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Hexamethylene diisocyanate, 2,4-toluene diisocyanate, 2,6-toluene diisocyanate, isophorone diisocyanate and 4,4′-methylene diphenyl diisocyanate – Determination of hexamethylenediamine, 2,4-toluenediamine, 2,6-toluenediamine, isophoronediamine and 4,4′-methylenedianiline in urine using gas chromatography-mass spectrometry

Biomonitoring Method – Translation of the German version from 2017

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Biomonitoring Methods

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Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area verified the present biomonitoring method. The described method allows the simultaneous determination of the metabolites of hexamethylene diisocyanate (HDI), 2,4-toluene diisocyanate and 2,6-toluene diisocyanate (TDI), isophorone diisocyanate (IPDI) and methylene diphenyl diisocyanate (MDI) in human urine. After acid hydrolysis the released amines, hexamethylenediamine (HDA), 2,4-toluenediamine, and 2,6-toluenediamine (TDA), isophorone diamine (IPDA) and 4,4′-methylenedianiline (4,4′-MDA) are extracted from urine, derivatised using heptafluorobutyric anhydride and quantified by NCI-GC-MS. The method was extensively validated and the reliability data were confirmed by independent laboratories, which have established and cross-checked the whole procedure.

Keywords

hexamethylene diisocyanate; HDI; 2,4-toluene diisocyanate; 2,4-TDI; 2,6-toluene diisocyanate; 2,6-TDI; isophorone diisocyanate; IPDI; methylene diphenyl diisocyanate; MDI; hexamethylenediamine; HDA; 2,4-toluenediamine; 2,4-TDA; 2,6-toluenediamine; 2,6-TDA; isophorone diamine; IPDA; 4,4′-methylene dianiline; MDA; urine; biomonitoring; analyses in biological materials; gas chromatography

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Hexamethylene diisocyanate, 2,4-toluene diisocyanate, 2,6-toluene diisocyanate, isophorone diisocyanate and 4,4′-methylene diphenyl diisocyanate – Determination of hexamethylenediamine, 2,4-toluenediamine, 2,6-toluenediamine, isophoronediamine and 4,4′-methylenedianiline in urine using gas chromatography-mass spectrometry

Matrix: Urine

Hazardous substances: Hexamethylene diisocyanate, 2,4- and 2,6-toluene diisocyanate, isophorone diisocyanate, 4,4′-methylene diphenyl diisocyanate

Analyt. principle: Gas chromatography with mass spectrometry (GC/MS)

Completed in: November 2011

Overview of the parameters that can be determined with this method and the corresponding hazardous substances:

Hazardous substance	CAS	Parameter	CAS
hexamethylene diisocyanate (HDI)	822-06-0	hexamethylenediamine (HDA)	124-09-4
hexamethylenediamine (HDA)	124-09-4		
2,4-toluene diisocyanate (2,4-TDI)	584-84-9	2,4-toluenediamine (2,4-TDA)	95-80-7
2,4-toluenediamine (2,4-TDA)	95-80-7		
2,6-toluene diisocyanate (2,6-TDI)	91-08-7	2,6-toluenediamine (2,6-TDA)	823-40-5
2,6-toluenediamine (2,6-TDA)	823-40-5		
isophorone diisocyanate (IPDI)	4098-71-9	isophoronediamine (IPDA)	2855-13-2
isophoronediamine (IPDA)	2855-13-2		
4,4′-methylene diphenyl diisocyanate (MDI)	101-68-8	4,4′-methylenedianiline (MDA)	101-77-9
4,4′-methylenedianiline (MDA)	101-77-9		

Summary

The described method allows the simultaneous determination of the metabolites of hexamethylene diisocyanate (HDI), 2,4-toluene diisocyanate and 2,6-toluene diisocyanate (TDI), isophorone diisocyanate (IPDI) and 4,4′-methylene diphenyl diisocyanate (MDI) in human urine. After acid hydrolysis the released amines hexamethylene diamine (HDA), 2,4- and 2,6-toluenediamine (TDA), isophorone diamine (IPDA) and 4,4′-methylenedianiline (MDA) are extracted from urine and derivatised using heptafluorobutyric anhydride. The derivatives are separated via gas chromatography on a capillary column and detected by a mass sensitive detector using the selected ion monitoring mode with negative chemical ionisation. Calibration is carried out with urine samples, spiked with the analytes, and processed and analysed in the same way as unknown samples.

Reliability data of the method

Hexamethylenediamine (HDA)

Within-day precision:	Standard deviation (rel.)	s_w = 3.3%
	Prognostic range	u = 7.3%
	at a mean concentration of 3.8 µg HDA per liter urine and where n = 12 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 5.6%
	Prognostic range	u = 12.7%
	at a mean concentration of 3.9 µg HDA per liter urine and where n = 10 determinations	
Accuracy[1)]:	Recovery rate (rel.)	r = 103% or 105%
	at a spiked concentration of 1.0 µg or 10 µg HDA per liter urine and where n = 6 determinations	
Detection limit:	0.2 µg HDA per liter urine	
Quantitation limit:	0.7 µg HDA per liter urine	

2,4-Toluenediamine (2,4-TDA)

Within-day precision:	Standard deviation (rel.)	s_w = 2.6%
	Prognostic range	u = 5.7%
	at a mean concentration of 3.3 µg 2,4-TDA per liter urine and where n = 12 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 7.1%
	Prognostic range	u = 16.1%
	at a mean concentration of 3.4 µg 2,4-TDA per liter urine and where n = 10 determinations	
Accuracy[1)]:	Recovery rate (rel.)	r = 104% or 105%
	at a spiked concentration of 1.0 µg or 10 µg 2,4-TDA per liter urine and where n = 6 determinations	
Detection limit:	0.1 µg 2,4-TDA per liter urine	
Quantitation limit:	0.4 µg 2,4-TDA per liter urine	

2,6-Toluenediamine (2,6-TDA)

Within-day precision:	Standard deviation (rel.)	s_w = 4.0%
	Prognostic range	u = 8.8%
	at a mean concentration of 4.1 µg 2,6-TDA per liter urine and where n = 12 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 6.2%
	Prognostic range	u = 14.0%
	at a mean concentration of 4.0 µg 2,6-TDA per liter urine and where n = 10 determinations	
Accuracy[1)]:	Recovery rate (rel.)	r = 95% or 109%
	at a spiked concentration of 1.0 µg or 10 µg 2,6-TDA per liter urine and where n = 6 determinations	
Detection limit:	0.1 µg 2,6-TDA per liter urine	
Quantitation limit:	0.4 µg 2,6-TDA per liter urine	

cis-/trans-Isophoronediamine (cis-IPDA, trans-IPDA)

Within-day precision:	Standard deviation (rel.)	s_w = 16.8%
	Prognostic range	u = 40.0%
	at a mean concentration of 4.9 µg IPDA per liter urine and where n = 12 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 33.6%
	Prognostic range	u = 76.0%
	at a mean concentration of 3.8 µg IPDA per liter urine and where n = 10 determinations	
Accuracy[1)]:	Recovery rate (rel.)	r = 92% or 89%
	at a spiked concentration of 1 µg or 10 µg IPDA per liter urine and where n = 6 determinations	
Detection limit:	0.2 µg IPDA per liter urine	
Quantitation limit:	0.5 µg IPDA per liter urine	

4,4′-Methylenedianiline (MDA)

Within-day precision:	Standard deviation (rel.)	s_w = 8.1%
	Prognostic range	u = 17.8%
	at a mean concentration of 5.7 µg MDA per liter urine and where n = 12 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 13.5%
	Prognostic range	u = 30.5%
	at a mean concentration of 5.4 µg MDA per liter urine and where n = 10 determinations	

Accuracy[1)]:	Recovery rate (rel.)	r = 105% or 101%
	at a spiked concentration of 1 µg or 10 µg MDA per liter urine and where n = 6 determinations	
Detection limit:	0.1 µg MDA per liter urine	
Quantitation limit:	0.4 µg MDA per liter urine	

General information on isocyanates and their diamines

Diisocyanates are colourless to pale yellow liquids or solids with a low melting point, which are characterized by their reactive isocyanate groups. Diisocyanates containing NCO groups react with polyols to produce polyurethanes, and with polyamines (or water) to form polyurea. The dimerization of isocyanates to uretdiones and the trimerization to isocyanurates are also of crucial importance [Römpp 2017].

Diisocyanates are commonly used in a wide variety of industrial applications. The most commonly used diisocyanates are toluene diisocyanate (TDI), 4,4′-methylene diphenyl diisocyanate (MDI), hexamethylene diisocyanate (HDI) as well as isophorone diisocyanate (IPDI). The aromatic isocyanates 2,4-, 2,6-TDI and MDI are used as starting substances in the manufacture of polyurethanes, special plastics, coating and adhesive raw materials, cast elastomers, polyurethane foams, two-component adhesives as well as parquet adhesives. The aliphatic isocyanate HDI is used, among other things, as a hardener in two-component polyurethane coatings and parquet sealers, whereas the cycloaliphatic IPDI is also used as a hardener in two-component polyurethane coatings and powder coatings [Römpp 2017].

Apart from the diisocyanates, their respective diamines are also used for industrial applications, for example as starting substances in the production of polyamides (HDA), as intermediates in the production of dyes (2,4-TDA, 2,6-TDA and MDA) and as hardeners for epoxy resins or binders for printing inks (IPDA) [Römpp 2017].

The occupational routes of exposure to diiscocyanates can either be inhalation or dermal absorption. The risk of inhalation exposure depends on the compound's vapour pressure, the processing temperature and the type of task performed. Apart from the sensitizing effect, respiratory tract irritation is a major health risk associated with exposure to diisocyanates. The irritating properties may lead to concentration dependent symptoms on the eyes and the respiratory tract. However, the acute irritant effect of the individual isocyanates varies significantly. Also with regard to chronic toxicity, the focus is on the effect on the respiratory tract [Hartwig 2013 a]

Moreover, 2,4-TDI, 2,6-TDI and MDI are classified as carcinogens. Table 1 provides a summary of the toxicological classification by the Commission [DFG 2016]. For details on the assessment of the individual compounds, please refer to the toxicological occupational documentations of the Commission [Greim 2003; Greim 2008; Hartwig 2013 a; Hartwig 2013 b; Hartwig 2013 c].

In order to reduce inhalation exposure, diisocyanates are often used as polyisocyanates. These polymerized compounds based on diisocyanates (e.g. in paints and coatings) are composed of dimers, trimers or short-chain polymers with terminal

1) Accuracy was evaluated by the examiner of the method by usage of internal standards d_4-2,6-TDA, d_4-2,4-TDA, d_4-MDA and d_8-1,6-HDA, which where synthesized in the own laboratory.

Table 1 MAK values and assignment of various diisocyanates and diamines to risk categories by the Commission [DFG 2016].

Hazardous substance	MAK [ppm (mg/m³)]	Carcinogen category	H, S designation	Biological assessment value
Hexamethylene diisocyanate (HDI)	0.005 (0.035)	–	Sah	***BAT value*** 15 µg HDA per g creatinine (after hydrolysis)
Tolulene diisocyanate (2,4-TDI and 2,6-TDI)	–	3 A	Sah	–
2,4-Tolulene diamine (2,4-TDA)	–	2	H Sh	***EKA*** 2,4-TDA in urine (after hydrolysis)
Isophorone diisocyanate (IPDI)	0.005 (0.046)	–	Sah	–
Isophorone diamine (IPDA)	–	–	Sh	–
4,4′-Methylene diphenyl diisocyanate (MDI)/ polymeric MDI	– (0.05 E)	4	H Sah	***BLW*** 10 µg MDI per liter urine (after hydrolysis)
4,4′-Diaminodiphenyl-methane (MDA)	–	2	H Sh	***BAR*** < 0,5 µg MDA per liter urine (after hydrolysis)

isocyanate groups. They are considerably less volatile, but often contain residual amounts of monomeric diisocyanates, so that there is also a risk of diisocyanate exposure caused by these products.

The metabolism of isocyanates in humans is quite complex and poorly understood. A characteristic common to diisocyanates is the very reactive isocyanate function (Figure 1), which reacts within the human body with nucleophiles to form adducts with peptides, proteins and nucleic acids. As proof of an exposure to diisocyanates, the respective diamine is determined in urine. These diamines used as biomarkers are probably not metabolites, but released within the sampling material from various isocyanate adducts through hydrolysis. The recovery of biomarkers depends on the hydrolysis method applied, the time of sampling and the route of exposure. Basically, the biomarkers used to assess exposure are not isocyanate-specific; for example, no distinction can be made between an exposure to diamines and the respective monomeric or polymeric diisocyanates.

Analysis of the isocyanate-derived diamines after acid hydrolysis of urine from volunteers inhaling known concentrations of isocyanate showed that for 2,4- and 2,6-TDI, 15 to 20% and 17 to 23% of the dose were found as 2,4-TDA and 2,6-TDA in urine, respectively. [Brorson et al. 1991]. Skarping et al. [1991] found similar values of 8 to 14% of the inhaled dose of 2,4-TDI and 14 to 18% of 2,6-TDI. Tinnerberg et al. [1995] found 39% (range 9 to 94%) of an inhaled dose of HDI and 27% (range 19 to 46%) of a dose of IPDI to be excreted in urine as hydrolysable adducts.

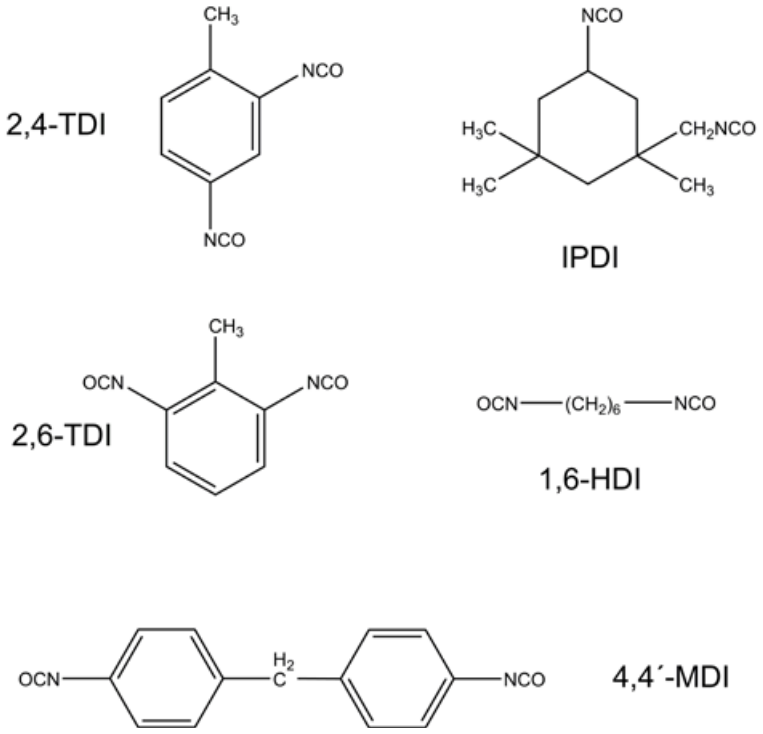

Figure 1 Structural formulae of the diisocyanates that can be assessed using this method.

The initial elimination half-life of TDI adduct fragments in urine is 1.9 hours for 2,4-TDI and 1.6 hours for 2,6-TDI, with both isomers having a second half-life of around 5 hours [Brorson et al 1991; Skarping et al 1991]. The half-life for the elimination of TDI-adducts in plasma is considerably longer, namely around 10 days and 21 days [Lind et al 1996, 1997]. After exposure to MDI, the elimination half-life of MDI adduct fragments in urine was up to 4 hours [Schütze et al. 1995; Sepai et al. 1995]. The mean elimination half-life of HDI adduct fragments in urine is 2.5 hours [Brorson et al. 1990; Hartwig 2013 b; Tinnerberg et al. 1995] and of IPDI adduct fragments in urine it is 2.8 hours [Tinnerberg et al. 1995].

Considering the short half-lifes, it is recommended that samples should be taken at the end of work/ shift for the biomonitoring of diiscocyanates.

Several volunteer and occupational studies show a good correlation between airborne concentrations of TDI and their hydrolysable adduct fragments in urine [Brorson et al. 1991; Kääriä et al. 2001 a; Maitre et al. 1996; Persson et al. 1993; Rosenberg and Savolainen 1986], HDI [Maitre et al. 1996; Tinnerberg et al. 1995] and IPDI [Tinnerberg et al. 1995].

Field studies have shown the utility of biological monitoring for detecting low-level exposure to isocyanates. Persson et al. [1993] were able to detect exposure to TDI, based on measurements of TDA in urine, for workers exposed to concentrations

between 0.4–4 μg/m³. Accordingly, MDA could be quantified in the urine of MDI exposed workers at air monitoring levels below the detection limit of 20–30 μg/m³ [Kääriä et al. 2001 b; Schütze et al. 1995].

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1 General principles

The described analytical method enables the simultaneous determination of the metabolites of hexamethylene diisocyanate (HDI), 2,4- and 2,6-toluene diisocyanate (TDI), isophorone diisocyanate (IPDI) and methylene diphenyl diisocyanate (MDI) in human urine. After acid hydrolysis the released amines hexamethylene diamine (HDA), 2,4- and 2,6-toluenediamine (TDA), isophorone diamine (IPDA), and methylenedianiline (MDA) are extracted from urine and derivatised using heptafluorobutyric anhydride. The derivatives are separated via gas chromatography on a capillary column and detected by a mass sensitive detector using the selected ion monitoring mode with negative chemical ionisation. Calibration is carried out with urine samples, spiked with the analytes, and processed and analysed in the same way as unknown samples.

2 Equipment, chemicals and solutions

2.1 Equipment

- Gas chromatograph 6890 Hewlett Packard coupled with a Hewlett Packard 5973 mass spectrometer and data processing system
- Gas chromatographic column: length: 30 m; inner diameter: 0.32 mm; film thickness 1 µm; stationary phase: 5% diphenyl 95% dimethyl polysiloxane (e.g. BP5, SGE)
- Analytical balance (e.g. Sartorius)
- Water bath with temperature control (e.g. Haake Messtechnik)
- Block thermostat (e.g. Grant Instruments Cambridge)
- Nitrogen evaporator (e.g. ReactiVap III, Pierce)
- Rotary tumbler (e.g. RM 5, CAT)
- Laboratory centrifuge (e.g. Heraeus)
- Vortex mixer (e.g. Scientific Industries)
- Various beakers (e.g. VWR)
- 10 mL and 100 mL volumetric flasks (e.g. Brand)
- 5 mL and 10 mL glass centrifuge tube with PTFE lined screw cap (e.g. Macherey-Nagel)
- 1.8 mL screw cap vials for GC with PTFE lined screw caps (e.g. VWR)
- 200 µL micro-inserts (e.g. VWR)
- Microlitre pipettes, adjustable between 10 and 100 µL or between 100 and 1000 µL with suitable pipette tips (e.g. Eppendorf)
- Polypropylene container for urine collection (e.g. Sarstedt)

2.2 Chemicals

Unless stated otherwise, the quality of all used chemicals must be at least p. a.

- Diethyl ether HPLC grade (e.g. EMD Millipore No. 1.00921)
- Toluene HPLC grade (e.g. Fisher Chemical No. T290SK-1)
- 4,4′-Methylenedianiline, 99% (e.g. Sigma-Aldrich No. 31640)
- 1,6-Hexamethylene diamine, 98% (e.g. Aldrich No. H11696)
- 1,6-$^{13}C_2$-Hexamethylene diamine (e.g. Aldrich No. 491160)
- 1,7-Heptamethylene diamine, 98% (e.g. Aldrich No. D17408)
- 2,4- and 2,6-Toluene diamine (e.g. Aldrich No. 442311 and 148113)
- d_3-2,4-Toluene diamine (e.g. CDN Isotopes No. D-5472)
- d_8-4,4′- Methylenedianiline (e.g. CDN Isotopes No. D-6240)
- Isophorone diamine (mixture of cis- and trans-, e.g. EMD Millipore No. 8.14123)
- Heptafluorobutyric anhydride, derivatisation grade, 99% (e.g. Aldrich, No. 394912)
- Hydrochloric acid 0,1 M (e.g. Aldrich)
- Sodium hydroxide solution 10 M (e.g. Fisher)
- Sulfuric acid, concentrated (e.g. EMD Millipore No. 07208-M)
- Citric acid (e.g. Sigma-Aldrich No. 33114)
- Urine (20 mL) from a person with no known exposure to diisocyanates

2.3 Solutions

Stock Solutions

- 1,6-Hexamethylene diamine (HDA) stock solution (116 mg/L, 1 mM)
  11.6 mg of HDA were weighed exactly and transferred quantitatively using 5 mL of 0.1 M HCl to a 100 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M HCl, mixed, then decanted into a glass bottle and stored frozen (at –20 °C).
- 2,4-Toluene diamine (2,4-TDA) stock solution (122 mg/L, 1 mM)
  12.2 mg of 2,4-TDA were weighed exactly and transferred quantitatively using 5 mL of 0.1 M HCl to a 100 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M HCl, mixed, then decanted into a glass bottle and stored frozen (at –20 °C).
- 2,6-Toluene diamine (2,6-TDA) stock solution (122 mg/L, 1 mM)
  12.2 mg of 2,6-TDA were weighed exactly and transferred quantitatively using 5 mL of 0.1 M HCl to a 100 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M HCl, mixed, then decanted into a glass bottle and stored frozen (at –20 °C).
- Isophorone diamine (IPDA) stock solution (170 mg/L, 1 mM)
  17 mg of IPDA were weighed exactly and transferred quantitatively using 5 mL of 0.1 M HCl to a 100 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M HCl, mixed, then decanted into a glass bottle and stored frozen (at –20 °C).
- 4,4′-Methylenedianiline (MDA) stock solution (198 mg/L, 1 mM)
  19.8 mg of MDA were weighed exactly and transferred quantitatively using 5 mL of 0.1 M HCl to a 100 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M HCl, mixed, then decanted into a glass bottle and stored frozen (at –20 °C).

The stock solutions stored at –20 °C are stable for at least two years without significant losses.

Working Solutions

- Diisocyanate biomarkers working solution (4 μM)
  A working solution of all five biomarkers was prepared by adding 400 μl of each stock solution into a 100 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M hydrochloric acid, mixed, then aliquoted (0.75 mL) into vials and stored frozen (at –20 °C).

The working solution currently used is stored at +4 °C in the refrigerator. At this temperature the solution is stable without significant losses.

2.4 Internal standards

Stock Solutions

- 1,7-Heptamethylene diamine (HpDA) stock solution (130 mg/L, 1 mM)
  13 mg of HpDA were weighed exactly and transferred quantitatively using 5 mL of 0.1 M HCl to a 100 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M HCl, mixed, then decanted into a glass bottle and stored frozen (at –20 °C).
- d_8-4,4′-Methylenedianiline (d_8-MDA) stock solution (206 mg/L, 1 mM)
  20.6 mg d_8-MDA were weighed exactly and transferred quantitatively using 5 mL of 0.1 M HCl to a 100 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M HCl, mixed, then decanted into a glass bottle and stored frozen (at –20 °C).
- 1,6-$^{13}C_2$-Hexamethylene diamine stock solution (^{13}C-HDA) (1000 mg/L)
  100 mg ^{13}C-HDA were weighed exactly and transferred quantitatively using 10 mL of 0.1 M HCl to a 100 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M HCl, mixed, then decanted into a glass bottle and stored frozen (at –20 °C).
- d_3-2,4-Toluene diamine stock solution (d_3-2,4-TDA) (1000 mg/L)
  10 mg d_3-2,4-Toluene diamine were weighed exactly and transferred quantitatively using 0.1 M HCl to a 10 mL Grade A volumetric flask. The solution was made up to the mark with 0.1 M HCl, mixed, then decanted into a glass bottle and stored frozen (at –20 °C).

Working solution

- d_3-2,4-Toluene diamine working solution (d_3-2,4-TDA) (1.5 mg/L)
  Pipette 150 µL of d_3-2,4-diaminotoluene stock solution into a 100 mL Grade A volumetric flask and made up to volume with 0.1 M HCl. The solution was mixed, then decanted into 20 mL screw cap vials and stored frozen at –20 °C. The working solution currently used is stored at +4 °C in the refrigerator. At this temperature the solution is stable without significant losses.
- Mixed Internal standard working solution (1.3 mg/L HpDA, 1.5 mg/L ^{13}C-HDA, 0.2 mg/L d_8-MDA, 0.075 mg/L d_3-2,4-TDA)
  A working solution of mixed internal standards is prepared as follows: 150 µL of ^{13}C-HDA, 1 mL of HpDA, 100 µL of d_8-MDA **stock** solutions and 5 mL of the d_3-2,4-diaminotoluene **working** solution are pipetted into a 100 mL Grade A volumetric flask and made up to volume with 0.1 M HCl. The working solution currently used is stored at +4 °C in the refrigerator. At this temperature the solution is stable without significant losses.

2.5 Calibration standards

Calibration standards are prepared before analysis in urine of persons with no known exposure to isocyanates or diamines. The aqueous working solution of the diisocyanate biomarkers according to Section 2.3 is mixed with urine according

Table 2 Pipetting scheme for the calibration standards.

Calibration solution	Spiked concentration [nM]	Volume of working solution [µL]	Volume of pooled urine [µL]
0	0	0	2000
1	50	25	1975
2	100	50	1950
3	150	75	1925
4	200	100	1900
5	250	125	1875
6	300	150	1850

to the pipetting scheme given in Table 2 to a final volume of 2 mL, resulting in calibration standards with concentrations between 0 and 300 nM. The calibration solutions are processed in the same way as the samples (see Section 3).

3 Specimen collection and sample preparation

3.1 Specimen collection

Urine samples should be collected at the end of a period of exposure to isocyanates or the respective diamines. 30 mL universal containers containing citric acid (0.5 g) are used for urine sampling. If transported at ambient temperature, samples should reach the laboratory within two days. Samples should be stored frozen at –20 °C prior to analysis.

3.2 Sample preparation

Before analysis, the urine samples are thawed at room temperature and then thoroughly mixed. For analysis, an aliquot of 2 mL of each sample is pipetted into a 10 mL tube. 100 µL of the internal standards working solution are added to each tube, followed by concentrated sulphuric acid (200 µL). The tubes are capped, thoroughly mixed and then placed into a water bath (>98 °C) for hydrolysis. After 90 min the tubes are removed and allowed to cool to room temperature. Sodium hydroxide solution (10 M, 2 mL) is added to each tube and the tube is mixed again. Afterwards, diethyl ether (4 mL) is added to each tube. Finally, the tubes are capped and mixed thoroughly on a rotary tumbler for 20 min followed by centrifugation (2200 × *g*) for 5 min. Approximately 3 mL of the supernatant is then transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen (<50 mL/min at 30–40 °C). The residue in each tube is dissolved in toluene (200 µL) and derivatised with heptafluorobutyric anhydride (20 µL) in capped tubes at 55 °C for 1 hour. After cooling, both, the toluene and the excess derivatising agent, are removed under a gentle stream of nitrogen with a flow rate <50mL/min at 30–40 °C

until the residue is dry. The residue is dissolved in toluene (100 µL), transferred to a micro-insert of a GC vial and measured by GC-MS.

4 Operational parameters

4.1 Gas chromatography

Capillary column:	Stationary phase:	BP5 (5% diphenyl 95% dimethyl polysiloxane)
	Length:	30 m
	Inner diameter:	0.32 mm
	Film thickness:	1 µm or equivalent
Temperatures:	Column:	Initial temperature 150 °C (2 min), then increase at a rate of 10 °C/min to 240 °C (0 min), then increase at a rate of 20 °C/min to 300 °C (5 min), then cool
	Injector:	350 °C
	Transfer line:	300 °C
Carrier gas:	Helium 5.0	
	Flow rate:	0.9 mL/min
Injection volume:	2 µL; splitless (30 sec)	

4.2 Mass spectrometry

Detection is performed in selected ion monitoring mode using negative chemical ionisation with methane reagent gas.

Ionisation mode:	negative chemical ionisation (NCI)
Ionisation energy:	70 eV
Source temperature:	150 °C
Dwell time:	100 ms
Solvent delay:	3 min
Detection mode:	Selected-Ion-Monitoring

All the other parameters have to be optimised according to the manufacturer's instructions.

5 Analytical determination

For the analytical determination of the urine samples, prepared according to Section 3.2, 2 µL of each sample are injected into the GC/MS system (splitless). The identification of the analytes is based on the retention times and on characteristic

Table 3 Characteristc ion traces and retention times of the analytes and their internal standards.

Analyte	Ions (m/z) quantifier, qualifier	Retention time (min)
2,6-TDA	495	10.2
HDA	488, 448, 449	10.4
^{13}C-HDA	490	10.4
d_3-2,4-TDA	498	10.6
2,4-TDA	495	10.7
cis-IPDA	542	11.4
trans-IPDA	542	11.8
HpDA	462	12.0
MDA	571	16.7
d_8-MDA	574	16.7

ion traces. The retention times of the individual analytes as well as the characteristic ion traces for the analytes, resulting from the stated operational parameters (see Section 4), are illustrated in Table 3.

The given retention times are intended as a rough guide only. Users of the method must ensure proper separation performance of the used GC column that may influence the resulting retention behaviour of the analyte. A chromatogram of a urine sample, spiked with the isocyanate metabolites is given in Figures 2 and 3 in the Appendix.

6 Calibration

The calibration standards in urine are processed in the same way as the urine samples to be analysed (see Section 3.2) and measured according to Sections 4 and 5.

A calibration curve is calculated for each analyte based on the ratio of the peak area of the analyte to the peak area of the respective internal standard for each of the known concentrations. HpDA is used as an internal standard for IPDA. ^{13}C-HDA is used for HDA, d_3-2,4-TDA is used for 2,4-TDA and 2,6-TDA and d_8-MDA is used as an internal standard for MDA. The linearity of the calibration curve was confirmed in the range between the detection limit and analyte levels of 300 nM for all of the analytes .The calibration is acceptable if there are no significant intercepts, and the correlation coefficient is ≥0.99.

7 Calculation of the analytical results

The concentration for each analyte in each sample is determined by dividing the peak-area ratio for the respective analyte by the slope of the appropriate calibration curve. Any value found above 300 nM should be diluted and re-analysed.

8 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the Bundesärztekammer (German Medical Association) and in a general chapter of the MAK-Collection for Occupational Health and Safety Part IV: Biomonitoring Methods [Bader et al. 2010; Bundesärztekammer 2008].

To check precision, urine spiked with a known and constant analyte level is analysed as a control within each analytical run. As material for quality control is not commercially available, it must be prepared in the laboratory.

Urine from a person with no known exposure to diisocyanates or diamines should be used to prepare the QC material. The urine is acidified with 5% 6 M HCl and then spiked at a nominal 50 nmol/L with each diamine. Vials, containing 5 mL aliquots of QC urine are stored frozen at –20 °C. Quality control samples (2 mL) should be run at the end of the calibration curve and after every 5th pair of samples. The acceptable range for the QC samples is defined as the mean +/– two standard deviations and is determined in a pre-analytical period [Bader et al. 2010].

9 Evaluation of the method

The reliability of the method was proved by comprehensive validation and by implementation and validation of the procedure in a second independent laboratory.

9.1 Precision

For determining the within-day precision, urine samples were spiked with the analytes, prepared and analysed. Based on the 12-fold determination of these urine samples the within-day precision was calculated (see Table 4).

For the deterimination of the precision from day-to-day spiked urine samples were analysed. Based on the 10-fold determination of these urine samples the within-day precision was calculated (see Table 5).

Table 4 Within-day precision for the determination of biomarkers for diisocyanates in urine (n = 12).

Analyte	Mean concentration [µg/L]	Standard deviation (rel.) [%]	Prognostic range [%]
HDA	3.8	3.3	7.3
2,4-TDA	3.3	2.6	5.7
2,6-TDA	4.1	4.0	8.8
cis-/trans-IPDA	4.9	16.8	40.0
MDA	5.7	8.1	17.8

Table 5 Day-to-day precision for the determination of biomarkers for diisocyanates in urine (n = 10).

Analyte	Mean concentration [µg/L]	Standard deviation (rel.) [%]	Prognostic range [%]
HDA	3.9	5.6	12.7
2,4-TDA	3.4	7.1	16.1
2,6-TDA	4.0	6.2	14.0
cis-/trans-IPDA	3.8	33.6	76.0
MDA	5.4	13.5	30.5

9.2 Accuracy

Recovery tests were carried out in order to determine the accuracy of the method. Therefore, urine was spiked with the analytes in levels of 1 µg/L or 10 µg/L, respectively, prepared and analysed by the examiner of the method. The relative recovery rates were found to range between 90–105% or 88–109% (Table 6).

Furthermore, accuracy was assessed by the developer of the method by participating in an external round robin of the FIOH.

Table 6 Accuracy data of the determination of biomarkers for diisocyanates in urine (n = 6).

Analyte	Spiked concentration [µg/l]	Recovery rate (rel.) [%]	Standard deviation (rel.) [%]
HDA	1	103	6.0
	10	105	11.8
2,4-TDA	1	104	2.3
	10	105	2.2
2,6-TDA	1	95	4.8
	10	109	1.5
cis-/tans-IPDA	1	92	16.8
	10	89	17.5
MDA	1	105	8.2
	10	101	3.4

9.3 Detection limits and quantitation limits

The detection limit was estimated using the 3-fold signal to noise-ratio. The quantitation limit was determined analogously (9-fold signal to noise-ratio). The detection limits and the quantitation limits of the analytes are presented in Table 7.

Table 7 Detection limits and quantitation limits of the investigated biomarkers for diisocyanates.

Analyte	Limit of detection [µg/L]	Limit of quantitation [µg/L]
HDA	0.2	0.7
2,4-TDA	0.1	0.4
2,6-TDA	0.1	0.4
cis-/tans-IPDA	0.2	0.5
MDA	0.1	0.4

9.4 Sources of error

HDA and HpDA are relatively volatile and losses of these analytes can take place during sample work up due to solvent evaporation. To avoid loss of analytes low temperature (<40 °C), low nitrogen flow (<50 mL/min) and abortion of the evaporation step, when dryness is reached, are important. Other methods of evaporating solvents will need investigating and may require the use of a 'keeper' solvent.

Moreover, HDA and HpDA show low extraction recoveries. This can significantly affect the precision and detection limits.

The usage of internal standards is important for excellent method precision and low detection limits. While the internal standards HpDA, ^{13}C-HDA, d_3-2,4-TDA and d_8-MDA were used for the method validation by the developer of the method, one examiner of the method used the own synthesized internal standards d_8-HDA, d_4-2,4-TDA, d_4-2,6-TDA and d_4-MDA for the examination of the method and the evaluation of accuracy data.

10 Discussion of the method

The described analytical method was developed to quantify and assess exposure to the common diisocyanates by hydrolysing their conjugates in urine and detecting the respective diamines. No free isocyanate-derived diamines are found in urine before hydrolysis. Thus, the hydrolysis stage is critical. Early stages of the method development attempted to use alkaline hydrolysis but problems were encountered with high 'background' levels in the HDA analysis. It was not clear if the hydrolysis was producing HDA from endogenous substances or whether the interference was simply a co-eluting peak. Acid hydrolysis reduced this interference and has been used since.

The chromatographic conditions described are optimised to reduce the run cycle time. A 0.2 or 0.25 mm column with a thinner film thickness will give better chromatography but will require a lower injection temperature and give a longer run time.

In many isocyanate-containing products, particularly HDI, the isocyanates are present as oligomers or polymers with little free isocyanate monomer. According to unpublished observations in initial studies of occupational exposure to isocyanate paints, containing HDI-oligomers (but little free HDI), the levels of HDA found in

urine seemed to be higher than might be expected from exposure to just the HDI monomer. This may suggest that the hydrolysis is releasing HDA from some of the oligomeric HDI-conjugates. If this is the case, changing the hydrolysis conditions may produce different results.

Occasionally (~ 1/100) HDA is detected when there is no obvious occupational source. The combinations of the correct ions in the correct ratio with the correct retention time suggest that this reflects cryptic exposure to HDI or HDA. As some reagents can contain detectable levels of HDA leading to blank values, the quality of the analytical reagents, in particular the heptafluorobutyric anhydride, is important.

The described method has been used extensively in studies of occupational exposure to diisocyanates in the UK. In some cases isocyanate-derived diamines, other than those expected, were found and reported. The subsequent investigations usually indicated previously unknown isocyanate exposure rather than analytical interference. The analytical method is intended to assess exposure to diisocyanates but by measuring diamines in urine it will also detect any exposure to the diamines. Co-exposure to both isocyanates and diamines should be considered when interpreting results.

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12 Appendix

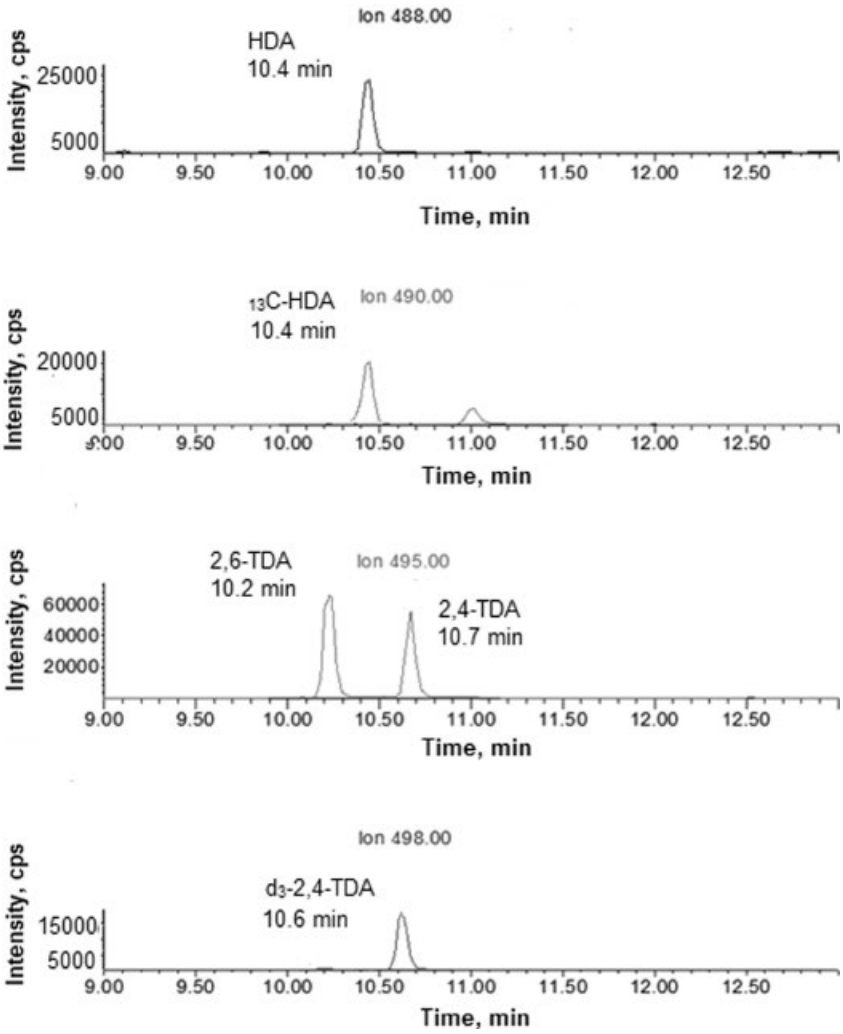

Figure 2 Chromatograms of urine spiked with 120 nmol/L HDA and 2,4- and 2,6-TDA.

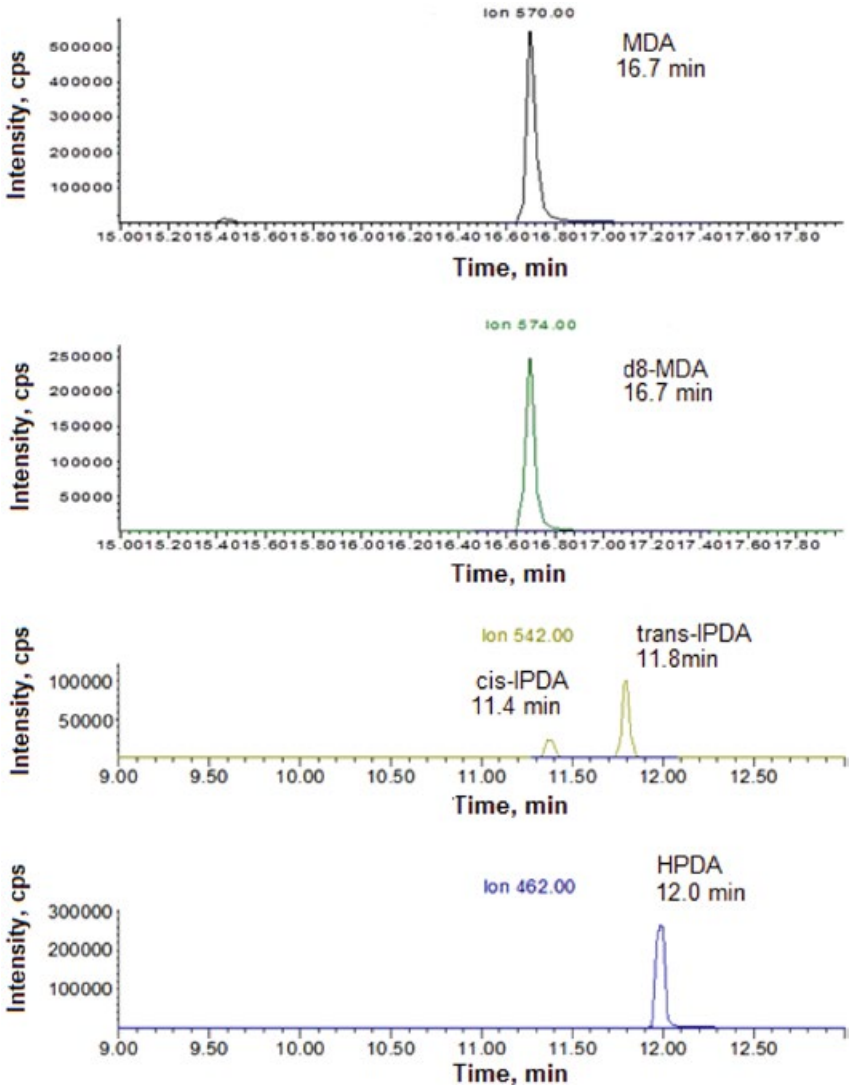

Figure 3 Chromatograms of urine spiked with 120 nmol/L MDA and IPDA.