Student's *t*-test: two-sample assuming unequal variances between Study I & II
** two-tail *p*-value <0.01 using Student's *t*-test: two-sample assuming unequal variances between Study I & II
*** two-tail *p*-value <0.001 using Student's *t*-test: two-sample assuming unequal variances between Study I & II

TAHI in urine and plasma show a positive linear correlation with paint time (r = 0.28 and 0.41, respectively), breathing-zone HDI monomer concentration (r = 0.22 and 0.54, respectively), and breathing-zone HDI isocyanurate concentration (r = 0.21 and 0.61, respectively) (Table 4). The correlation between daily total breathing-zone HDI isocyanurate concentration and the daily average urine TAHI concentration was consistent for the entire study cohort (i.e., Study I and Study II). A stronger linear correlation was observed between the measured total daily breathing-zone HDI isocyanurate and the daily plasma TAHI (r = 0.61) while no correlation was observed between HDI monomer and plasma HDA (r = -0.01). Interestingly, we observed little to no correlation between the well accepted biomarker for HDI monomer exposure, HDA in urine or plasma, to measured personal HDI exposure concentrations. Neither biomarker showed a strong correlation with respect to the measured skin concentrations. The results also indicate that the daily average urine TAHI concentrations correlate better with the measured total daily breathing-zone HDI isocyanurate concentration without creatinine adjustment (r = 0.21) than with creatinine adjustment (r = 0.10). The respective correlation for HDI monomer and urine HDA without creatinine adjustment was r = -0.03 and with creatinine adjustment r = -0.02. Our results confirm the earlier reported observation that measured biomarker levels of HDI monomer exposure (i.e., HDA levels in urine of blood) do not correlate with HDI oligomer exposure (Liu et al., 2004). Our results also confirm that TAHI is a suitable biomarker for HDI isocyanurate exposure and will allows us to distinguish between HDI isocyanurate and HDI monomer exposure and, thus, provide a major advance in characterizing both exposures through multiple exposure routes. Because HDI isocyanurate inhalation and skin exposure levels are significantly higher than levels of HDI monomer in the spray-painting environment (Fent et al., 2009a; Fent et al., 2009b; Fletcher, 2015; Kim, 2015; Reeb-Whitaker et al., 2012) and HDI isocyanurate is potentially more potent sensitizing agent (Aalto-Korte et al., 2010; Zissu et al., 1998), TAHI can be used as a direct biomarker of HDI isocyanurate dose.

Table 4. Linear correlations (r) between biological HDA and TAHI measurements and external exposure measurements for HDI monomer and HDI isocyanurate. Paint time represents the total paint time during the day (min). Air HDI and Air Isocyanurate represent the sum of concentrations for all tasks during the day multiplied by the total paint time (µg/m^3). Skin HDI and Skin Isocyanurate represent the sum of concentrations during the day from tape-strip sampling (ng/mm^3). HDA and TAHI in urine and plasma represent average daily concentrations (µg/L) and average daily concentrations adjusted for grams of creatinine (µg/g).

	Paint Time (min)	**Air HDI (µg/m^3)**	**Air Isocyanurate (µg/m^3)**	**Skin HDI (µg/mm^3)**	**Skin Isocyanurate (µg/mm^3)**
Urine					
HDA (µg/L)	0.01	-0.03	-0.02	-0.01	0
HDA (µg/creatinine)	-0.02	-0.02	-0.03	-0.01	0.01
TAHI (µg/L)	***0.28***	***0.22***	***0.21***	0	0.06
TAHI (µg/creatinine)	***0.11***	***0.19***	***0.10***	***0.14***	0.04
Plasma					
HDA (µg/L)	-0.01	-0.01	0	0.02	0.03
TAHI (µg/L)	***0.41***	***0.54***	***0.61***	-0.02	0.09

Current Ongoing Investigations

We have finished analysis of TAHI in urine and blood samples collected from the workers in Study I & II. However, we are currently re-analysis of plasma samples from Study II for HDA because the MDL (0.08 µg/L) was significantly higher than previously reported for Study I (0.02 µg/L) (Flack et al., 2010b). It is estimated the sample processing and analysis will take 1 – 2 months. We are continuing our efforts to develop the linear mixed-effects models to investigate the relationship between the biomarkers (*i.e.*, TAHI and HDA in urine and blood) and inhalation and skin exposure to HDI isocyanurate and HDI monomer as well as personal and work environment factors. Furthermore, we are expanding our genetic analyses (NIOSH R21 OH010203 "Influence of Genetic Markers on Exposure Assessment Models") to include these biomarkers to further investigate and to understand the impact of individual genetic and epigenetic variation on the observed biomarker levels (Sun, 2016; Taylor, 2017).

Due to unforeseen difficulties and time required for the development of robust analyses for TAHI biomarker

in urine and blood, the unexpected requirement to use a sophisticate nano-UPLC-ESI-MS/MS machine for these analyses, and the delays in availability to use this machine, we experienced significant delays during the granting period to perform the work proposed in the project. However, because most of the samples are now analyzed, we are able to proceed quickly in the data analyses and model developments to accomplish the goals outlined in Aim 2 and several manuscripts are being prepared for publication.

Conclusions

We were the first to develop a protocol for the analysis of trisaminohexyl isocyanurate (TAHI) as a biomarker of HDI isocyanurate exposure in the urine and blood of workers occupationally exposed to 1,6-hexamethylene diisocyanate (HDI)-containing spray-paints. HDI isocyanurate represents the predominant isocyanate exposure during automotive spray-painting, however, biological monitoring of spray-paint exposures has been limited to HDA, the hydrolysis metabolite of HDI monomer. Our goal was to develop an assay to detect and quantify TAHI in the urine and blood of exposed workers, and utilize this data to investigate how personal and workplace factors affect the exposure-dose relationship. To develop this assay, we synthesized standards and internal standards in-house, and evaluated three extraction methods and two derivatization methods over the first year of the project. The novel sample treatment involved acid-hydrolysis of urine or blood, liquid-liquid extraction with dichloromethane, and derivatization with acetic anhydride. The derivatized product, trisacetamidohexyl isocyanurate (TAAHI), was analyzed using an innovative nano-UPLC-ESI-MS/MS method for small molecule quantitation that we developed during this project. Our method is sensitive and specific for analysis of TAHI in urine and blood of exposed workers with method detection limits (MDL) of 0.03 µg/L and 0.02 µg/L, respectively.

With this developed assay, we analyzed urine samples from 72 exposed workers (N = 607) and plasma samples from 69 exposed workers (N = 173). TAHI was detected above the MDL in 195 (32%) urine samples (55 of 72 workers) and in 26 (15%) plasma samples (15 of 69 workers). We observed positive linear correlations between paint time or daily total breathing-zone HDI isocyanurate with TAHI in urine or plasma. The strongest linear correlation was observed between the total daily breathing-zone HDI isocyanurate and the daily plasma TAHI (r = 0.61). Comparatively, it is noteworthy that no linear correlations were observed between paint time or breathing-zone HDI monomer with HDA in urine or plasma. Using single factor ANOVA and Student's t-test (assuming unequal variances), we determined that there were statistically significant differences between the Study I and Study II data sets for paint time ($p < 0.05$), breathing-zone HDI monomer and HDI isocyanurate levels ($p < 0.01$), and skin HDI isocyanurate levels ($p < 0.001$). The differences in exposures between Study I and Study II may contribute to the difference in the measured mean exposure levels and the number of detected values for TAHI in urine and plasma. We are currently developing our sophisticated exposure modelling tools to evaluate the relative contributions of HDI isocyanurate and HDI monomer exposures to the observed biomarker levels. Our data clearly shows that the 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. Additionally, analysis of HDI isocyanurate exposure and urine TAHI will allow us to determine whether TAHI follows a similar bi-phasic elimination pattern to urine HDA. Our results confirm that TAHI is a suitable biomarker for HDI isocyanurate exposure and will allows us to distinguish between HDI isocyanurate and HDI monomer exposure and, thus, provide a major advance in characterizing both exposures through multiple exposure routes. The identification and quantification of TAHI as a biomarker of HDI isocyanurate exposure is critical for improvement of diisocyanate exposure assessment through characterization of exposure-dose relationships for both HDI monomer and HDI isocyanurate in occupationally exposed populations. Because HDI isocyanurate inhalation and skin exposure levels are significantly higher than levels of HDI monomer in the spray-painting environment (Fent et al., 2009a; Fent et al., 2009b; Fletcher, 2015; Kim, 2015; Reeb-Whitaker et al., 2012) and HDI isocyanurate is potentially more potent sensitizing agent (Aalto-Korte et al., 2010; Zissu et al., 1998), TAHI can be used as a direct biomarker of HDI isocyanurate dose. We expect this knowledge will provide useful input in setting exposure limits, which are currently lacking for HDI oligomers.

Publications

Robbins Z, Bodnar W, Zhang Z, Gold A, Nylander-French LA: [2017] Trisaminohexyl Isocyanurate, a Urinary Biomarker of HDI Isocyanurate Exposure. *J Chromatogr B Analyt Technol Biomed Life Sci*, under review.

Taylor, L.: [2017] Influence of Genetic Variance on Occupational Exposure to 1,6-Hexamethylene Diisocyanate Isocyanurate, MS Thesis, University of North Carolina at Chapel Hill.

Robbins Z, Bodnar W, Zhang Z, Gold A, Nylander-French LA: [2016] Trisaminohexyl Isocyanurate, a Biomarker for HDI Isocyanurate Exposure. 26th Annual International Society of Exposure Science Conference, Utrecht, The Netherlands, October 9-13.

Additional scientific manuscripts presenting the findings included in this report are currently being prepared for publication.

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Cumulative Inclusion Enrollment Report Table

View Burden Statement

PHS Inclusion Enrollment Report

This report format should NOT be used for collecting data from study participants.

OMB Number: 0925-0001 and 0925-0002
Expiration Date: 10/31/2018

***Study Title (must be unique):** Quantifying Determinants of Spray Painters' Isocyanurate Exposure

* Delayed Onset Study? ☐ Yes ☒ No

If study is not delayed onset, the following selections are required:

Enrollment Type ☐ Planned ☒ Cumulative (Actual)

Using an Existing Dataset or Resource ☒ Yes ☐ No

Enrollment Location ☒ Domestic ☐ Foreign

Clinical Trial ☐ Yes ☒ No **NIH-Defined Phase III Clinical Trial** ☐ Yes ☐ No

Comments:

Racial Categories	Ethnic Categories									
	Not Hispanic or Latino			Hispanic or Latino			Unknown/Not Reported Ethnicity			**Total**
	Female	**Male**	**Unknown/ Not Reported**	**Female**	**Male**	**Unknown/ Not Reported**	**Female**	**Male**	**Unknown/ Not Reported**	
American Indian/ Alaska Native	0	1	0	0	0	0	0	0	0	1
Asian	0	1	0	0	0	0	0	0	0	1
Native Hawaiian or Other Pacific Islander	0	0	0	0	0	0	0	0	0	0
Black or African American	0	4	0	0	0	0	0	0	0	4
White	1	48	0	0	9	0	0	0	0	58
More than One Race	0	5	0	0	4	0	0	0	0	9
Unknown or Not Reported	0	0	0	0	0	0	0	0	0	0
Total	1	59	0	0	13	0	0	0	0	73

Inclusion of Gender and Minority Study Subjects
The spray painters in our study cohort included 72 males and one female. The self-reported ethnic subgroups were divided between 60 Caucasian, 9 Hispanic, 4 African-American, 1 Asian, 1 Native American, and 9 of more than one ethnic group.

Inclusion of Children
Not Applicable. All the workers who agreed to participate in the study and provided informed consent as well as environmental and biological samples were older than 21 years.

Materials Available for Other Investigators
No data or research materials are available to be shared with other investigators at this time. Sharing research resources is not possible. The study materials consist of individual human samples obtained by an IRB approval from the investigators institutions. The IRB approval specifically states that samples and data derived from human subjects cannot be made available to other investigators. However, scientific findings will be shared with other researchers in the larger scientific community and the public via scientific publications and submission of accepted manuscripts to PubMed Central. The dissemination of results and information to the public will be done according to the published NIH guidelines for dissemination of research results (grants.nih.gov/grants/policy/data_sharing). All computational tools, mathematical and statistical models will be shared with other investigators after these have been validated internally and through a peer-review process.