A validated UPLC-MS/MS method for the determination of aliphatic and aromatic isocyanate exposure in human urine

Maggy Lépine[1, 2], Lekha Sleno[1*], Jacques Lesage[1] and Sébastien Gagné[2*]

1) *Université du Québec à Montréal*, Chemistry Department, PO Box 8888 Downtown Station, Montreal, H3C 3P8, Canada, sleno.lekha@uqam.ca

2) *Institut de recherche Robert-Sauvé en santé et en sécurité du travail*, 505 boul. De Maisonneuve Ouest, Montréal, Québec, H3A 3C2, Canada, (514) 288-1551, sebastien.gagne@irsst.qc.ca

*co-corresponding authors

ABSTRACT

4,4'-methylenediphenyldiisocyanate (MDI), toluenediisocyanate (2,4-TDI and 2,6-TDI), and 1,6'-hexamethylenediisocyanate (HDI) are all commonly used in the production of polyurethane-containing materials in different application areas. Workers exposed occupationally to these compounds may develop sensitization with the potential to lead to asthma. Isocyanates are metabolized *in vivo* by conjugation to macromolecules and/or by acetylation prior to being eliminated into urine. The hydrolysis of urine samples releases free amine compounds from these metabolites as biomarkers of exposure, specific to each parent isocyanate: 4,4'-methylenedianiline (MDA), toluenediamine (2,4-TDA and 2,6-TDA) and hexamethylenediamine (HDA). To address the need for a validated method that could be used for the simultaneous determination of biomarkers of aliphatic and aromatic isocyanates to monitor occupational exposure based on recommended thresholds, we have developed an UPLC-MS/MS method for the quantitation of MDA, TDA isomers and HDA following acid hydrolysis, solid-phase extraction and derivatization of urine samples. Free amine compounds were derivatized with acetic anhydride to augment chromatographic retention and signal intensity. The method was developed considering the biological guidance value (BGV) of MDA at 10 $\mu g \cdot L^{-1}$, and biological exposure indices (BEI) of TDA isomers and HDA at 5 $\mu g \cdot g^{-1}$ and 15 $\mu g \cdot g^{-1}$ creatinine, respectively. Limits of detection allowed monitoring down to 6% of BGV/BEI, with precision within 8%. Using inter-laboratory reference samples, the accuracy and reliability of the method, were assessed and deemed acceptable based on three rounds of measurements. This novel method has therefore been proven as useful for occupational safety and health assessments.

Keywords: Isocyanate, Urinary biomarkers, Biological monitoring, Quantitative analysis, UHPLC-MS/MS, Acetylation

1. INTRODUCTION

Isocyanates are reactive molecules used in the manufacturing of polyurethanes (PURs). PURs are used in a large variety of applications and therefore found in various materials. Aromatic isocyanates, such as toluenediisocyanate (TDI) and 4,4-methylenediphenyldiisocyanate (MDI), are mainly used in the production of insulating foams, flexible foams, glues and adhesives [1-3] while aliphatic isocyanates, such as hexamethylenediisocyanate (HDI), are often found in paints [4, 5]. MDI, TDI and HDI contain two isocyanate groups (N=C=O) in their structures [6]. Isocyanates have the ability of forming polyisocyanate molecules, especially for MDI and HDI. The high reactivity of isocyanate makes them toxic, causing adverse health effects. These compounds can cause skin and eye irritation[7], as well as respiratory sensitization [1, 8] Different acute and chronic effects ranging from dermatitis and irritation of mucous membranes [1, 9] to more severe effects on the respiratory system, such as occupational asthma [9-14], hypersensitivity pneumonitis [9, 14, 15] and reduced lung function [1] may result from isocyanate exposure.

In order to ensure workplace safety, it is important to properly monitor the exposure to isocyanates by using environmental air monitoring combined with biological monitoring. Environmental monitoring does not allow the real absorbed dose in the body (or exposure) to be evaluated, while biological monitoring can measure all possible routes of exposure. Biological monitoring is essential for the evaluation of personal protective equipment, the contribution of dermal and oral absorption, and also to assess if the workload can be increased for a given

individual. It allows isocyanate exposure to be measured using the known urinary metabolites of each isocyanate as biomarkers of exposure [11].

Isocyanates absorbed in the body, mainly by inhalation and skin contact, are metabolized through bimolecular conjugation (forming protein and glutathione adducts) or acetylation reactions. The low molecular weight conjugates and acetylated metabolites formed are eliminated into urine [15-18]. Urine samples containing those metabolites must be hydrolysed to release free amine compounds as biomarkers of exposure [1, 10] corresponding to 4,4'-methylenedianiline (MDA), 2,4-toluenediamine (2,4-TDA), 2,6-toluenediamine (2,6-TDA) and hexamethylenediamine (HDA) for MDI, 2,4-TDI, 2,6-TDI and HDI, respectively.

Recommended values have been established by internationally-recognized institutions to evaluate biological biomarkers of exposure to TDI (isomers), MDI and HDI in urine after an eight-hour work shift. The German Research Foundation (DFG) has fixed a biological guidance value (BGV) of hydrolysed MDA at 10 $\mu g \cdot L^{-1}$ (≈50 nM) for monitoring MDI exposure [19]. The American Conference of Governmental Industrial Hygienists (ACGIH) has established TDI and HDI biological exposure indices (BEI) values of hydrolysed TDA (2,4-TDA and 2,6-TDA) and hydrolysed HDA at 5 $\mu g \cdot g^{-1}$ creatinine (≈20–125 nM for a range of 4.4-26.5 mM creatinine) and 15 $\mu g \cdot g^{-1}$ creatinine (≈60–400 nM for a range of 4.4-26.5 mM creatinine) respectively [20].

Multiple methods have been developed for the determination of isocyanate biomarkers using liquid chromatography-mass spectrometry (LC-MS) [15, 21-23] and gas chromatography-mass spectrometry (GC-MS) [24]. The use of mass spectrometry for detection gives good selectivity and acceptable sensitivity for the analysis of these biomarkers in a complex matrix, such as urine. Various published methods suggest different strategies to evaluate biomarkers of exposure

to isocyanates in human urine. The hydrolysis time proposed can be relatively short, for example 1 hour[24], or rather much longer (16 hours) [10, 23]. The extraction of hydrolysed compounds in urine samples can be performed using a liquid-liquid extraction (LLE) with toluene[10, 23], dichloromethane[22] or ethyl ether[24], or using a solid-phase extraction (SPE)[15, 21]. Some of these methods also use derivatization of the molecules followed by analysis by GC-MS or LC-MS. These derivatization steps require either a long heating step [24] or an additional extraction step to remove excess reagent [10, 23]. To be able to evaluate adequately the biological exposure to isocyanates in the workplace, a method needs to be reliable but also developed considering the recommended values based on worker exposure levels. Published analytical methods have not previously allowed the simultaneous measurement of these four isocyanates biomarkers to monitor occupational exposure based on recommended threshold values [10, 22, 23], or have only been developed to assess exposure in the general population [15, 21]. In addition, the published methods have not assessed their performance with an inter-laboratory proficiency program in order to ensure the reliability of the method for occupational safety and health (OSH) requirements. For this purpose, a new and validated quantitative assay has been developed in human urine using a simple solid phase extraction (SPE) and derivatization followed by UPLC-MS/MS analysis, employing multiple reaction monitoring on a triple quadrupole instrument, for assessing isocyanate exposure. The newly developed method was tested with reference samples from the inter-laboratory program provided from the German External Quality Assessment Scheme (G-EQUAS).

2. EXPERIMENTAL

2.1 Chemicals

4,4'-methylenedianiline (MDA), 1,6-hexamethylenediamine (HDA), 2,4-toluenediamine (2,4-TDA), 2,6-toluenediamine (2,6-TDA), 1,6-diaminohexane-1,1,6,6-d_4 (d_4-HDA), and N,N′-hexamethylene bis(acetamide) (diacetyl-HDA) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). 2,6-diaminotoluene-α,α,α-d3 (2,6-d_3-TDA) and 2,4-diaminotoluene-α,α,α-d3 (2,4-d_3-TDA) were purchased from C/D/N isotopes (Pointe-Claire, Canada). 4,4'-methylenedianiline-[$^{15}N_2$,^{13}C] ($^{13}C^{15}N_2$-MDA) was obtained from IsoScience (Ambler, PA, USA). Acetonitrile (ACN), methanol (MeOH) and water, all LC-MS grade, and ammonium hydroxide (30%, NH_4OH) were all purchased from Fisher Scientific (St-Laurent, Canada) with. Sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), sodium tetraborate decahydrate, acetic anhydride, and LC-MS grade formic acid were also obtained from Sigma-Aldrich (Milwaukee, WI, USA).

2.2 Collection of human urine

Non-occupationally exposed human urine was obtained by anonymous donation and kept at 4ºC for less than 30 days as control urine. Inter-laboratory urine samples were provided from the German External Quality Assessment Scheme (G-EQUAS) for analyses in biological materials (Erlangen, Germany). All urine samples used in this work were collected in accordance with IRSST's Research Ethics Committee validated procedures. Humans were not directly involved in the present study.

2.3 Creatinine and density levels

Measurement of creatinine and density levels were evaluated in urine samples the same day. Urinary creatinine was measured by the spectrometric method based on Jaffe's reaction [25]. An Architect c4000 system spectrophotometer from Abbott (Abbott Park, IL, USA) was used to perform the analysis. The density measurements were determined by refractometry [25] with a Densitometer DMA 38 from Anton-Paar (Saint-Laurent, Canada).

2.4 Calibration curve solutions

Stock solutions of 500 µM of MDA, $^{13}C^{15}N_2$-MDA, 2,4-TDA, 2,6-TDA, 2,4-d_3-TDA and 2,6-d_3-TDA were prepared in 100% acetonitrile. HDA and d_4-HDA stocks were prepared in water. Seven working standard solutions of MDA, 2,4-TDA, 2,6-TDA and HDA were prepared from stocks in water. The first working solution were at 0.2 µM and contained MDA and TDA isomers only. The six other solutions at 0.5, 1, 2, 5, 10 and 20 µM contained all analyte compounds. An internal standard (IS) working solution was prepared at 2 µM for $^{13}C^{15}N_2$-MDA, 2,4-d_3-TDA, 2,6-d_3-TDA and d_4-HDA. Calibration samples were prepared by adding 10 µL of standard working solution and 10 µL of IS working solution in 180 µL of urine. Final concentrations of MDA and TDA isomers were 10, 25, 50, 100, 250, 500 and 1000 nM (2 – 200 $\mu g \cdot L^{-1}$ for MDA and 1.2 – 122 $\mu g \cdot L^{-1}$ for TDA isomers) while the final concentration for HDA were 25, 50, 100, 250, 500 and 1000 nM (2,9 – 116 $\mu g \cdot L^{-1}$). The final concentration of IS was 100 nM for all compounds (19.8 $\mu g \cdot L^{-1}$ for MDA, 12.2 $\mu g \cdot L^{-1}$ for TDA isomers and 11.6 $\mu g \cdot L^{-1}$ for HDA).

2.5 Sample preparation

IS working solution (10 µL) was spiked into 190 µL of urine, followed by the addition of 200 µL of 3 M sulfuric acid. The mixture was heated at 80ºC for overnight (≥16h) using a Eppendorf thermomixer (Fisher Scientific, Nepean, Canada) and cooled to room temperature followed by the addition of 200 µL of 5 N NaOH and centrifuge with a Sorvall Legend Micro 21 centrifuge (Fisher Scientific, St-Laurent, Canada) at 20 215 x g (14 500 RPM) for 3 minutes to remove any insoluble material prior to solid-phase extraction (SPE). SPE were performed using an extraction manifold (with 20 cartridge positions) (Waters, Mississauga, Canada). MCX (30 mg, 1 mL) mixed-mode cation exchange cartridges from Waters (Waters, Dublin, Ireland) were first conditioned with 1 mL of methanol followed by equilibration with 1 mL of water. Samples were then loaded on the cartridge, then 1 mL of 2% FA in water followed by 1 mL of methanol were added to wash the cartridge. Samples were eluted using 500 µL of 5% NH_4OH in 83% methanol and 2 x 500 µL of 15% NH_4OH in 50% methanol. Eluents were then evaporated to dryness under nitrogen at 60ºC with a TurboVap LV evaporation system (Biotage, Charlotte, NC, USA). Extracts were reconstituted with 198 µL of borate buffer (50 mM, pH 8.5) and instantly derivatized with the addition of 2 µL of pure acetic anhydride at room temperature. Samples were finally centrifuged at 20 215 x g (14 500 RPM) for 3 minutes to remove any insoluble material and transferred to a HPLC vial with insert. A hydrolysis test was performed using diacetyl-HDA (commercially available) while diacetyl-MDA and diacetyl-TDA isomers were produced using anhydride acetic derivatization of the free amine versions of each. Urine sample spiked with double acetylated MDA, TDA and HDA, and IS at 5 µM as final concentration were treated with 200 µL of 3 M sulfuric acid at 80°C. Aliquots were taken at 0, 1, 2, 4, 6, and 24h time points for TDI isomers and MDI metabolites while aliquots were taken at 0, 1, 2, 4, 6, 8, 16

and 24 h for HDI metabolites. Sodium hydroxyde (200 µL, 5N) was added to each aliquot prior to the SPE step described above.

2.6 LC-MS/MS analysis

Samples were injected (5 µL) onto an Acquity UPLC HSS T3 1.7 µm, 2.1 x 50 mm column (Waters, Dublin, Ireland) using a Waters ACQUITY I-class UPLC. Elution was performed at 40ºC with a flow rate of 0.6 $mL \cdot min^{-1}$ using a gradient of water (A) and acetonitrile (B), both containing 0.1% formic acid. The gradient started at 2% B for 1 min, increased first to 17% in 3 min, to 40% in 1.5 min and to 90% in 0.5 min and held for 1 min. MRM was performed on a Waters Xevo TQ triple quadrupole mass spectrometer (Waters, Milford, MA, USA) in electrospray positive ion mode. Source parameters were as follows: source and desolvation temperature, respectively of 150°C and 500°C, and desolvation flow of nitrogen gas at 1000 $L \cdot hr^{-1}$. MRM transitions and conditions for each compound and internal standard are listed in table 1.

For the hydrolysis, acetylation and extraction efficiency tests, samples (5 µL) were injected onto the same LC conditions described above using a Nexera UHPLC system (Shimadzu, Columbia, MD, USA). A TripleTOF 5600 (quadrupole–time-of-flight) mass spectrometer from Sciex (Concord, Canada) was employed in positive electrospray mode for high-resolution mass spectrometry (HRMS) analysis. Source conditions were as follows: ionspray voltage at 5000V, temperature of 450°C and source gases (GS1 and GS2) both at 50 psi with curtain gas at 30 psi. The general method procedure has been described in previous work published by our group [26].

2.7 Data analysis

Data were processed using Masslynx 4.1 (Waters, Milford, MA, USA), with automatic peak integration. Curve calibration used a quadratic equation with a weighting of 1/x. For hydrolysis, acetylation and extraction efficiency tests, Analyst TF 1.7.1 (Sciex, Framingham, MA, USA) were used for data acquisition and data were processed using PeakView 2.2 (Sciex, Framingham, MA, USA) and MultiQuant 3.0.2 (Sciex, Framingham, MA, USA).

2.8 Analytical performance

Analytical performance has been evaluated for each measured isocyanate biomarker. Analytical parameters included limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy and recovery. They all have been validated according to the criteria established by the accreditation organization of the American Industrial Hygiene Association (AIHA-LAP LLC). The estimation of LOD and LOQ measurements were based on a signal-to-noise (S/N) ratio of 3 and 10, respectively, using the measurement of 10 replicates of the lowest standard for each compound. The intra-day precision was calculated by analysing on the same day six different samples of four concentrations of 25, 50, 250 and 500 nM (n=24) for MDA and TDA isomers (9.9, 19.8, 49.5 and 99 $\mu g \cdot L^{-1}$ for MDA, and 3, 6.1, 30.5 and 61 $\mu g \cdot L^{-1}$ for TDA isomers) and 50, 100, 250 and 500 nM (n=24) for HDA (5.8, 11.6, 29 and 58 $\mu g \cdot L^{-1}$). The inter-day precision was measured on six different days by the analysing of four concentrations of 25, 50, 250 and 500 nM (n=24) for MDA and TDA isomers (9.9, 19.8, 49.5 and 99 $\mu g \cdot L^{-1}$ for MDA, and 3, 6.1, 30.5 and 61 $\mu g \cdot L^{-1}$ for TDA isomers) and 50, 100, 250 and 500 nM (n=24) for HDA (5.8, 11.6, 29 and 58 $\mu g \cdot L^{-1}$). Method accuracy was evaluated by analysing reference urine samples from the G-EQUAS program. Finally, recovery and matrix effect were evaluated by comparing six

replicates at four concentrations levels spiked in urine with replicates spiked in water. The concentrations were set at 25, 50, 250 and 500 nM (n=24) for MDA and TDA isomers (9.9, 19.8, 49.5 and 99 $\mu g \cdot L^{-1}$ for MDA, and 3, 6.1, 30.5 and 61 $\mu g \cdot L^{-1}$ for TDA isomers) and 50, 100, 250 and 500 nM (n=24) for HDA (5.8, 11.6, 29 and 58 $\mu g \cdot L^{-1}$).

3. RESULTS AND DISCUSSION

3.1 Samples preparation

3.1.1 Acetylation efficiency

A recent method from our lab showed the possibility to determine MDA levels in human urine without derivatization [26]. However, 2,4-TDA, 2,6-TDA and HDA are more polar molecules, making their analysis by reverse-phase LC-MS challenging, especially in a complex matrix as urine. With the objective to simultaneously measure MDA, TDA and HDA in the same sample, derivatization was necessary to have appropriate chromatographic retention and sensitivity for TDA isomers and HDA. Derivatization also yielded more specific MRM transitions, than would be possible with the underivatized compounds. The reaction with acetic anhydride was investigated since it was known to yield a simple and fast reaction with free amines [27], without the need to remove excess of reagent. Analytes were derivatized in a borate buffer pH 8.5 using acetic anhydride. The efficiency of derivatization was evaluated to ensure that all compounds were quantitatively doubly acetylated. High-resolution extracted ion chromatograms, in Figure 1, show that only di-acetylated analyte was observed, confirming the efficiency of the derivatization step. No underivatized or mono-derivatized analytes were detected.

3.3.2 Hydrolysis efficiency

In order to assess isocyanate exposure, biological monitoring is necessary to evaluate the amount absorbed into the body. The conjugates and acetylated metabolites formed *in vivo* after exposure are eliminated in urine. The free amines released after acid hydrolysis serve as biological biomarkers to determine the level of exposure of the parent isocyanate. Hydrolysis efficiency was evaluated using metabolites found in urine after exposure to MDI, HDI and TDI corresponding to: diacetyl-MDA, diacetyl-HDA, and diacetyl-TDA [16-18, 28, 29]. The hydrolysis time was varied from 1 to 24 h. After 4 h of hydrolysis at 80ºC, MDI and TDI metabolites were completely hydrolysed, while 16 h of hydrolysis at 80 ºC was necessary to completely hydrolysed HDI metabolites (Fig 2). With the objective to have a method to simultaneously measure MDI, TDI isomers and HDI exposure biomarkers, the hydrolysis step was set at 80°C for 16h in order to allow sufficient time to run the method within an 8h working day for a lab technician, however it is noted here that 4h would be sufficient for MDI and TDI isomers.

3.1.3 Extraction efficiency

To extract isocyanate biomarkers from hydrolysed urine samples, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) were compared for the four compounds. These preliminary results showed that SPE allowed better extraction efficiency than LLE with toluene for all analytes, especially for HDA with a 50% intensity increase in urine samples. Solid-phase extraction was therefore selected. Elution conditions were tested using a mixed-mode cation exchange polymer-based cartridge (Oasis MCX) with first a relatively low concentration of NH_4OH corresponding to 5% NH_4OH in 83% MeOH) . MDA and TDA were well eluted, but

only 32% were recovery for HDA (Table 2). Increasing the percentage of NH_4OH gave a better recovery for HDA with an increase of 33% but led to a decrease in the recovery for MDA. Indeed, MDA need a higher ratio of organic solution to be well eluted while HDA need a higher ration of basic solution. A double elution has then investigated to provide a compromise for each compound. Analytes were eluted with 500 µL of 5% NH_4OH: 83% MeOH) followed by 2 x 500 µL 15% NH_4OH: 50% MeOH. This double elution allows all analytes to be well recovered in the same extract. A good recovery for MDA, 2,6-TDA, 2,4-TDA and HDA of 96%, 88%, 83% and 73% were obtained, respectively (Table 2).

3.2 LC-MS/MS method development

MRM parameters were optimized for the doubly-acetylated MDA, TDA and HDA protonated molecules as precursor ions. The most intense product ion was chosen for quantitation, and a secondary ion was used for confirmation (Fig. 3). Chromatographic separation was optimized to ensure retention and minimize matrix effects for the four derivatized analytes. The HSS T3 column showed good chromatographic separation and all analytes were well separated in less than 5 minutes with a total run time of 8 min including column re-equilibration time (Fig. 4).

3.3 Analytical parameter validation

3.3.1 Dynamic range, LOD/LOQ and precision

According to the reference exposure values (BGV or BEI) for each compound, the dynamic range was set from 10 to 1000 nM for MDA and TDA isomers, and 25 to 1000 nM for HDA (Table 3). Validation parameters were evaluated by using reference samples with known concentrations of MDA, TDA isomers and HDA. Considering the fact that TDA isomers and

HDA have BEI values according to urinary creatinine concentration, a level corresponding to an average creatinine of 12.3 mM was used to evaluate all analytical parameters [25]. This value corresponds to an average found for women and men representative of a working population. Coefficients of determination (R^2) for all calibration curves were > 0.99 for each compound. The % accuracy and % CV values of each concentration level for each compound shown in Table 4 insured the good reliability of all result. The sensitivity was determined by evaluating the limits of detection (LOD) and quantification (LOQ), based on the method of the AIHA-LAP, LLC accreditation program (American Industrial Hygiene Association Laboratory Accreditation Programs). Good LOD and LOQ values of less than 5% and 20% BGV/BEI, respectively, were obtained for each of the measured analytes (Table 3). Finally, the robustness of the method was also evaluated by the measurement of intra-day and inter-day precisions (Table 3).

3.3.2 Recovery and Matrix effect

It's important to consider the complexity and variability of human urine, as well as all potential interferences during the analysis. Creatinine and density levels were measured to confirm that urine samples were in the acceptance range between 4.4-26.5 mM for creatinine and 1.010-1.030 $g \cdot mL^{-1}$ for density [20]. Solid-phase extraction followed by derivatization was employed to reduce potential variability resulting from the biological matrix. An isotopically labeled internal standard was also used for each analyte to minimize the variability in sample preparation and instrumental sensitivity. The matrix effect was evaluated by comparing peak areas in spiked urine and water samples with the same sample preparation (see method section). This method is based on the requirement of the AIHA-LAP, LLC accreditation program. A total recovery of

100,8 %, 103.9 %, 99.8 % and 96.6 %, average date of four concentrations levels (see method section) was obtained for MDA, 2,4-TDA, 2,6-TDA and HDA, respectively (Table 3).

3.3.3 Accuracy (G-EQUAS program)

The accuracy of the method was evaluated using an inter-laboratory reference program provided by the German External Quality Assessment Scheme. The success of this program makes it possible to certify the performances of the sample preparation method, the analysis and then the quantification of the samples. Three rounds of duplicate control urine samples provided from the G-EQUAS were evaluated. All measured values were in the tolerance range provided by the program (Table 5).

LC-MRM chromatograms of a representative control sample (62B) showed the good separation and accuracy obtained for real samples (fig 4). The success in this test confirms the reliability of our newly developed method. No current published method [10, 15, 21-23] has used this inter-laboratory test to evaluate their method. This step is necessary for OSH requirements, to ensure the method is adequate for OSH practitioners conducting workplace investigations.

4. CONCLUSIONS

A new method has been developed for the determination of simultaneous aliphatic and aromatic isocyanate exposure in human to evaluate occupationally-relevant exposure levels. This method is based on the quantification of urinary biomarkers of MDI, TDI (2,4 and 2,6) and HDI exposure, corresponding to MDA, (2,4- and 2,6-)TDA and HDA, respectively, and can evaluate all possible route of exposure. The developed method has been validated in order to monitor the biological guidance values established by the DFG for MDA and the biological exposure indices

by the ACGIH for TDA isomers and HDA. Sample preparation has been optimized to provide a method with good accuracy, robustness and reliability. This biological monitoring method is based upon recommended threshold values and can be combined with environmental (air) monitoring to ensure workplace safety.

Declaration of conflict of interest: The authors have no conflict of interest to declare.

5. REFERENCES

1. Cocker J. Biological monitoring for isocyanates. Occup Med (Lond). 2007;57(6):391-3. doi:10.1093/occmed/kql148.

2. European Commission. Methylenediphenyl diisocyanate (MDI): Institute for Health and Consumer Protection2005.

3. Brorson T, Skarping G, Sango C. Biological monitoring of isocyanates and related amines. IV. 2,4- and 2,6-toluenediamine in hydrolysed plasma and urine after test-chamber exposure of humans to 2,4- and 2,6-toluene diisocyanate. Int Arch Occup Environ Health. 1991;63(4):253-9. doi:10.1007/bf00386374.

4. Gaines LG, Fent KW, Flack SL, Thomasen JM, Ball LM, Richardson DB et al. Urine 1,6-hexamethylene diamine (HDA) levels among workers exposed to 1,6-hexamethylene diisocyanate (HDI). Ann Occup Hyg. 2010;54(6):678-91. doi:10.1093/annhyg/meq041.

5. Brorson T, Skarping G, Nielsen J. Biological monitoring of isocyanates and related amines. II. Test chamber exposure of humans to 1,6-hexamethylene diisocyanate (HDI). Int Arch Occup Environ Health. 1990;62(5):385-9. doi:10.1007/bf00381369.

6. Kreis K, Aumann-Suslin I, Ludeke A, Wegewitz U, Zeidler J, Graf von der Schulenburg JM. Costs of isocyanate-related occupational diseases: A systematic review. J Occup Environ Hyg. 2019;16(7):446-66. doi:10.1080/15459624.2019.1609005.

7. Sekizawa J, Greenberg MM. Diphenylmethane diisocyanate (MDI). Geneva: World Health Organization2000 Contract No.: 27.

8. Thorne PS, Hillebrand JA, Lewis GR, Karol MH. Contact sensitivity by diisocyanates: potencies and cross-reactivities. Toxicology and Applied Pharmacology. 1987;87(1):155-65. doi:10.1016/0041-008x(87)90093-7.

9. Rosenberg C, Nikkila K, Henriks-Eckerman ML, Peltonen K, Engstrorm K. Biological monitoring of aromatic diisocyanates in workers exposed to thermal degradation products of polyurethanes. J Environ Monit. 2002;4(5):711-6. doi:10.1039/b206340a.

10. Marand A, Karlsson D, Dalene M, Skarping G. Determination of amines as pentafluoropropionic acid anhydride derivatives in biological samples using liquid chromatography and tandem mass spectrometry. Analyst. 2004;129(6):522-8. doi:10.1039/b403439b.

11. Cocker J. Biological monitoring for isocyanates. Ann Occup Hyg. 2011;55(2):127-31. doi:10.1093/annhyg/meq083.

12. Jones K, Johnson PD, Baldwin PEJ, Coldwell M, Cooke J, Keen C et al. Exposure to Diisocyanates and Their Corresponding Diamines in Seven Different Workplaces. Ann Work Expo Health. 2017;61(3):383-93. doi:10.1093/annweh/wxx006.

13. Creely KS, Hughson GW, Cocker J, Jones K. Assessing isocyanate exposures in polyurethane industry sectors using biological and air monitoring methods. Ann Occup Hyg. 2006;50(6):609-21. doi:10.1093/annhyg/mel024.

14. Bello A, Xue Y, Gore R, Woskie S, Bello D. Assessment and control of exposures to polymeric methylene diphenyl diisocyanate (pMDI) in spray polyurethane foam applicators. Int J Hyg Environ Health. 2019;222(5):804-15. doi:10.1016/j.ijheh.2019.04.014.

15. Bhandari D, Ruhl J, Murphy A, McGahee E, Chambers D, Blount BC. Isotope Dilution UPLC-APCI-MS/MS Method for the Quantitative Measurement of Aromatic Diamines in Human Urine: Biomarkers of Diisocyanate Exposure. Anal Chem. 2016;88(21):10687-92. doi:10.1021/acs.analchem.6b03191.

16. Sabbioni G, Dongari N, Kumar A. Determination of a new biomarker in subjects exposed to 4,4'-methylenediphenyl diisocyanate. Biomarkers. 2010;15(6):508-15. doi:10.3109/1354750X.2010.490880.

17. Bolognesi C, Baur X, Marczynski B, Norppa H, Sepai O, Sabbioni G. Carcinogenic risk of toluene diisocyanate and 4,4'-methylenediphenyl diisocyanate: epidemiological and experimental evidence. Crit Rev Toxicol. 2001;31(6):737-72. doi:10.1080/20014091111974.

18. Gledhill A, Wake A, Hext P, Leibold E, Shiotsuka R. Absorption, distribution, metabolism and excretion of an inhalation dose of [14C] 4,4'-methylenediphenyl diisocyanate in the male rat. Xenobiotica. 2005;35(3):273-92. doi:10.1080/00498250500057591.

19. DFG. List of MAK and BAT values 2016: Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area2016.

20. ACGIH. Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices. Cincinnati, OH: 2017.

21. Bhandari D, Bowman BA, Patel AB, Chambers DM, De Jesus VR, Blount BC. UPLC-ESI-MS/MS method for the quantitative measurement of aliphatic diamines, trimethylamine N-oxide, and beta-methylamino-l-alanine in human urine. J Chromatogr B Analyt Technol Biomed Life Sci. 2018;1083:86-92. doi:10.1016/j.jchromb.2018.02.043.

22. Sakai T, Morita Y, Kim Y, Tao YX. LC-MS determination of urinary toluenediamine in workers exposed to toluene diisocyanate Toxicology letters. 2002;134(1-3):259-64.

23. Skarping G, Dalene M, Brunmark P. Liquid-Chromatography and Mass-Spectrometry Determination of Aromatic-Amines in Hydrolyzed Urine from Workers Exposed to Thermal-Degradation Products of Polyurethane. Chromatographia. 1994;39(9-10):619-23. doi:Doi 10.1007/Bf02268290.

24. Cocker JG, W. and Wilson, H. K. Assessment of occupational exposure to 4,4'-diaminophenylmethane (methylene dianiline) by gas chromatography-mas spectrometry analysis of urine. British Journal of Industrial Medecine. 1986;43(9):620-5.

25. Sauve JF, Levesque M, Huard M, Drolet D, Lavoue J, Tardif R et al. Creatinine and specific gravity normalization in biological monitoring of occupational exposures. J Occup Environ Hyg. 2015;12(2):123-9. doi:10.1080/15459624.2014.955179.

26. Lepine M, Sleno L, Lesage J, Gagne S. A validated liquid chromatography/tandem mass spectrometry method for 4,4'-methylenedianiline quantitation in human urine as a measure of 4,4'-methylene diphenyl diisocyanate exposure. Rapid Commun Mass Spectrom. 2019;33(6):600-6. doi:10.1002/rcm.8380.

27. Baker GB, Coutts RT, Holt A. Derivatization with acetic anhydride: applications to the analysis of biogenic amines and psychiatric drugs by gas chromatography and mass spectrometry. J Pharmacol Toxicol Methods. 1994;31(3):141-8. doi:10.1016/1056-8719(94)90076-0.

28. DFG. 4,4′ - Methylene diphenyl diisocyanate (MDI) [101 - 68 - 8] and “polymeric” MDI (PMDI) [9016 - 87 - 9] [MAK Value Documentation, 2008]. The MAK-Collection for Occupational Health and Safety. Wiley-VCH Verlag GmbH & Co. KGaA; 2015.

29. Sepai O, Henschler D, Sabbioni G. Albumin adducts, hemoglobin adducts and urinary metabolites in workers exposed to 4,4'-methylenediphenyl diisocyanate. Carcinogenesis. 1995;16(10):2583-7. doi:10.1093/carcin/16.10.2583.

Table 1 Structures of analytes and internal standards with MRM parameters

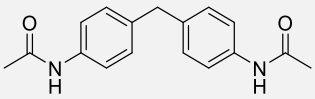
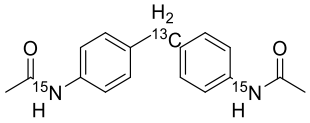
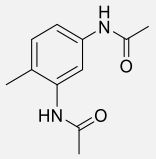
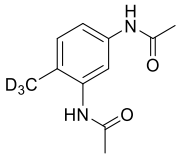
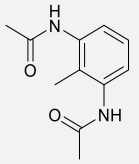
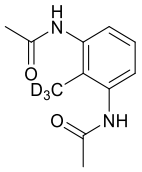
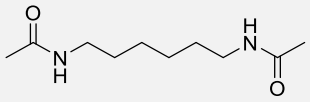
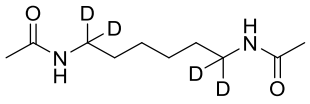
Analytes	Structure	Cone voltage (V)	Ions transitions, *m/z* (Collision energy, eV)
MDA-di-Ac		25	283.1→ 106.0 (25) 283.1→ 148.0 (15)
$^{13}C^{15}N_2$-MDA-di-Ac		25	286.3→ 108.0 (25) 286.3→ 150.0 (15)
2,4-TDA-di-Ac		25	207.1→ 123.0 (25) 207.1→ 165.0 (15)
2,4-d_3-TDA-di-Ac		25	210.1→ 126.0 (25) 210.1→ 168.0 (15)
2,6-TDA-di-Ac		25	207.1→ 123.0 (25) 207.1→ 165.0 (15)
2,6-d_3-TDA-di-Ac		25	210.1→ 126.0 (25) 210.1→ 168.0 (15)
HDA-di-Ac		20	201.1→100.0 (20) 201.1→142.0 (15)
d_4-HDA-di-Ac		20	205.1→ 104.0 (20) 205.1→ 146.1 (15)

Table 2 Recovery (%) of Solid-Phase extraction

Analytes	% Recovery (average ± SD)		
	5% NH_4OH, 83% MeOH (1.0 mL)	15% NH_4OH, 50% MeOH (1.0 mL)	5% NH_4OH, 83% MeOH + 15% NH_4OH, 50% MeOH (0.5 + 1.0 mL)
MDA	99.3 ± 4.6	65.7 ± 3.8	95.5 ± 0.6
2,6-TDA	82.0 ± 0.1	83.8 ± 4.0	87.7 ± 0.4
2,4-TDA	72.1 ± 0.1	75.2 ± 1.7	83.3 ± 2.4
HDA	32.2 ± 2.2	65.3 ± 3.8	73.4 ± 4.2

Table 3 Analytical parameters obtained by the method validation

Parameters	MDA	2,6-TDA	2,4-TDA	HDA
Biological guidance value (BGV) or Biological exposure indices (BEI)	10 $\mu g \cdot mL^{-1}$ (≈50 nM)	5 $\mu g \cdot g^{-1}$ cr (≈20–125 nM)	5 $\mu g \cdot g^{-1}$ cr (≈20–125 nM)	15 $\mu g \cdot g^{-1}$ cr (≈60–400 nM)
DYNAMIC RANGE (NM)	10 – 1000	10 – 1000	10 – 1000	25 – 1000
COEFFICIENT OF DETERMINATION (R^2)	>0.99	>0.99	>0.99	>0.99
LIMIT OF DETECTION (LOD) (NM) (N=10)	2.0 (4.0% BGV)	3.2 (5.6% BEI)	1.8 (3.2% BEI)	5.0 (2.8% BEI)
LIMIT OF QUANTITATION (LOQ) (NM) (N=10)	6.7 (13.4% BGV)	10.7 (18.8% BEI)	6.2 (10.9% BEI)	16.7 (9.3% BEI)
INTRA-DAY PRECISION (% CV) (N=24)	1.2	2.5	5.2	3.2
INTER-DAY PRECISION (% CV) (N=24)	2.2	5.0	7.5	7.6
% RECOVERY (AVERAGE ± CV)	100.8 ± 2.6	99.8 ± 4.6	103.9 ± 4.6	96.6 ± 7.1

***cr = creatinine**

Table 4 Accuracy (%) and CV (%) values from calibration curves of each compound

Standards (nM)	% accuracy (%CV) (n=3)			
	MDA	2,6-TDA	2,4-TDA	HDA
10	97.0 (0.3)	94.7 (0.3)	105.3 (0.2)	-
25	99.6 (1.0)	98.7 (2.4)	91.3 (1.5)	116.5 (1.2)
50	102.3 (1.0)	97.5 (2.8)	96.5 (1.3)	87.4 (1.9)
100	102.0 (2.5)	109.9 (6.3)	109.1 (8.9)	91.3 (6.1)
250	100.2 (5.1)	101.1 (10.9)	97.4 (8.8)	97.9 (0.6)
500	98.9 (4.1)	97.1 (6.0)	99.2 (6.4)	103.5 (0.7)
1000	100.2 (1.1)	100.6 (1.8)	100.2 (2.8)	99.4 (1.7)

Table 5. Quantitative results from samples provided by the G-EQUAS program

Samples	MDA		HDA	
	Reference values (tolerange range) (nM)	Measured values (nM)	Reference values (tolerange range) (nM)	Measured values (nM)
59A	31.2 (23.5 – 38.9)	27.8	36.9 (21.9 – 54.2)	34.9
59B	62.8 (49.2 – 76.4)	58.7	75.4 (58.1 – 92.7)	81.7
61A	14.3 (9.4 – 19.1)	11.2	20.3 (10.0 – 30.6)	25.2
61B	69.3 (54.5 – 84.2)	62.9	94.1 (71.2 – 117.1)	98.1
62A	26.7 (19.1 – 34.3)	25.2	37.2 (24.0 – 50.3)	33.0
62B	105.9 (79.6 –132.3)	107.0	105.0 (83.6 – 126.4)	119.7

Samples	2,6-TDA		2,4-TDA	
	Reference values (tolerange range) (nM)	Measured values (nM)	Reference values (tolerange range) (nM)	Measured values (nM)
59A	15.4 (10.2 – 20.5)	16.8	32.7 (23.4 – 42.1)	40.1
59B	40.8 (29.5 – 52.1)	40.8	72.1 (57.4 – 86.8)	71.4
61A	14.7 (9.2 – 20.1)	14.6	10.1 (6.4 – 20.1)	6.5
61B	72.4 (48.8 – 94.9)	82.0	53.8 (35.9 – 71.7)	45.7
62A	42.8 (30.8 – 58.0)	50.3	44.2 (30.4 – 58.0)	51.6
62B	78.1 (55.3 –100.9)	91.9	80.1 (59.5 – 100.8)	92.8

FIGURE CAPTIONS

Fig 1 High-resolution extracted ion chromatogram of derivatized TDA isomers, HDA and MDA (a), and mass spectra of derivatized 2,6-TDA (b), 2,4-TDA (c), HDA (d) and MDA (e) showing the double acetylation product (measured by high-resolution mass spectrometry)

Fig 2 Hydrolysis profiles of di-acetylated versions of 2,6-TDA (a), 2,4-TDA (b), HDA (c) and MDA (d) metabolites spiked into urine

Fig 3 MS/MS spectra of di-acetylated 2,6-TDA (a) 2,4-TDA (b), HDA (c) and MDA (d) showing the most intensive ion fragmentation choose for MRM transition

Fig 4 Representative LC-MRM chromatograms for sample 62B from the G-EQUAS inter-laboratory program