

**A validated LC/MS/MS method for 4,4'-methylenedianiline quantitation in human urine as a measure of 4,4'-methylene diphenyl diisocyanate exposure**

Maggy Lépine<sup>1,2</sup>, Lekha Sleno<sup>1\*</sup>, Jacques Lesage<sup>1</sup> and Sébastien Gagné<sup>2\*</sup>

<sup>1</sup>Université du Québec à Montréal, Chemistry Department, PO Box 8888 Downtown Station, Montreal, H3C 3P8, Canada

<sup>2</sup>Institut de recherche Robert-Sauvé en santé et en sécurité du travail, 505 boul. De Maisonneuve Ouest, Montréal, Québec, H3A 3C2, Canada,  
([sebastien.gagne@irsst.qc.ca](mailto:sebastien.gagne@irsst.qc.ca))

\*co-corresponding authors: [sleno.lekha@uqam.ca](mailto:sleno.lekha@uqam.ca) and [sebastien.gagne@irsst.qc.ca](mailto:sebastien.gagne@irsst.qc.ca)

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/rcm.8380

## Abstract

### Rationale

4,4'-methylene diphenyl diisocyanate (MDI) is a highly reactive isocyanate used in the production of polyurethanes. Workers exposed to these products may develop sensitization to the diisocyanate compounds, leading to occupational asthma. Quantifying MDI levels is necessary to ensure workplace safety. MDI is metabolized by acetylation and/or conjugation to macromolecules for excretion into urine. All metabolites can be chemically hydrolysed to form the free diamine 4,4'-methylenedianiline (MDA) as a urinary biomarker of MDI exposure. Current methods involve long sample preparation, or have been designed using costly automation. There is therefore a need to develop a new practical method for assessing exposure to MDI.

### Method

Urine samples were acidified and heated to form MDA, followed by neutralization and liquid-liquid extraction. Extracts were separated by reverse-phase chromatography on a HSS T3 column followed by analysis on a triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode.

### Results

$^{13}\text{C}^{15}\text{N}$ -MDA was selected as the internal standard (IS) of choice following an investigation of internal standard stability. The hydrolysis efficiency, forming free MDA from conjugated metabolites *in vivo*, was evaluated using 4,4'-methylenebis(acetanilide) spiked into urine and complete hydrolysis occurred after one hour. A dynamic range of 5 to 500 nM was achieved, and was useful for monitoring MDI exposure considering the biological guidance value (BGV) of 10 µg/L (~50 nM) proposed by the German Research Foundation (DFG). The LOD and LOQ of the method were 0.8 and 2.7 nM, respectively. The intra-day and inter-day

precisions were 4.33% and 4.27%, respectively. Finally, the method was tested with inter-laboratory samples coming from the German External Quality Assessment Scheme (GEQUAS) program and the results submitted were all within in the allowable tolerance range.

## Conclusion

A practical and validated method for the analysis of small to medium-sized batches of samples has been developed for the biological monitoring of MDI exposure in human urine.

**Keywords:** 4,4'-methylene diphenyl diisocyanate, 4,4'-methylenedianiline, UHPLC-MS/MS, Biological monitoring, Quantitative analysis

## INTRODUCTION

4,4'-methylene diphenyl diisocyanate (MDI) is a highly reactive isocyanate used in the manufacturing of polyurethanes (PURs)<sup>1</sup>. PURs composed of MDI are found in various materials such as insulating foam, glue and adhesives<sup>2</sup>. Workers using these products can therefore be exposed to isocyanates. The high reactivity of isocyanates makes them toxic, with acute or chronic effects. MDI is an irritant and an important respiratory sensitizer, which can cause severe respiratory diseases such as occupational asthma<sup>3-5</sup>, hypersensitivity pneumonitis or alveolitis<sup>6</sup>. MDI is also a skin<sup>7</sup> and eye irritant<sup>8</sup>.

MDI can be absorbed in the body by inhalation or by dermal contact as predominant routes of exposure<sup>9</sup>. After being absorbed, MDI is conjugated to biomolecules (forming protein and glutathione adducts)<sup>2,4,10-13</sup> and/or acetylated<sup>2,9,13-15</sup>. The low molecular weight conjugates and acetylated metabolites are eliminated in urine<sup>10,16</sup>. To analyze these metabolites in a urine sample, hydrolysis is performed to release free MDA, as the biological biomarker of MDI exposure<sup>6,9,12,17</sup>.

Various strategies have been taken by industries to better protect workers from isocyanates and to comply with governmental regulations. To assess the adequacy of exposure control, exposure measurements for the various work operations are taken and compared with occupational exposure limits (OELs). The approach most commonly used for the evaluation of MDI exposure is environmental air monitoring. Most countries have adopted an occupational exposure limit of 5 ppb in air for an eight hour work shift<sup>18,19</sup>. However, this approach does not allow the evaluation of the real dose absorbed and biological monitoring is therefore necessary to properly monitor exposure<sup>6</sup>. A recommended value of 10 µg/L (50 nM)<sup>20</sup> for hydrolysed MDA has been proposed by the DFG. This value is established for carcinogens, sensitizers and allergens, or substances with limited toxicological information based on occupational-medical information and can be used for exposure assessment or controlling hygienic measures<sup>20</sup>.

Over the years, different experimental approaches have been developed for the determination of MDA as a urinary biomarker of exposure. Ideally, a method not involving derivatization<sup>21</sup> is favored in order to simplify sample preparation. Current methods are either not measuring free MDA<sup>5,6,17,22,23</sup> or are designed to analyze large numbers of samples using costly automation<sup>10</sup>. The majority of methods involve a long hydrolysis step of over 16 hours<sup>3,6,16,17,23</sup>. There is therefore a need to develop a novel method for the analysis of small to medium batches of samples, while ideally considering the need to be environmentally-friendly. For this purpose, we have validated a novel method for MDA determination in human urine by LC/MS/MS in multiple reaction monitoring (MRM) mode.

## EXPERIMENTAL

### Chemicals

4,4'-Methylenedianiline (MDA) and 4,4'-methylenebis(acetanilide) (AcMDA) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). 4,4'-Methylene- $d_8$ -dianiline ( $d_8$ -MDA) and 4,4'-Methylene- $d_2$ -dianiline ( $d_2$ -MDA) were purchased from C/D/N isotopes (Pointe-Claire, Qc, Canada). 4,4'-Methylenedianiline- $[^{15}\text{N}_2, ^{13}\text{C}]$  ( $^{13}\text{C}^{15}\text{N}_2$ -MDA) was obtained from IsoScience (Ambler, PA, USA). Methanol (MeOH), toluene and water were purchased from Fisher Scientific (St-Laurent, Qc, Canada) and were all HPLC grade. Sodium hydroxide (NaOH), sulfuric acid ( $\text{H}_2\text{SO}_4$ ) and acetic acid, all ACS grade, were also obtained from Sigma-Aldrich.

### Collection of human urine

Human urine of control subjects (not exposed to isocyanates) was obtained by anonymous donation and kept at 4°C for less than 30 days. Inter-laboratory urine samples came from the German External Quality Assessment Scheme (G-EQUAS) for analyses in biological materials (Erlangen, Germany).

### Creatinine and density levels

Urinary creatinine was analyzed by a spectrometric method based on Jaffe's reaction<sup>24</sup> using an Architect c4000 system spectrophotometer from Abbott (Abbott Park, IL, USA). The density measurements were determined by refractometry<sup>24</sup> using a Densitometer DMA 38 from Anton-Paar (Saint-Laurent, Qc, Canada). Both measurements were performed on the same day.

### Calibration curve solutions

Stock solutions of 1 mM MDA and 500  $\mu\text{M}$   $^{13}\text{C}^{15}\text{N}_2\text{-MDA}$  were prepared in water. Seven working standard solutions of MDA were prepared in water at 0.1, 0.2, 0.4, 1, 2.5, 5 and 10  $\mu\text{M}$ . An internal standard (IS) working solution ( $^{13}\text{C}^{15}\text{N}_2\text{-MDA}$ ) was made at 1  $\mu\text{M}$ . Calibration samples were prepared by adding 10  $\mu\text{L}$  of standard working solution and 10  $\mu\text{L}$  of IS working solution in 180  $\mu\text{L}$  of urine. The final concentrations of MDA were 5, 10, 25, 50, 100, 250 and 500 nM while the final concentration of IS was 50 nM.

### Sample preparation

The following general workflow was applied for all samples including the calibration curve standards. The working IS solution (10  $\mu\text{L}$ ) was spiked into 190  $\mu\text{L}$  of urine, followed by the addition of 20  $\mu\text{L}$  of 18 M sulfuric acid and incubation of the resulting solution at 100°C for 1h. The sample was then neutralized with 140  $\mu\text{L}$  of 10 N NaOH prior to extraction with 400  $\mu\text{L}$  of toluene. During extraction, the samples were mixed vigorously and then centrifuged for 20 min at 14,500 rpm before the organic layer (approximately 315  $\mu\text{L}$ ) was transferred to a clean tube. The extracts were evaporated to dryness in a Speedvac at 50°C and reconstituted with 200  $\mu\text{L}$  of water.

The internal standard degradation test was performed using solutions of 1  $\mu\text{M}$  for each isotope-labeled MDA compound in water. Samples (1 mL) were treated with 1.5 mL of 3 M  $\text{H}_2\text{SO}_4$  at 100°C, and aliquots were taken for 0, 1, 2, 4, 6 and 16 h time points. The samples were neutralized with  $\text{NH}_4\text{OH}$  to obtain an approximate pH of 7.

## LC/MS/MS analysis

Samples were injected (5  $\mu$ L) onto an Acquity UPLC HSS T3 1.8  $\mu$ m, 2.1 x 50 mm column (Waters, Dublin, Ireland) using a Waters ACQUITY I-class UPLC system. Elution was performed at 40°C with a flow rate of 0.6 mL/min using a gradient of water (A) and methanol (B), both containing 0.1% acetic acid. The gradient started at 5% B (held for 0.5 min) followed by an increase to 90% in 3 min and was held at 90% for one additional minute. The LC/MS/MS spectra were then acquired on a Waters Xevo TQD triple quadrupole mass spectrometer (Milford, MA, USA) in electrospray positive ion mode, where MDA and the IS yielded  $[M+H]^+$  ions at  $m/z$  199 and 202, respectively. The source parameters were as follows: source temperature of 150°C, desolvation temperature of 500°C and desolvation flow of nitrogen gas at a rate of 1000 L/hr. The monitored MRM transitions were  $m/z$  199.0 $\rightarrow$ 106.0 (quantitation ion) and  $m/z$  199.0 $\rightarrow$ 79.0 (qualifier ion) for the MDA and  $m/z$  202.1 $\rightarrow$ 108.1 for the IS with a collision energy (CE) of 25 eV.

For the internal standard degradation test, samples (2  $\mu$ L) were injected onto the same column as described above using a Nexera UHPLC system (Shimadzu, Columbia, MD, USA). An elution at 40°C with a flow rate of 0.5 mL/min was performed using the same mobile phases and gradient. A TripleTOF 5600 (quadrupole–time-of-flight) mass spectrometer from Sciex (Concord, ON, Canada) with a DuoSpray ion source in positive mode was used for high-resolution mass spectrometry (HRMS) analysis of internal standard samples. The ion source parameters were as follows: nebulizer (nitrogen) and drying (dry air) gases (GS1 and GS2) at 50 psi, pressure curtain gas (CUR) pressure at 35 psi, source temperature of 450°C and ionspray voltage of 5000 V. TOF-MS data was acquired between  $m/z$  80 and 925 with accumulation time of 600 ms and information-dependent acquisition (IDA) was used to

acquire TOF MS/MS data of the three highest intensity ions after each TOF-MS spectrum, with an accumulation time of 150 ms. The total cycle time was 1.1 s.

### **Data analysis**

LC-MS/MS data from samples and calibration standards were processed using Masslynx 1.0 software (Waters), with automatic peak integration. For the internal standard degradation test, Analyst TF 1.7.1 software (Sciex) was used for data acquisition and data were processed using PeakView 2.2 software (Sciex).

### **Analytical performance**

Limit of detection (LOD) and limit of quantitation (LOQ) measurements were based on a signal-to-noise (S/N) ratio of 3 and 10, respectively. The intra-day precision was calculated by analysing six different samples of four concentrations (5, 25, 50 and 500 nM) analysed within the same day (n=24). The inter-day precision was calculated by analysing four concentrations (5, 25, 50 and 500 nM) analysed on six different days (n=24). Method accuracy was evaluated by analysing reference urine samples from G-EQUAS program. Finally, matrix effect and recovery was investigated by comparing six replicates at four concentrations levels (5, 25, 50 and 500 nM) spiked in urine with replicates spiked in water.

## RESULTS AND DISCUSSION

### Sample preparation

#### *Internal standard stability tests*

Three different internal standards (IS) were evaluated to test their stability during the hydrolysis step:  $d_8$ -MDA,  $d_2$ -MDA and  $^{13}C^{15}N_2$ -MDA. For the  $d_8$ -MDA, there is a loss of deuterium labels on the amine groups and therefore, this internal standard is rapidly converted to  $d_4$ -MDA in solution. The structures of these IS compounds are shown in Figure 1. Hydrolysis was performed over a time course of 16 h and the results are presented in Figure 2A. Interestingly, it was found that the deuterium labels on the ring carbons of  $d_4$ -MDA were lost during the hydrolysis step; however,  $d_2$ -MDA and  $^{13}C^{15}N_2$ -MDA were found to be stable.  $^{13}C^{15}N_2$ -MDA was chosen as the better choice due to the 3 Da mass difference in molecular weight from the analyte molecule, therefore offering less potential for interference or cross-talk in the mass spectrometer.

The degradation of  $d_4$ -MDA was investigated in detail and the results are shown in Figures 2B and 2C. The hypothesis for this loss of deuterium label is related to the protonation of the amine during the hydrolysis step. This IS has four deuterium atoms located ortho to the two amino groups on the aromatic rings (Figure 1). Due to the strong hydrolysis step with sulfuric acid at 100°C, 50 % of the IS was degraded after 6 h of hydrolysis and we see eventual loss of all four deuterium labels, down to the unlabeled MDA compound, which would also interfere directly with the analyte to be measured in this assay.

### *Hydrolysis efficiency*

Biological monitoring is important for the determination of the real dose of MDI absorbed in humans. Since the metabolites formed are eliminated, urine samples containing those metabolites must be hydrolysed to form free MDA. To provide an efficient and sensitive method, the hydrolysis needed to be optimized. The hydrolysis efficiency was evaluated using 4',4'-methylenebis(acetanilide) (AcMDA) (Figure 1), a commercially-available metabolite found in urine<sup>2,9,14,15</sup>. The time of hydrolysis was varied from 1 to 16 h. After 1h of hydrolysis, only MDA (hydrolysis product) was detected. It was established that in this case complete hydrolysis of MDI metabolites in urine occurred after 1 h.

### *Liquid-liquid extraction*

A liquid-liquid extraction procedure has been optimized for small to medium batches of samples while limiting solvent volumes, to be more environmentally friendly than previous methods<sup>3,16,17,21,25</sup>. The use of toluene rather than dichloromethane<sup>21</sup> as the extraction solvent is a major advantage as it allows the collection of the top layer. Extraction was efficient for the MDA according to the LOD and LOQ being measured. By reducing the starting volume of urine, we were able to limit solvent usage so that only 400  $\mu$ L per sample was used for extraction, as well as making the procedure more practical by using 1.5-mL polypropylene tubes instead of larger glass tubes or vials.

## LC/MS/MS method development

The MRM method was developed using the protonated molecule ( $[M+H]^+$ ) as the precursor ion, which fragment by the loss of an aniline molecule (Figure 3A). A chromatographic separation was developed using acetic acid in both aqueous and organic mobile phases with a gradient elution in 3 minutes. The overall run time including the equilibration period was 6 minutes.

## Analytical parameter validation

### *Dynamic range, LOD/LOQ and precision*

The method was applied to samples of known concentrations of MDA to determine various validation parameters. The dynamic range was adjusted to cover 10% to 1000% of the biological guidance value (BGV) of  $10 \mu\text{g/L}^{20}$  (~50 nM (Figure 3C)) corresponding to concentrations between 5 nM (Figure 3D) and 500 nM (Figure 3E). Coefficients of determination ( $R^2$ ) > 0.998 were obtained for all calibration curves. The % accuracy and % CV values for each concentration level, shown in Table 2, confirm the reliability of our results. Acceptable sensitivities of 0.8 nM (1.6 % of BGV) and 2.7 nM (5.4 % of BGV) were obtained for the LOD and LOQ, respectively. These results show that in this method, derivatization was not necessarily required to provide good sensitivity based on the BGV. Indeed, this method measuring free MDA allows the evaluation of a low exposure that corresponds to 5.4% of the target value. The intra-day and inter-day precisions shown are approximately 4 % and also show the robustness of the method.

### *Recovery and Matrix effect*

A liquid-liquid extraction step was employed in order to reduce potential variability of the method resulting from the biological matrix<sup>26</sup>. The method used to evaluate the matrix effect is inspired by the AIHA-LAP, LLC accreditation program (American Industrial Hygiene Association Laboratory Accreditation Programs). The matrix effect was studied by comparing peak areas in urine and water. A total recovery of 95 % was obtained (Table 2). The use of an internal standard helps to minimize variability of results from sample preparation and ionization efficiencies.

### *Control urine samples*

Urine samples obtained by eleven anonymous donations from people not known to be exposed to MDI were evaluated as a negative control. Creatinine and density levels were first measured to confirm that the urine samples were in the tolerance range between 4.4 and 26.5 mM and between 1.010 and 1.030 g/mL, respectively<sup>18</sup>. All the control urine samples were analysed using the developed method and no signal above the LOD signal to noise threshold of 3 was obtained. These results demonstrate that no interference is present at the retention time of the analyte.

### *Accuracy (G-EQUAS program)*

The inter-laboratory performance was used to evaluate the accuracy of the method after development and optimization. The method was used to test ten reference samples purchased from the G-EQUAS program. All measured values were within the tolerated ranges provided by the program. The results are presented in Table 3 and show the reliability of the method for urine samples.

## CONCLUSIONS

A practical and validated method has been developed to determine MDA levels in human urine, the urinary biomarker of MDI exposure. The developed method has been optimized in terms of hydrolysis time, accuracy, robustness and reliability according to the biological guidance values established by the DFG. The sample preparation was simplified and shortened to obtain analytical results more quickly than with current methods which involve analyte derivatization<sup>6,17</sup> and/or longer hydrolysis<sup>3,6,10,16,17</sup>. All possible routes of exposure to MDI are measured with this biological monitoring method. Moreover, this method could be combined with environmental monitoring (in air) and used by professionals to evaluate exposure to MDI in the workplace.

## REFERENCES

1. Creely KS, Hughson GW, Cocker J, Jones K. Assessing isocyanate exposures in polyurethane industry sectors using biological and air monitoring methods. *The Annals of occupational hygiene*. 2006;50(6):609-621.
2. Sabbioni G, Dongari N, Kumar A. Determination of a new biomarker in subjects exposed to 4,4'-methylenediphenyl diisocyanate. *Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals*. 2010;15(6):508-515.
3. 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):522-528.
4. Cocker J. Biological monitoring for isocyanates. *Occup Med (Lond)*. 2007;57(6):391-393.
5. Jones K, Johnson PD, Baldwin PEJ, et al. Exposure to Diisocyanates and Their Corresponding Diamines in Seven Different Workplaces. *Annals of work exposures and health*. 2017;61(3):383-393.

6. Rosenberg C, Nikkila K, Henriks-Eckerman ML, Peltonen K, Engstrom K. Biological monitoring of aromatic diisocyanates in workers exposed to thermal degradation products of polyurethanes. *J Environ Monit.* 2002;4(5):711-716.
7. Thorne PS, Hillebrand JA, Lewis GR, Karol MH. Contact sensitivity by diisocyanates: potencies and cross-reactivities. *Toxicol Appl Pharmacol.* 1987;87(1):155-165.
8. European Commission (EC). *Methylenediphenyl diisocyanate (MDI)*. Institute for Health and Consumer Protection;2005.
9. Deutsche Forschungsgemeinschaft (DFG). *4,4' - Methylene diphenyl diisocyanate (MDI) [ 101 - 68 - 8] and "polymeric" MDI (PMDI) [ 9016 - 87 - 9] [MAK Value Documentation, 2008]*. Wiley-VCH Verlag GmbH & Co. KGaA; 2015.
10. 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):10687-10692.
11. Gaines LG, Fent KW, Flack SL, et al. Urine 1,6-hexamethylene diamine (HDA) levels among workers exposed to 1,6-hexamethylene diisocyanate (HDI). *The Annals of occupational hygiene.* 2010;54(6):678-691.
12. Cocker J. Biological monitoring for isocyanates. *The Annals of occupational hygiene.* 2011;55(2):127-131.
13. 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; the fate of foreign compounds in biological systems.* 2005;35(3):273-292.
14. 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):2583-2587.
15. Bolognesi C, Baur X, Marczyński 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):737-772.

16. Peterson JC, Estiva EC, Lyttle DS, Harris RM. High-performance liquid chromatographic determination of 4,4'-methylenedianiline in human urine. *J Chromatogr.* 1991;564(1):205-212.
17. Skarping G, Delene M, P. B. Liquid Chromatography and Mass Spectrometry Determination of Aromatic Amines in Hydrolysed Urine from Workers Exposed to Thermal Degradation Products of Polyurethane. *Department of Occupational and Environmental Medicine.* 1994;39:619-623.
18. American Conference of Governmental Industrial Hygienists (ACGIH). *Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices.* Cincinnati, OH 2017.
19. National Institute for Occupational Safety and Health (NIOSH). *Criteria for a recommended standard occupational exposure to diisocyanates.* Cincinnati, OH: US Department of Health, Education, and Welfare; 1978.
20. Deutsche Forschungsgemeinschaft (DFG). *List of MAK and BAT values 2016: Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area.* 2016.
21. Sakai T, Morita Y, Ka Y, Tao YX. LC-MS determination of urinary toluenediamine in workers exposed to toluene diisocyanate *Toxicology Letters.* 2002;134(1-3):259-264.
22. Jones K, Cocker J, Piney M. Isocyanate exposure control in motor vehicle paint spraying: evidence from biological monitoring. *The Annals of Occupational Hygiene.* 2013;57(2):200-209.
23. Dalene M, Skarping G, Brorson T. Chromatographic determination of amines in biological fluids with special reference to the biological monitoring of isocyanates and amines. IV. Determination of 1,6-hexamethylenediamine in human urine using capillary gas chromatography and selective ion monitoring. *J Chromatogr.* 1990;516(2):405-413.
24. Sauve JF, Levesque M, Huard M, et al. Creatinine and specific gravity normalization in biological monitoring of occupational exposures. *Journal of Occupational and Environmental Hygiene.* 2015;12(2):123-129.
25. Robert A, Ducos P, Francin JM. Determination of urinary 4,4'-methylenedianiline and its acetylated metabolites by solid-phase extraction and HPLC analysis with UV and electrochemical detection. *Int Arch Occup Environ Health.* 1995;68(1):44-51.

26. Schlittenbauer L, Seiwert B, Reemtsma T. Matrix effects in human urine analysis using multi-targeted liquid chromatography-tandem mass spectrometry. *J Chromatogr A*. 2015;1415:91-99.

Accepted Article

**TABLE 1** Analytical parameters obtained by the method validation

|                                                        |            |
|--------------------------------------------------------|------------|
| <b>Biological guidance value (BGV)</b>                 | 50 nM      |
| <b>Dynamic range</b>                                   | 5 – 500 nM |
| <b>Coefficient of determination (<math>R^2</math>)</b> | >0.998     |
| <b>Limit of detection (LOD) (n=10)</b>                 | 0.8 nM     |
| <b>Limit of quantitation (LOQ) (n=10)</b>              | 2.7 nM     |
| <b>Intra-day precision (n= 24)</b>                     | 4.33%      |
| <b>Inter-day precision (n=24)</b>                      | 4.27%      |
| <b>Recovery (%)</b>                                    | 95 +/- 5.6 |

**TABLE 2** Accuracy (%) and CV (%) values from each standard measured in three different calibration curves

| Levels<br>concentration<br>(nM) | % Accuracy |         |         | % CV<br>(n= 3) |
|---------------------------------|------------|---------|---------|----------------|
|                                 | Curve 1    | Curve 2 | Curve 3 |                |
| 5                               | 106.8      | 103.4   | 106.8   | 1.9            |
| 10                              | 94.3       | 101.4   | 93.9    | 4.4            |
| 25                              | 99.2       | 97.3    | 100.6   | 1.7            |
| 50                              | 98.2       | 94.2    | 98.3    | 2.4            |
| 100                             | 101.6      | 104.1   | 100.0   | 2.0            |
| 250                             | 99.9       | 99.7    | 100.8   | 0.5            |
| 500                             | 100.0      | 100.0   | 99.8    | 0.1            |

**TABLE 3** Quantitative results from urine samples provide from the G-EQUAS program  
(inter-laboratory performance)

| Samples    | Reference values<br>(tolerance range)<br>(nM) | Measured values<br>(nM) |
|------------|-----------------------------------------------|-------------------------|
| <u>57A</u> | 28.7<br>(20.2 – 37.2)                         | <u>21.1</u>             |
| <u>57B</u> | 118.4<br>(90.4 – 146.4)                       | <u>106.0</u>            |
| <u>58A</u> | 14.9<br>(10.5 – 19.3)                         | <u>16.8</u>             |
| <u>58B</u> | 103.7<br>(80.3 – 126.9)                       | <u>106.4</u>            |
| <u>59A</u> | 31.2<br>(23.5 – 38.9)                         | <u>30.7</u>             |
| <u>59B</u> | 62.8<br>(49.2 – 76.4)                         | <u>67.9</u>             |
| <u>60A</u> | 7.6<br>(5.0 – 10.2)                           | <u>6.7</u>              |
| <u>60B</u> | 68.0<br>(50.8 – 85.4)                         | <u>65.2</u>             |
| <u>61A</u> | 14.3<br>(9.4 – 19.1)                          | <u>15.8</u>             |
| <u>61B</u> | 69.3<br>(54.5 – 84.2)                         | <u>72.8</u>             |

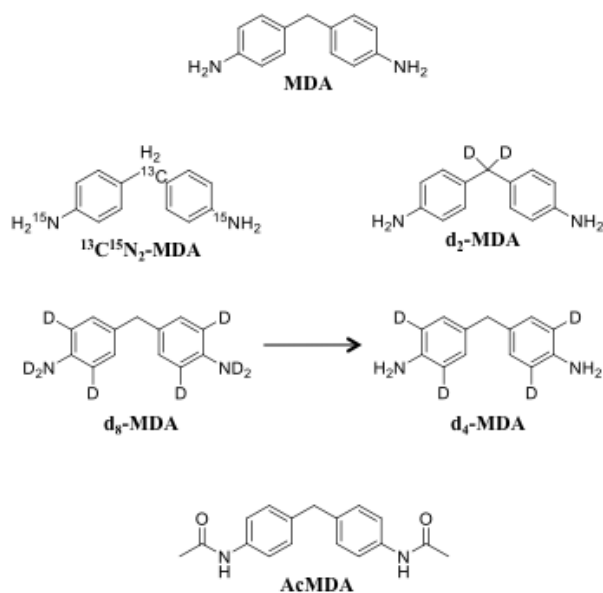


**FIGURE 1** Structures of MDA,  $\text{d}_4$ -MDA (via  $\text{d}_8$ -MDA),  $\text{d}_2$ -MDA,  $^{13}\text{C}^{15}\text{N}_2$ -MDA and AcMDA

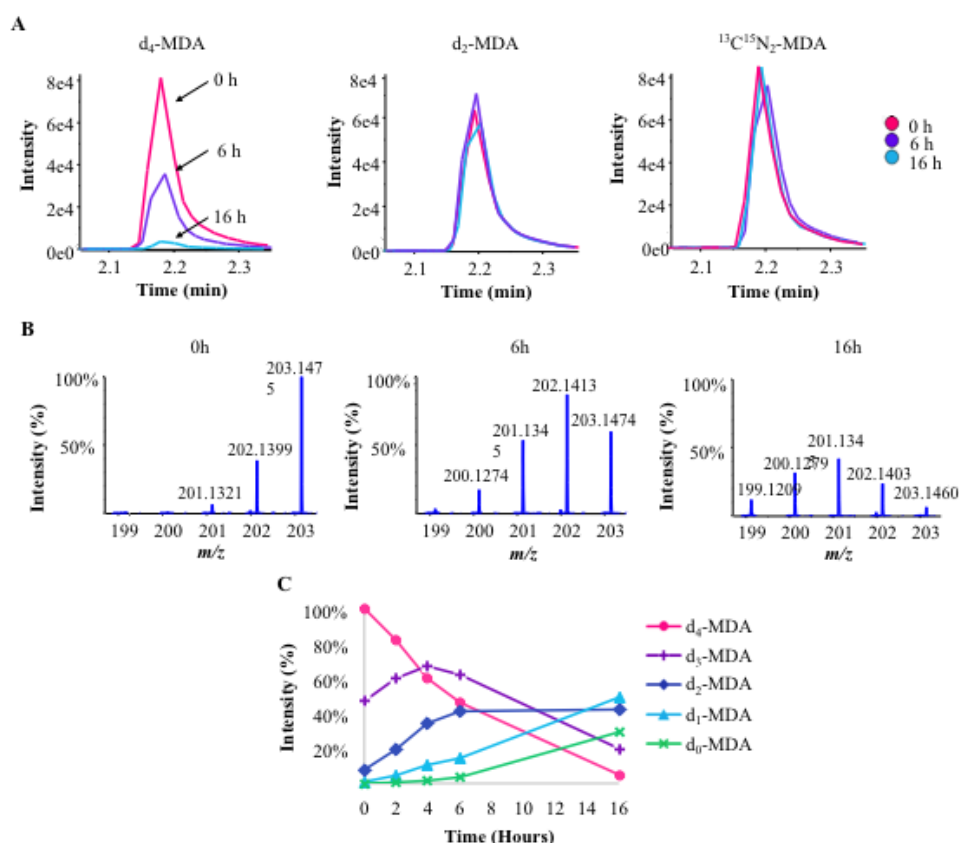


**FIGURE 2 (A)** For each internal standard studied, the extracted ion chromatogram of the protonated precursor (measured by high-resolution mass spectrometry  $\pm 0.01$   $m/z$  units) is shown after 0, 6 and 16 h of hydrolysis time. The signal for  $d_4$ -MDA is seen to decrease substantially upon hydrolysis, whereas the other two IS compounds are stable. **(B)** Mass spectra showing  $m/z$  region from  $d_0$ -MDA to  $d_4$ -MDA at 0, 6 and 16 h of hydrolysis, showing loss of deuterium labels over time **(C)** Relative peak areas of  $d_0$  to  $d_4$ -MDA, exhibiting the concerted losses of deuterium labels

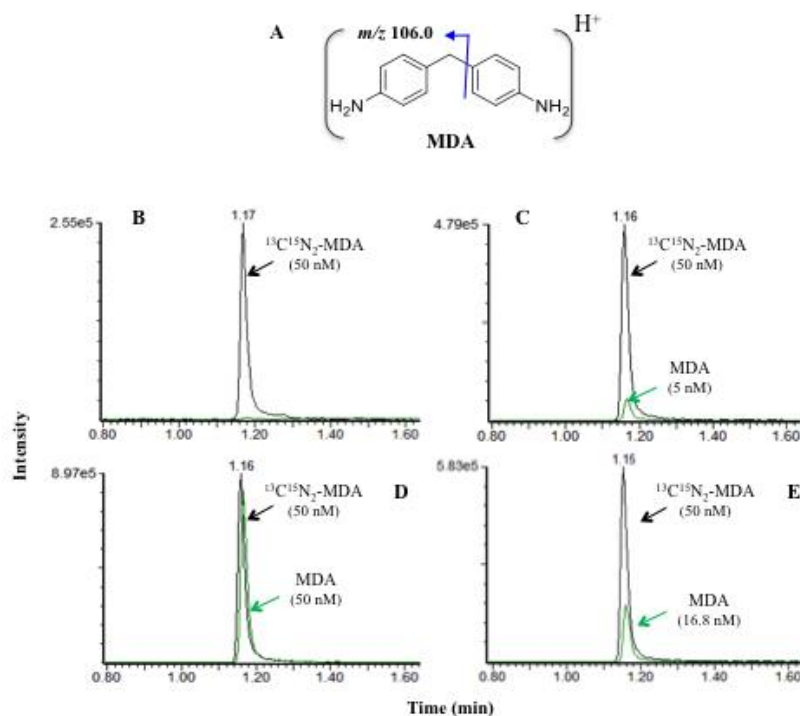


**FIGURE 3** MDA MRM fragmentation (A). MRM chromatograms of blank sample (B), lowest standard (5 nM) (C), mid-range standard (50 nM) (D), and sample 58A from the G-EQUAS (16.8 nM) (E)