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Determination of 4,4′-methylenediphenyldianiline (MDA) and identification of isomers in technical-grade MDA in hydrolysed plasma and urine from workers exposed to methylene diphenyldiisocyanate by gas chromatography–mass spectrometry

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Abstract

Gas chromatography–mass spectrometry using chemical ionization with ammonia as reagent gas monitoring both positive and negative ions was applied. Negative-ion monitoring using ammonia and the pentafluoropropionic anhydride (PFPA) derivatives were chosen owing to low detection limits and good separation for the isomers studied. Technical-grade methylenediphenyldiisocyanate (MDI) was analysed and three isomers, 4,4′-, 2,4′- and 2,2′-methylenediphenyldianiline (MDA), were determined in addition to methylated MDA. Plasma and urine from an exposed worker were hydrolysed and analysed and the MDA isomers were identified in the biological samples.

1. Introduction

Isocyanates are mainly used for the production of polyurethane (PUR). Their environmental and occupational hazards makes this group of chemicals very interesting as many workers are potentially exposed to them [1–4]. One of the most frequent used isocyanates is methylene diphenyldiisocyanate (MDI). The main component of commercially used MDI is 4,4′-methylene diphenyldiisocyanate but several other isomers are also present. Owing to the low threshold limit value (TLV), the normal exposure to isocyanates is low. However, in infrequent incidents in industry high exposure can be expected. The exposure during these occasions is difficult to monitor as they occur only by accident.

When PUR is thermally degraded, numerous different isocyanate, aminoisocyanate and amine compounds are formed, some of which may be toxic [5]. These compounds are of great toxicological interest as workers are exposed. Thermally degraded PUR can give high concentrations of other isocyanate compounds, other than the monomer used in the production of the polymer. Amino derivatives of toluene diisocyanate (TDI), e.g., 2-6-aminotoluene-2-isocyanate, have been demonstrated in thermal

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degradation products of TDI–PUR [6]. Air measurements of isocyanates, based on sampling using impingers and derivatization reagents followed by chromatographic determination, will only determine the monomer (that used for PUR manufacture) concentration. Other forms of isocyanates will show up in the chromatogram as spurious peaks and baseline drift.

It is important from the occupational safety point of view to measure these compounds. However, for analytical purposes reference compounds are necessary, and these are not available for most of the compounds formed during the thermal degradation of PUR. Determining only 4,4′-MDI in air therefore gives limited information. Relevant biological markers, indicating the exposure, would therefore be very useful. Biological markers have further advantages as it may be possible to observe the levels of compounds many days after the exposure based on the plasma protein adducts formed with MDI [7].

Methods have been developed for the biological monitoring of exposure to HDI (1,6-hexamethylene diisocyanate) [8], 2,6- and 2,4-TDI [9] and MDI by determination of the corresponding amine in hydrolysed biological fluids such as blood and urine [10–15]. We have previously studied a worker exposed to MDI–PUR [16,17]. The half-life of MDA-hydrolysed urine was 70–80 h and in serum 21 days. Recently Brunmark et al. [12] presented a GC–MS method with negative-ion chemical ionization (NCI) for the determination of low concentrations of MDA in hydrolysed urine and plasma [12]. The coefficient of variation for urine and plasma between assays was 6% and the instrumental detection limit was less than 10 ng/l. In this work, the presence of several MDA isomers in hydrolysed plasma and urine from MDI-exposed workers were investigated.

2. Experimental

2.1. Apparatus

A VG-Quattro triple quadruple mass spectrometer (Fisons Instruments, VG-Biotech, Altrincham, Cheshire, UK) connected to a Carlo Erba GC-8000 gas chromatograph equipped with a splitless injector (Fisons Instruments, Milan, Italy) was employed. The column oven temperature was held isothermal at 100°C for 1 min, then raised at 10°C/min to 300°C, where it was kept for 2 min. The temperature of the ion source was 250°C and the GC–MS interface temperature was 300°C. The capillary inlet pressure of helium was 0.8 kg/m^2. The solvent delay was set to 3 min. The instrument was used in the chemical ionization mode using ammonia as the reagent gas and monitoring positive (PCI) and negative ions (NCI). Quantitative measurements were made monitoring the m/z 470 and 472 negative ions corresponding to the (M − 20) ions of the 4,4′-MDA and the dideuterated 4,4′-MDA (MDDA) pentafluoropropionic amide (PFPA) derivatives, respectively. The dwell time for each of the ions was 0.1 s and the inter-scan delay was 0.02 s. The pressure in the ion source, in the case of NCI with ammonia, was kept at ca. $2 \cdot 10^{-4}$ mbar (source readout pressure, not the actual pressure in the ionization cavity). The emission current was 100 mV and the electron energy 70 eV. Mass spectra were obtained by scanning ions between 50 and 600 u for 0.7 s with an inter-scan delay of 0.1 s.

A Sigma 3E-1 centrifuge (Sigma, Hartz, Germany) was used for phase separation in the work-up procedure. The samples were evaporated in a Speed-Vac 290 centrifuge (Savant, Farmingdale, NY, USA).

2.2. Columns

Fused-silica capillary columns with a DB-5 chemically bonded stationary phases (J & W Scientific, Folsom, CA, USA) (25 m × 0.25 mm I.D.) with a film thickness of 0.25 μm were used.

2.3. Chemicals

We obtained 4,4′-MDA from Fluka (Buchs, Switzerland), pentafluoropropionic anhydride (PFPA) from Pierce (Rockford, IL, USA), toluene from Janssen (Beerse, Belgium), acetonitrile from Lab-Scan (Dublin, Ireland), dideuterated 4,4′-MDA [$C^2H_2(C_6H_4NH)_2$]

(MDDA) and 4,4'-MDA–PFPA derivative from Synthelec (Lund, Sweden) and technical-grade MDA from ICI (Brussels, Belgium).

2.4. Procedure

Preparation of standard solutions

Standard solutions of the investigated samples were prepared by dissolving ca. 25 mg of the amines in 5 ml of acetonitrile. A 1-ml volume of PFPA was then added and the excess of reagent and the liberated acid were removed in a vacuum centrifuge. The dry residue was diluted in 100 ml of acetonitrile. The solutions were further diluted with toluene to the appropriate concentrations.

Sampling, handling and storage of biological samples

Urine samples were collected in polyethylene bottles. A 10-ml volume of venous blood was taken on each occasion. The urine and serum samples were stored in a freezer at −18°C until analysis.

Work-up procedure for biological samples

To a 1-ml urine or serum sample, 1.5 ml of 3 *M* H_2SO_4 and 100 μl of 1 *M* HCl containing 5.9 μg/l MDDA as internal standard were added. The sample was then hydrolysed at 100°C overnight. A 5-ml volume of saturated NaOH and 2 ml of toluene were added and the mixture was shaken for ca. 10 min and centrifuged at 1500 *g* for 15 min. A 1.5-ml volume of the organic layer was transferred to a test-tube, 20 μl of PFPA were added and the mixture was immediately shaken vigorously for ca. 10 min. The excess of the reagent and the acid formed were removed by extraction with 2 ml of 1 *M* phosphate buffer solution (pH 7.5). A 1-ml volume of the toluene layer containing the amide derivative and the internal standard were transferred into a 1.5-ml vial and was then ready to be injected into the GC–MS system.

Work-up procedure for technical MDA samples

To a test-tube containing 10 ml of acetonitrile, 25 mg MDA were added and 1.0 ml of PFPA was added. The mixture was immediately shaken vigorously for ca. 10 min, then the solvent and the excess of the reagent and the acid formed were removed under vacuum. The dry residue was dissolved in 100 ml of acetonitrile. The sample was then diluted 1:10 000 with toluene. A 1-ml volume of the toluene solution containing the amide derivatives was transferred into a 1.5 ml vial and then injected into the GC–MS system.

3. Results

3.1. Storage and treatment of samples

Urine samples spiked with 4,4'-MDA were found to be stable after acidification. No noticeable degradation of the samples was found when stored for several weeks in the dark at room temperature. Plasma samples were kept frozen until analysis. A study of the nature of the MDA-forming compound is currently in progress, and indicates the MDI is covalently bonded in plasma and urine. These bondings are expected to break up under acidic hydrolysis.

3.2. Chromatography

Technical-grade MDA was derivatized using PFPA. All the amide derivatives formed showed excellent chromatographic behaviour, as can be seen in the GC–PCI-MS (Fig. 1) and GC–NCI-MS (Fig. 2) traces. For GC–PCI-MS the major components found were three MDA isomer peaks (I–III, Fig. 1B) monitored at *m/z* 508 (*m/z* = M + 18) and five peaks (Fig. 1A, a–e) of compounds with a molecular mass 14 u higher (*m/z* 522) than MDA. In the PCI traces the *m/z* 522 ions represents less than 5% of the total *m/z* 508 area. With knowledge of the MDI manufacturing process, one can assume that these peaks represents methylated forms of MDA. The three MDA peaks and the five methyl-MDA peaks are also seen in Fig. 2A and B. Then sensitivity is at least 50–100 times better but the relative sensitivity between isomers varies. Using the DB-5 capillary column, baseline separation of the MDA amides were obtained. In Fig. 3 the

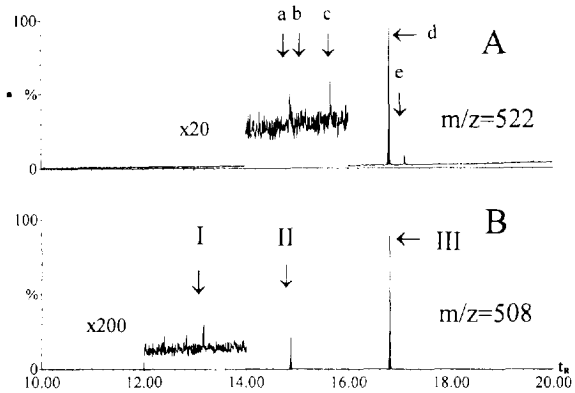


Fig. 1. GC–mass fragmentogram of technical-grade MDA–PFPA derivatives in toluene using PCI with ammonia (A) m/z 522 ions. Peaks a–d are most likely isomers of methyl-MDA. The chromatogram is magnified 20 times between 14 and 16 min. (B) m/z 508 ions. The chromatogram demonstrates peaks of different MDA isomers. Peak III is the 4,4'-MDA isomer.

structures of the investigated MDA isomers and methyl-MDA are shown.

Chromatograms for pooled urine and plasma samples obtained from ten MDI-exposed workers are shown in Fig. 4. Analysis of blank plasma and urine samples did not show any interfering peaks.

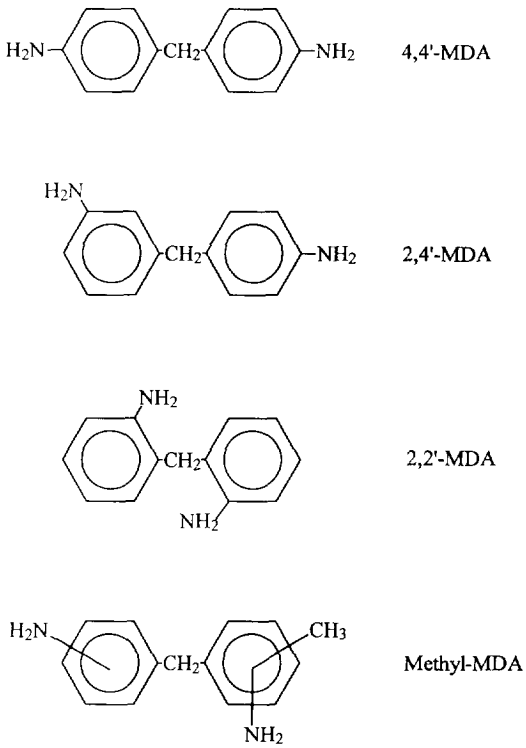


Fig. 3. Structures of MDA isomers and methylated MDA.

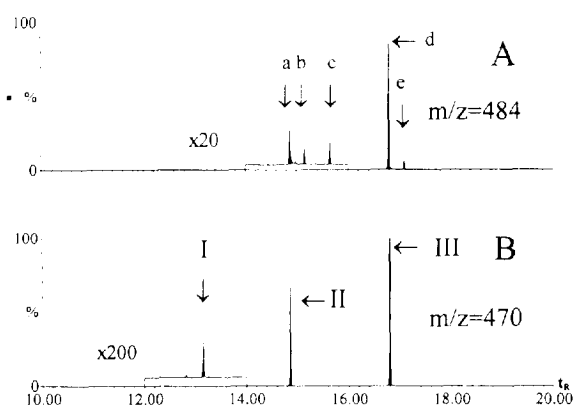


Fig. 2. GC–mass fragmentogram of technical-grade MDA as PFPA derivatives in toluene using NCI with ammonia (A) m/z 484 ions. Peaks a–d are most likely isomers of methyl-MDA. The chromatogram is magnified 20 times between 14 and 16 min. (B) m/z = 470 ions. Peak identities as in Fig. 1.

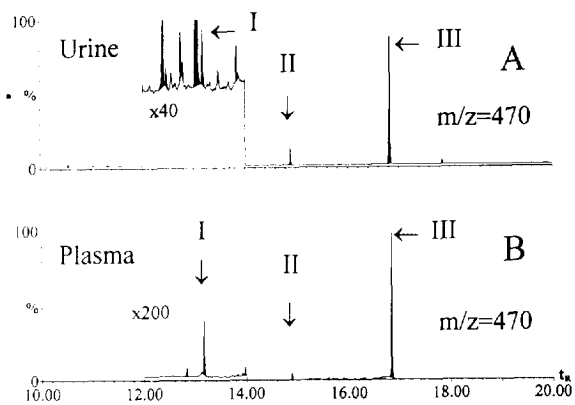


Fig. 4. GC with selected-ion monitoring of PFPA derivatives of MDA isomers in toluene using NCI with ammonia, monitoring m/z 470 ions. (A) Pooled hydrolysed urine samples from ten workers exposed to MDI. (B) Pooled plasma samples from the same workers. Peak identities as in Fig. 1.

3.3. Mass spectrometry

Technical-grade MDA contains many compounds and sensitive and selective detection methods are needed. Capillary GC with MS detection makes these determinations possible. When investigating low concentrations it was not possible to obtain chromatograms with all peaks completely resolved. For the biological samples GC–NCI-MS determinations were made monitoring the $m/z = (M - 20)^-$ fragments from the MDA–PFPA and the [$^2H_2$]MDA–PFPA derivatives. Urine and serum from the MDI-exposed patients were analysed. The chromatograms in Fig. 4 obtained for both pooled urine and pooled plasma samples demonstrates good separation of the MDA–PFPA derivative and the internal standard from the matrix.

Electron impact (EI) and PCI and NCI with ammonia as reagent gas were studied for technical-grade MDA containing several isomers of MDA and other aromatic amine compounds.

EI

The relative abundances of the molecular ions of the MDI-related MDA–PFPA derivatives are very low. Initial attempts to investigate the biological samples using EI were unsuccessful owing to the relatively low concentration of MDA and the complicated matrix.

PCI

As can be seen in Fig. 5, positive ions formed with ammonia give simple and easy to interpret spectra and very little fragmentation is seen. Spectrum A (4,4′-MDA–PFPA) shows the m/z $508 = (M + 18)^+$ ion as the most abundant ion with a relative abundance of the molecular ion about 1.5%. Spectra B and C most likely represent the 2,4′-MDA and 2,2′-MDA isomers and the spectra are very similar to that obtained for the 4,4′-MDA isomer. The most abundant ions in spectrum D are the m/z 522 and the 472 ions. In the chromatogram from technical-grade MDA many compounds at low concentrations are seen and not all peaks are completely resolved. In spectrum D the peak with the highest abundance is the m/z 522 ion and the fragment at m/z 472 probably represents an unresolved peak. The m/z 522 ion indicates the presence of a methylated analogue of MDA.

NCI

The spectra obtained using NCI with ammonia are shown in Fig. 5E–H. Spectrum E (4,4′-MDA–PFPA) shows m/z 450 and 470 ions as the most abundant ions. We have previously described a fragmentation pattern for toluenediamines (TDA) derivatized with PFPA, which showed the typical fragments of $m/z = (M - n \times 20; n = 1\text{–}4)^-$ due to the neutral loss of HF [9]. The negative fragments formed varied with the isomer and perfluoro fatty acid derivative studied and a more complicated fragmentation pattern is seen compared with positive ion monitoring. As expected, the MDA–PFPA also shows the same fragmentation pattern. The relative abundance of the molecular ion was less than 0.6% and the most abundant fragment was the $m/z = (M - 20)^-$ and the $m/z = (M - 40)^-$ fragments. Spectra F and G (the same peaks as in spectra B and C monitoring positive ions) probably represents the 2,4′-MDA and 2,2′-MDA isomers and the spectra are similar to that obtained for the 4,4′-MDA isomer. The most abundant ions in spectrum H are the m/z 484 and 434 ions. The m/z 484 ion indicates a methylated analogue of MDA and the m/z 434 ion the same unresolved compound as described for the positive-ion spectrum D. When analysing other peaks in the chromatogram at concentrations below 1% several other compounds were present. Aromatic monoamines such as aniline, methylaniline and dimethylaniline were observed. Aromatic diamines such as dimethyl-MDA and trimethyl-MDA were also seen.

3.4. Quantitatification

Calibration graph

Human urine samples were spiked with 4,4′-MDA at five different concentrations in the range 0.0–10.0 μg/l. The work-up procedure was then performed and the $m/z = (M - 20)^-$ fragments of MDA and the internal standard

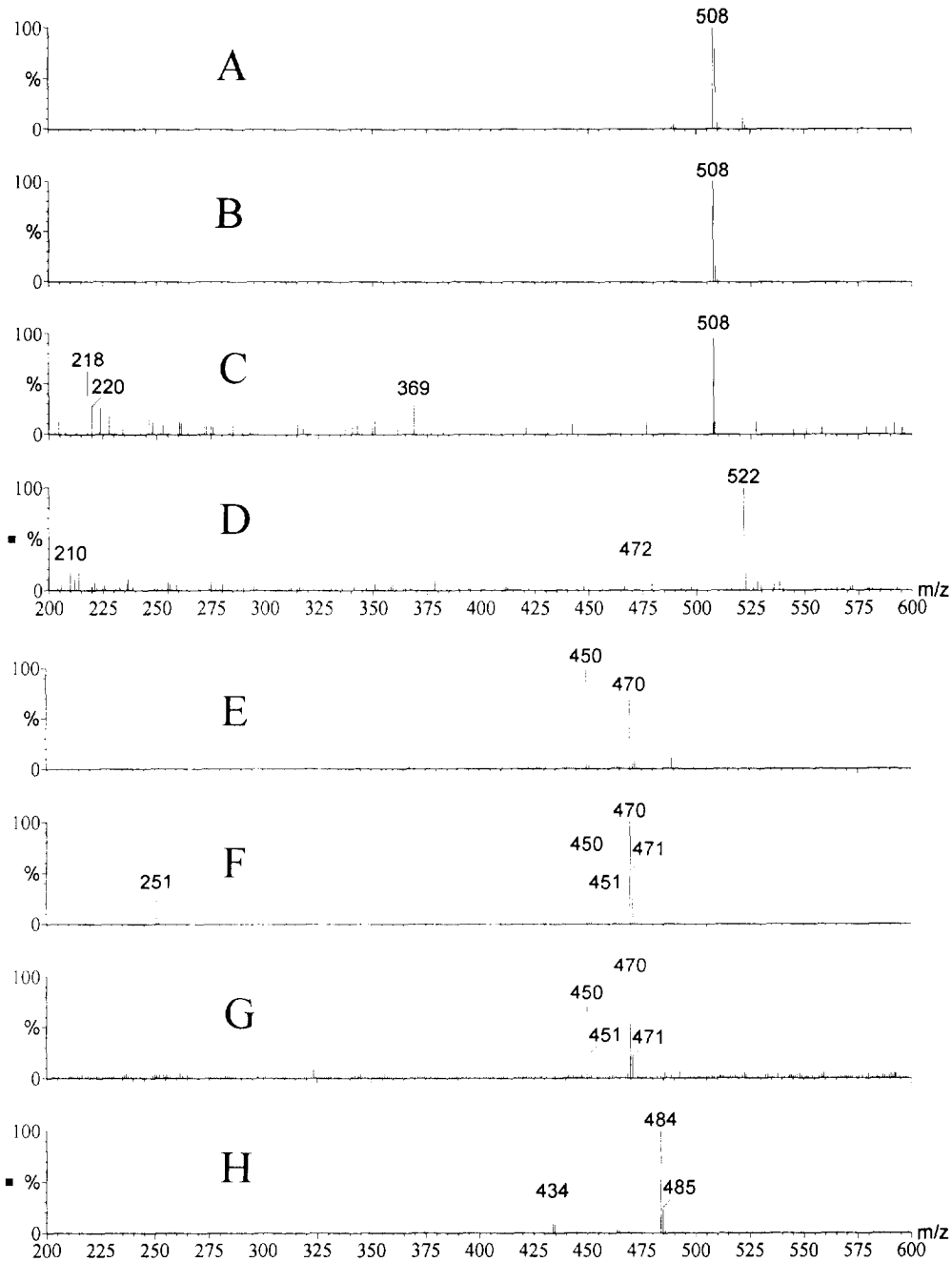


Fig. 5. Mass spectra of technical-grade MDA as PFPA derivatives. The mass spectra were obtained using ammonia. Positive-ion (A–D) and negative-ion (E–H) spectra are shown. Ions of m/z 100–600 were monitored in less than 1.0 s with an inter-scan delay of 0.1 s. PCI: (A) 4,4′-MDA (compound III); (B) MDA isomer II; (C) MDA isomer I; (D) methyl-MDA (compound d). NCI: (E) 4,4′-MDA (compound III); (F) MDA isomer II; (G) MDA isomer I; (H) methyl-MDA (compound d). A 1-μl volume of toluene containing ca. 25 ng of technical-grade MDA was injected.

dideuterated 4,4′-MDA were monitored. The ratios of the peak areas were calculated. For a typical calibration plot, the slope was 0.29 and the intercept was 0.064. Duplicate work-up procedures with duplicate injections were made for each concentration. The linear calibration plots obtained passed virtually through the origin and the correlation coefficients were typically 0.9994.

Reproducibility

Two human urine samples were spiked at a concentration of 10 μg/l on five different occasions during a period of 6 weeks. The samples were subsequently worked-up and analysed. The reproducibility was 5% ($n = 12$).

Detection limit

For the GC–MS system the detection limit, defined as twice the signal-to-noise ratio, is in the range 1–5 fg. The detection limit for biological samples is defined by the matrix. The detection limit defined as the blank plus three standard deviations of the blank was 0.05 μg/l of both human urine and plasma.

3.5. Application

Ten workers in a polyurethane factory were exposed to MDI. The route of MDI exposure has not yet been established but a study in more detail is in progress. Urine and plasma samples from the workers were pooled and analysed. The concentrations of 4,4′-MDA in hydrolysed pooled urine and plasma samples were 0.9 and of 2.3 μg/l, respectively.

The chromatograms are shown in Fig. 4. Three MDA isomers are seen in both chromatograms. It is interesting that the relative concentration of the three isomers varies between plasma and urine. Compounds I and II were not determined owing to the lack of reference substances. The total study includes about 200 workers and will be described in more detail in a forthcoming paper [18].

4. Discussion

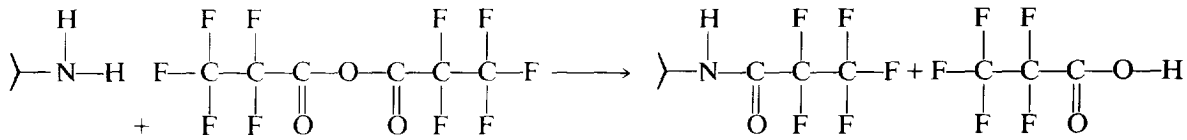
The production of MDI is an advanced chemical process. The first step is the nitrification of benzene followed by the reduction of the nitro group formed. Treatment with formaldehyde results in various condensation products with the main component being the 4,4′-MDA isomer. Aniline and methylaniline are present at very low concentrations. MDA isomers such as 2,2′-MDA and 2,4′-MDA are also formed and, as described above, also methyl-MDA with several isomers. MDI is formed by the reaction of MDA with phosgene, whereupon all the amine groups are converted into isocyanate groups. During hydrolysis of isocyanates, corresponding amines are formed. When investigating the hydrolysis products of MDI and MDI-exposed biological materials, MDA can be used as a biological marker. It is known that the composition of the isomers in MDA and MDI varies between batches and factories. Further, there are many different commercially available MDI qualities with major differences in the isomeric composition and the polymerization grade.

In this work, GC–MS has been demonstrated to be a powerful tool for investigating technical MDA and MDI.

Anhydride reagents reacts with amines with the formation of amides:

The perfluorofatty acid anhydride derivatization reagent was chosen owing to the enhanced sensitivity obtained when monitoring negative ions. PFPA was selected as it has been used most frequently in our earlier studies on the determination of aliphatic and aromatic amines.

It is not possible to determine compounds without having access to reference compounds and only semi-quantitative results are obtained. The isomers show different sensitivities and there are many components which are not resolved. NCI is by far the most sensitive technique but the spectra can be difficult to interpret as the fragmentation varies between isomers and the conditions in the ion source. When analysing biological materials only NCI gives satisfactory sensitive determinations. PCI has the advantage



that all the studied aromatic amine perfluorofatty acid derivatives give virtually the same type of mass spectra with the most abundant ion $m/z = (M + 18)^+$.

MDI has a very low TLV, e.g., 5 ppb in Sweden. In Sweden all isocyanates have the same TLV. The toxicity between different isocyanates may vary however. Complete knowledge of the variation in toxicity with the chemical structure is lacking. Further, the uptake, metabolism and toxicology of even the widely used 4,4'-MDI (main component in technical-grade MDI) is not known in detail, and the toxicological knowledge of other isocyanate components in technical-grade MDI even less. When evaluating hazards with isocyanates in the work environment, the actual content of the used (technical-grade) isocyanate must therefore be considered. Another route of exposure often observed is by dermal contact.

Earlier we have presented essential differences in the toxicokinetics for 2,4-TDI, 2,6-TDI and 1,6-HDI. The excretion in urine after exposure varies with the isomer studied. We have also seen an indication of the formation of protein adducts in plasma of workers and exposed volunteers. The different isomers of MDA in technical-grade MDA described in this paper may show different toxicology and toxicokinetics. To estimate the individual exposure to MDI, biological monitoring methods have great potential. The nature of the protein adducts in plasma with MDI is not known at present and further research is necessary.

More than five analogues of MDA, as their PFPA derivatives, in hydrolysed human urine and plasma samples were observed. The MDA isomers are not commercially available.

Acknowledgements

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References

[1] *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, No. 19, 4,4'-Methylene Diphenyldiisocyanate and Polymethylene Polyphenylisocyanate*, International Agency for Research on Cancer, Lyon, 1974, p. 314.

[2] A.L. Kennedy and W.E. Brown, *Isocyanates and Lung Disease: Experimental Approaches to Molecular Mechanisms (Occupational Medicine: State of the Art Review* Vol. 7, No. 2), Hanley and Belfus, Philadelphia, 1992, p. 301.

[3] *Kirk–Othmer Encyclopedia of Chemical Technology*, Vol. 2, Wiley-Interscience, New York, 1978, pp. 272–376.

[4] L. Renman, C. Sangö and G. Skarping, *Am. Ind. Hyg. Assoc. J.*, 47 (1986) 621–628.

[5] C. Rosenberg and H. Savolainen, *J. Chromatogr.*, 358 (1986) 385–391.

[6] G. Skarping, L. Renman, C. Sangö, L. Mathiasson and M. Dalene, *J. Chromatogr.*, 346 (1985) 191–204.

[7] A.G. Bailey, I. Brooks, Bird, P.B. Farmer and B. Street, *Anal. Biochem.*, 190 (1990) 175–181.

[8] M. Dalene, G. Skarping and H. Tinnerberg, *Chromatographia*, 38 (1994) 776–780.

[9] G. Skarping, M. Dalene and P. Lind, *J. Chromatogr.*, 663 (1994) 199–210.

[10] A. Tiljander, G. Skarping and M. Dalene, *J. Chromatogr.*, 479 (1989) 145–152.

[11] P. Brunmark, P. Persson and G. Skarping, *J. Chromatogr.*, 579 (1992) 350–354.

[12] P. Brunmark, M. Dalene and G. Skarping, *Analyst*, in press.

[13] J. Cocker, W. Gristwood and H.K. Wilson, *Br. J. Ind. Med.*, 43 (1986) 620–625.

[14] J. Cocker, A.R. Boobis and D.S. Davis, *Biomed. Environ. Mass Spectrom.*, 17 (1988) 161–167.

[15] C. Peterson, E.C. Estiva, D.S. Lyttle and R.M. Harris, *J. Chromatogr.*, 564 (1991) 205.

[16] E. Benfenati, M. Natangelo, R. Fanelli, G. Lualdi and R. Tridico, *Microchem. J.*, 46 (1992) 352.

[17] M. Littorin, L. Truedsson, H. Welinder, G. Skarping, U. Mårtensson and A. Sjöholm, *Scand. J. Work Environ. Health*, 20 (1994) 216–222.

[18] G. Skarping, M. Dalene and M. Littorin, *Int. Arch. Occup. Environ. Health*, submitted for publication.