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Trisaminohexyl isocyanurate, a urinary biomarker of HDI isocyanurate exposure

Zachary Robbins, **Wanda Bodnar**, **Zhenfa Zhang**, **Avram Gold**, **Leena A. Nylander-French***

Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, NC 27599, USA

Abstract

Biological monitoring of occupational exposure to 1,6-hexamethylene diisocyanate (HDI)-containing spraypaints is limited to analysis of metabolites of HDI monomer although polymeric HDI isocyanurate constitutes the predominant inhalation and skin exposure for workers in the automotive paint industry. A novel method using nanoflow ultra-performance liquid chromatography coupled to nano-electrospray ionization tandem mass spectrometry (nano-UPLC-ESI-MS/MS) was developed to quantify trisaminohexyl isocyanurate (TAHI), a hydrolysis product of HDI isocyanurate, in the urine of spray-painters. Analytical and internal standards were synthesized in-house and weighted linear regression calibration curves were generated using spiked control urine from non-exposed persons (0.06–7.98 μg/L; $N = 13$; $w = x^{-2}$; $r = 0.998$). Urine samples collected from 15 exposed workers ($N = 111$) were subjected to acid hydrolysis and extracted with dichloromethane, then derivatized with acetic anhydride. The derivatized product, trisacetamidohexyl isocyanurate (TAAHI), was analyzed using nano-UPLC-ESI-MS/MS. The protocol was sensitive and specific for analysis of TAHI in the urine of exposed workers with a method detection limit at 0.03 μg/L. TAHI was detected in 33 of 111 urine samples and in 11 of 15 workers. This biomarker for HDI isocyanurate is critical to determine the relative potency and dose-relationships between the monomer and oligomer exposure on the development of diisocyanate induced health effects in future studies.

Keywords

Biomarker; Exposure; Isocyanate; 1,6-Hexamethylene diisocyanate; Isocyanurate; Liquid chromatography–mass spectrometry

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 [1]. They are used in the manufacturing of many common products containing polyurethane such

*Corresponding author: leena_french@unc.edu (L.A. Nylander-French).

Declaration of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

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as adhesives, spray paints, foams, insulation, resins, sealants, and surface coatings [2,3]. One of the most commonly used isocyanates is 1,6-hexamethylene diisocyanate (HDI), comprised of the monomer and oligomers[1] (Fig. 1) [4]. Occupational exposure occurs during industrial production or during spray-painting operations such as auto-body refinishing or application of marine coatings [2]. 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 [5–11]. Exposures to aerosols and vapors of HDI monomer and oligomers, including HDI isocyanurate, are associated with a high risk of contact dermatitis and asthma [12–16]. Acute exposure can cause shortness of breath, rhinitis, irritation of the skin, eyes, and mucous membranes, and pulmonary edema [8,9,14,17].

Significant levels of inhalation and skin exposure to HDI monomer and its oligomers have been reported in spray-painters [18–22]. The predominant inhalation and skin exposure in automotive spray-painting is to HDI isocyanurate [20–22], 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 [16,23], and occupational asthma has been linked to HDI oligomer exposure without an immune response to the monomer [24]. Furthermore, it has been shown that HDI isocyanurate also penetrates skin at much faster rates (approximately 350 to 500 times) than HDI monomer [25]. 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 [18,19,26–31]. However, it has been shown that measured biomarker levels of HDI monomer exposure do not correlate with HDI oligomer exposure [32]. 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.

[1]Oligomers of isocyanates, which are indicated with different terms (prepolymers, polyisocyanates, adducts) in the literature, will be referred to as oligomers in this article.

2. Experimental

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. 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 Fig. 2. Composition and purity of the four standards were confirmed by NMR and LC–MS/MS in-house (see below).

2.2.1. Trisaminohexyl isocyanurate (MW = 426.3 g/mol; Fig. 2A)—Commercially available Desmodur® N 3300 (Bayer Material Science, 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 (Fig. S1). 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 (Fig. S2) and 35 eV (Fig. S3).

2.2.2. Trisacetamidohexyl isocyanurate (MW = 552.4 g/mol; Fig. 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) (Fig. S4). ^{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 (Fig. S5). Fragmentation spectra of precursor ion [M +

H]$^+$ for TAAHI (*m*/*z* 553.3) were obtained by direct injection ESI-MS/MS at collision energies 25 eV (Fig. 3A) and 50 eV (Fig. 3B).

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 (Fig. S6). ^{13}C NMR, (100 MHz, $CDCl_3$), 149.1, 119.8, 43.0, 28.4, 27.7, 26.1, 25.4, 17.3 ppm (Fig. S7).

2.2.4. Trisaminoheptyl isocyanurate (MW = 468.4 g/mol; Fig. 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 (Fig. S8). 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 (Fig. S9) and 35 eV (Fig. S10).

2.2.5. Trisacetamidoheptyl isocyanurate (MW = 594.4 g/mol; Fig. 2D)—TAHpI trichloride was acetylated with *N*,*N*′-dicyclohex-ylcarbodiimide 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 (Fig. 4A) and 50 eV (Fig. 4B).

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).

2.3. Study population

Urine samples were collected from 15 male spray-painters (*N* = 111) at 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 [20,21,27,30]. HDI monomer and oligomer exposures were quantified using personal breathing-zone and skin tape-strip sampling [20,21], and HDA levels were quantified in plasma and urine [27,30].

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 25M sodium hydroxide (2 mL) prior to liquid–liquid extraction with dichloromethane (3 × 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 mono-basic 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.

2.5. Chromatographic and mass spectrometric conditions

Urine samples were analyzed by 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 S1). 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 (24 eV), m/z 553.3 → 212.1 (46 eV), and m/z 553.3 → 130.0 (52 eV) (Fig. 5A), 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) (Fig. 5B).

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 ≥ 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. [33]. 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 < 15% for each quality control standard. The method detection limit (MDL) was calculated using the procedure established by the US EPA [34]. 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.

3. Results

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 Fig. 6. Control urine from a non-exposed volunteer (Fig. 6A) and urine sample 8 from worker #7 (Fig. 6B) 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 (Fig. 7A–B) and urine sample 3 from worker #14 (Fig. 7C–D). The fragmentation spectra for both spray-painters' urine samples closely resembled the spectra obtained from the purified standard (Fig. 3A–B). 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 Fig. 8 for control urine from a non-exposed volunteer spiked with 0.25 μg/L TAHI and 2.0 μg/L TAHpI (Fig. 8A) and urine sample 8 from worker #7 spiked with 2.0 μg/L TAHpI (Fig. 8B). 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.

3.2. TAHI in urine of spray painters

Table 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-painters' breathing-zone and skin HDI isocyanurate exposures were measured previously [20,21]. The mean and standard deviation for the paint-time adjusted breathing-zone exposure ranged from 70 ± 39 to 34,304 ± 27,191 $\mu g/m^3$ and for the skin exposure from 3 ± 4 to 3857 ± 3882 $\mu g/mm^3$. 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 0.06.

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 [18,20–22,27,29,30]. However, biological monitoring has been limited to the metabolites of HDI monomer exposure [18,19,26,27,29–31,35,36] even though HDI isocyanurate constitutes the largest portion of isocyanate exposure for spray-painters [20–22]. 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 [19,26,27,29–32,35–39]. However, the derivatizing agents commonly used for HDA analysis, heptafluorobutyric acid (HFBA) or perfluoropentanoic acid (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 [26,40–42]. LC–MS has also been used in analysis of

biomarkers of exposure to methylene diphenyl diisocyanate (MDI) [43,44] and toluene diisocyanate (TDI) [45–47]. 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 [18,19,26,27,30–32,35,37,39,48–50]. Acid hydrolysis is non-selective with a higher yield of total amine from acetylated, protein-conjugated, as well as unconjugated species [27,28,30,37] and is preferable to alkaline hydrolysis which selectively releases mono- and di-acetylated HDA [28,37,51–53]. Dichloromethane, an extraction solvent reported in the analytical literature [28,29,50–52,54,55], 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 [45]. 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 [18,19,26,27,30–33,35,39,42,48], hexane, and ethyl acetate [45,52,56]. 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) [30].

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 66,637 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 (15,946 μg/m^3 and 65 μg/m^3, respectively) and the

mean skin concentration (670 μg/mm^3 and 3 μg/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 [30]. 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.

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 note-worthy 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 [30]. 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 a more potent sensitizing agent [16,23], 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

^{13}C NMR	carbon-13 nuclear magnetic resonance spectroscopy
ESI	electrospray ionization
GC–MS	gas chromatography–mass spectrometry
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
HDA	1,6-diaminohexane
HDI	1,6-hexamethylene diisocyanate
HFBA	heptafluorobutyric acid
LC–MS	liquid chromatography-mass spectrometry
LLE	liquid–liquid extraction
MAPE	mean absolute percentage error
MDI	methylene diphenyl diisocyanate
MDL	method detection limit
PFPA	perfluoropentanoic acid
SPE	solid-phase extraction
SRM	selected reaction monitoring
TAAHI	trisacetamidohexyl isocyanurate
TAAHpI	trisacetamidoheptyl isocyanurate
TAHI	trisaminohexyl isocyanurate
TAHpI	trisaminoheptyl isocyanurate
TDA	toluenediamine
TDI	toluene diisocyanate
UPLC	ultra-performance liquid chromatography

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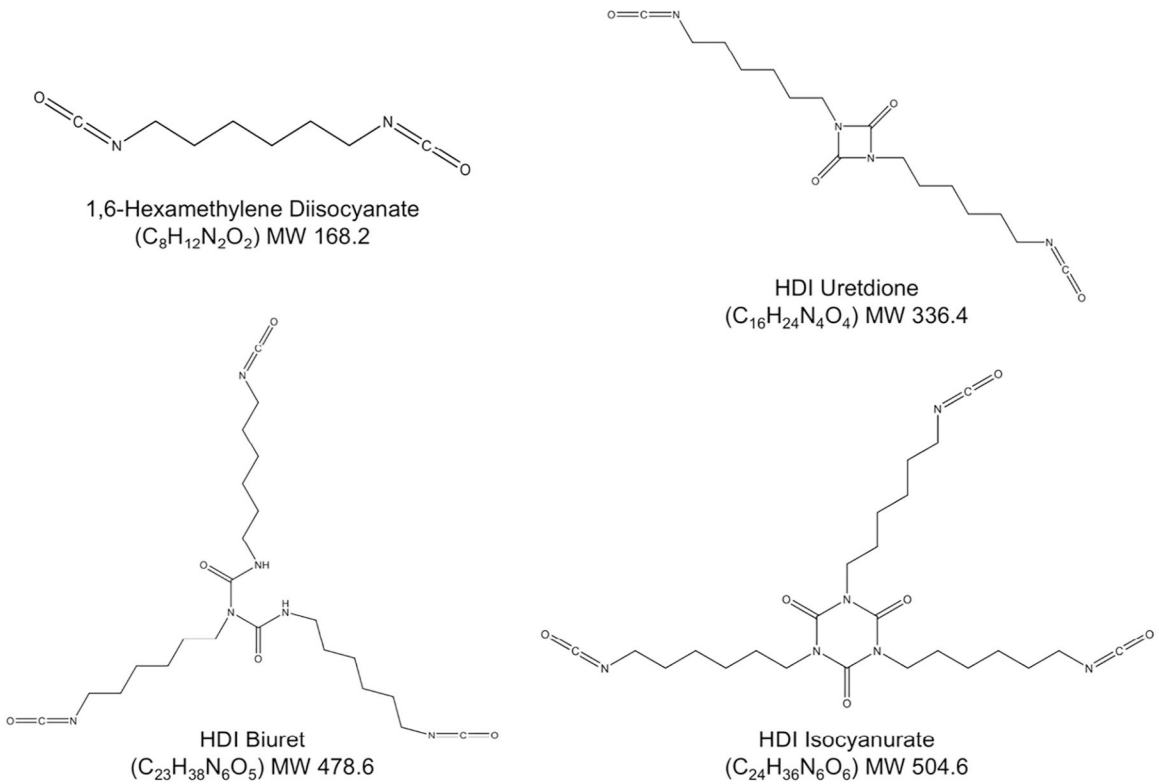


Fig. 1.

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

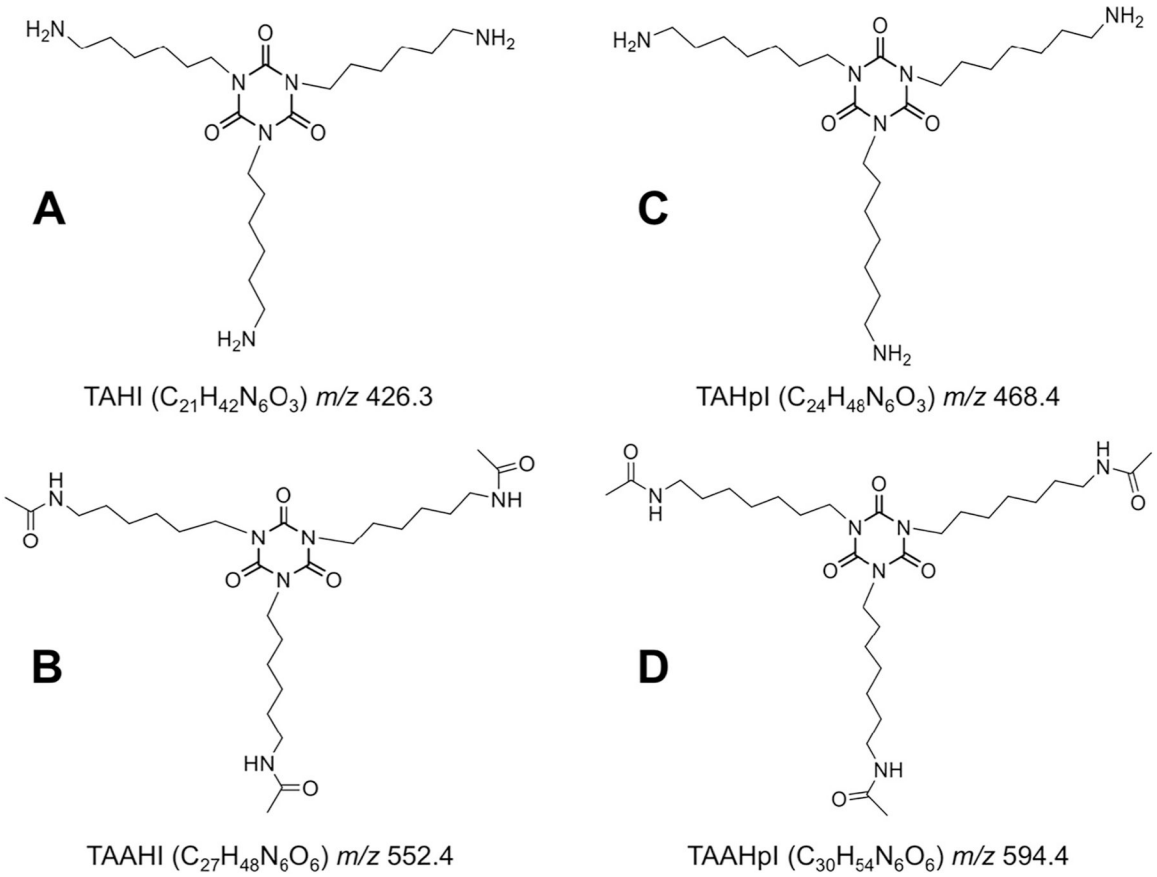


Fig. 2.

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

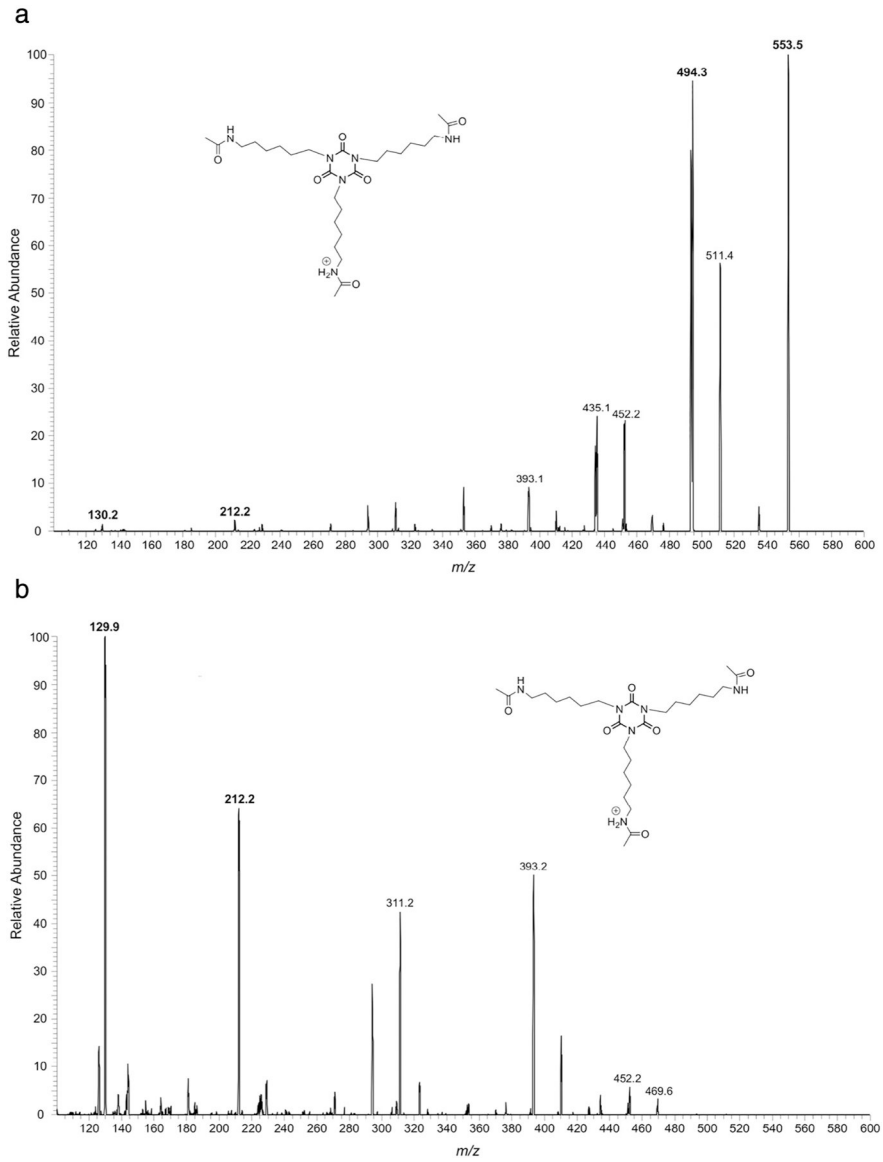


Fig. 3.

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

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

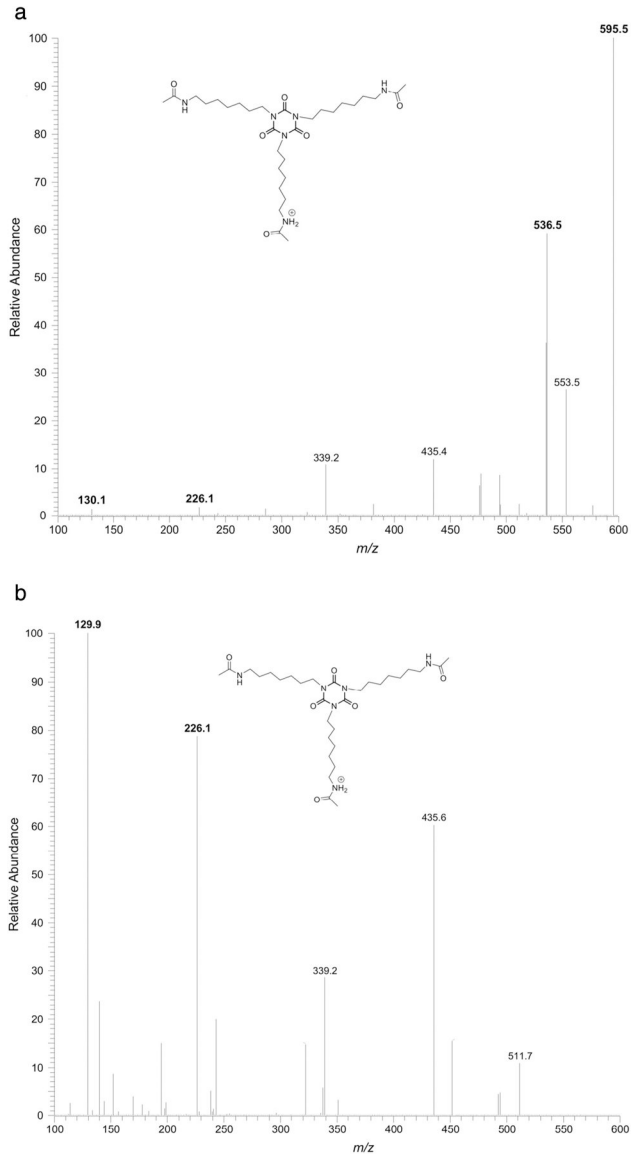


Fig. 4.

A: 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).

B: 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).

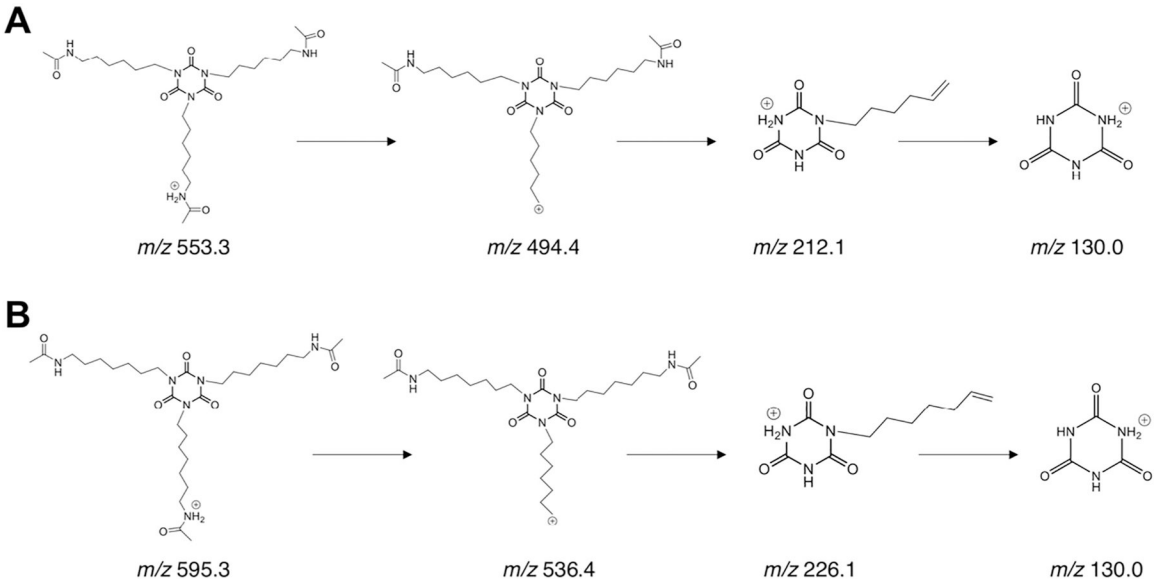


Fig. 5.
[A] TAAHI mass spectral fragments and [B] TAAHpI mass spectral fragments.

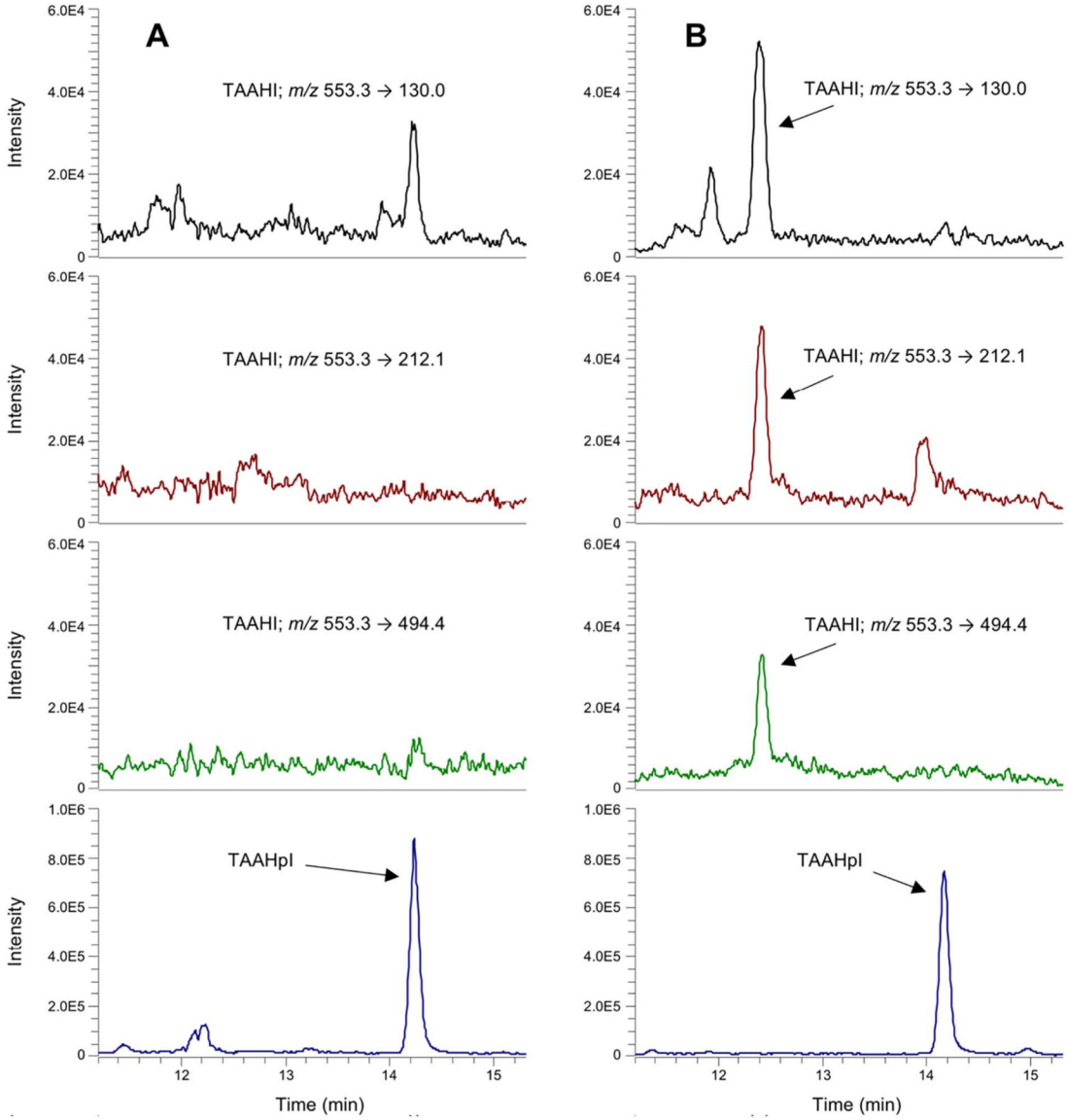
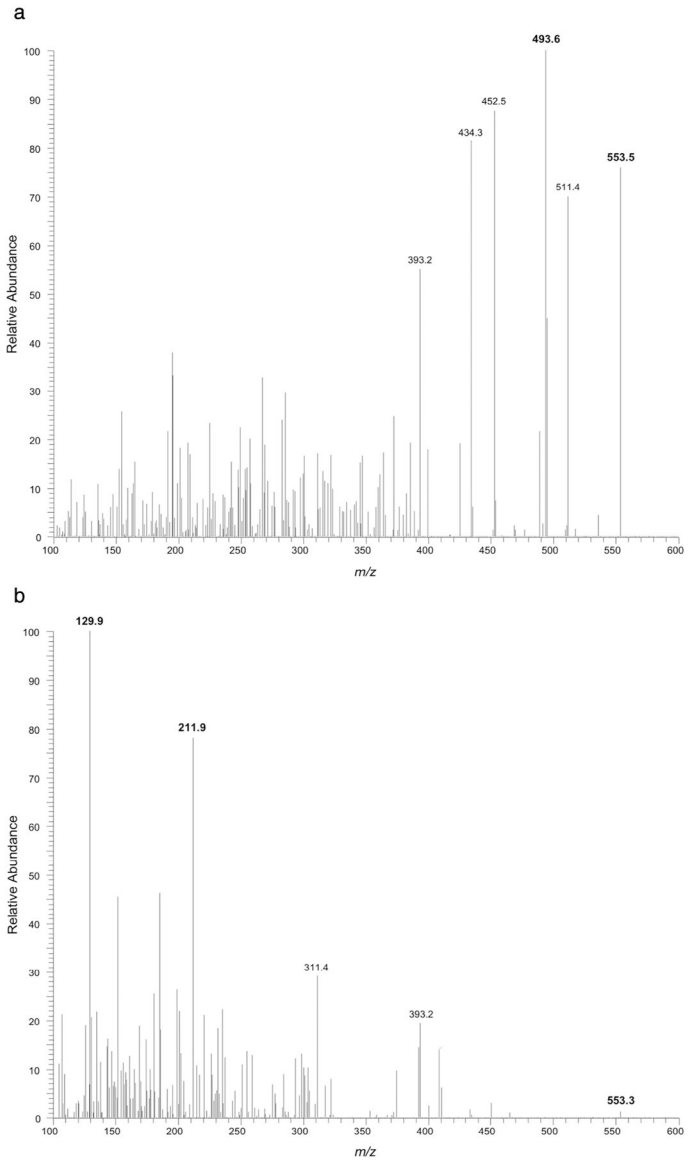


Fig. 6.

[A] 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 (combined *m*/*z* 595.3 → 130.0, 226.1, and 536.4); obtained for control urine spiked with TAHpI (2.0 μg/L).

[B] 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 (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.



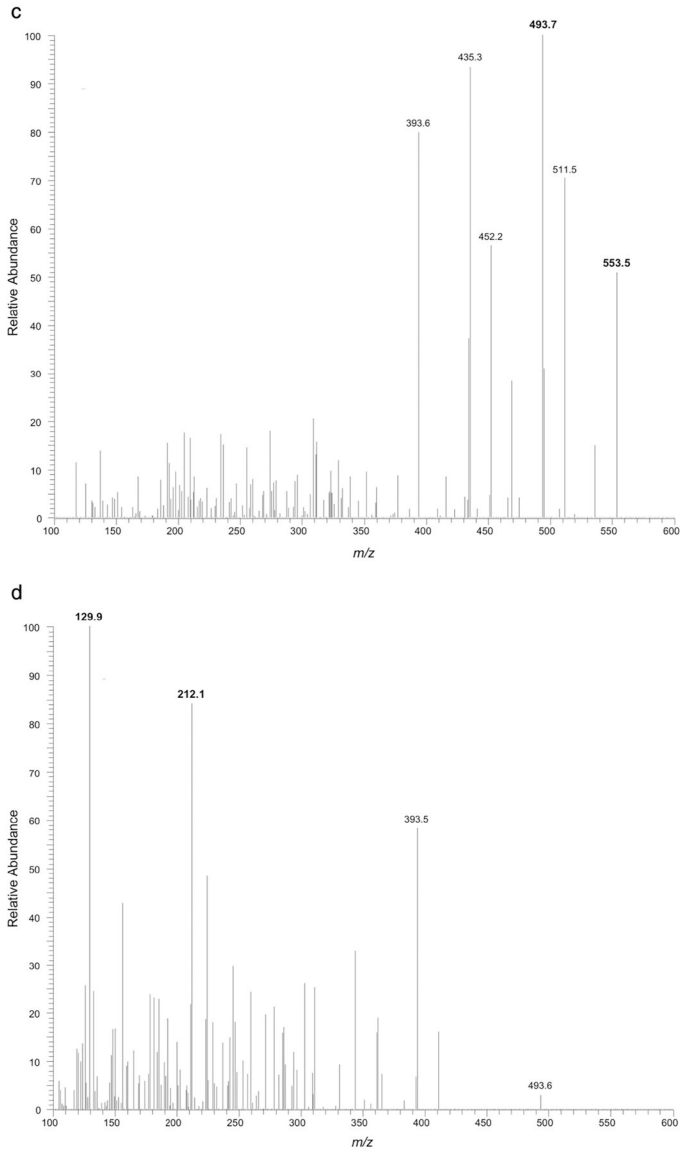


Fig. 7.

A: 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).

B: 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).

C: 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).

D: 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).

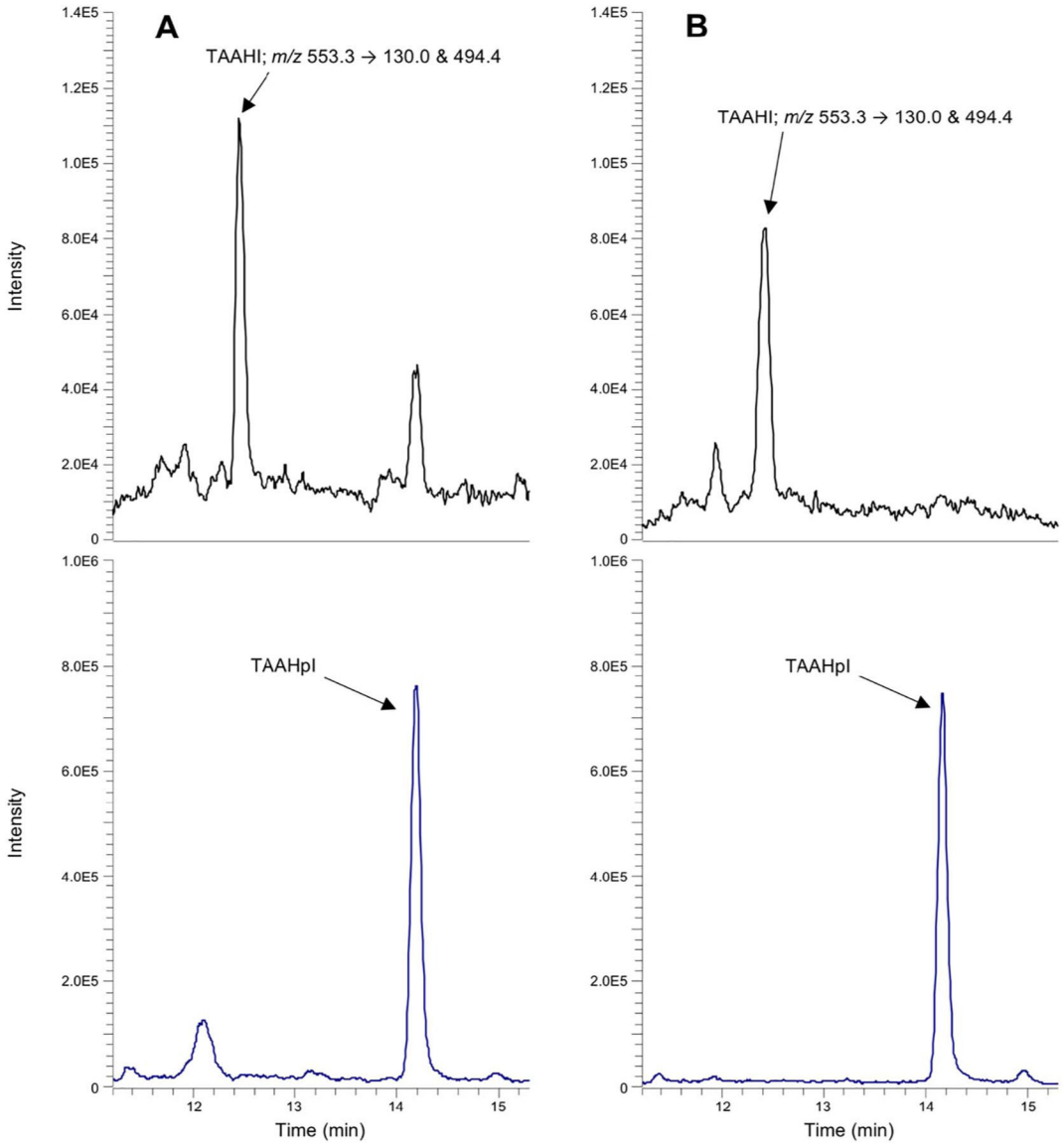


Fig. 8.

[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.

Table 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	11,802 ± 11,460	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	10,232 ± 6570	313 ± 277	12	0.22 ± 0.38	0.32 ± 0.24
4	1	2	8.0 ± 1.4	10,752 ± 12,539	1387 ± 1816	3	0.06 ± 0.06	< MDL
5	1	1	19.5	21,931	637	3	0.07 ± 0.12	0.04 ± 0.07
6	2	6	6.2 ± 2.5	34,304 ± 27,191	1181 ± 570	8	0.34 ± 0.42	0.14 ± 0.18
7	3	12	5.2 ± 3.3	17,101 ± 14,805	730 ± 502	19	0.42 ± 0.68	0.14 ± 0.23
8	2	3	5.8 ± 2.8	16,418 ± 4785	676 ± 520	5	0.27 ± 0.15	0.06 ± 0.13
9	2	4	9.0 ± 5.4	12,870 ± 20,501	635 ± 836	6	0.19 ± 0.12	< MDL
10	2	5	3.4 ± 1.7	18,970 ± 29,236	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	27,618 ± 32,774	3857 ± 3882	6	0.32 ± 0.13	0.34 ± 0.55
13	1	1	1.5	20,927	339	2	0.18 ± 0.26	1.99 ± 2.81
14	3	10	7.6 ± 3.1	20,435 ± 22,563	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 = below method detection limit.

[b] 5 of 6 urine samples were below MDL.