DETERMINATION OF ALIPHATIC AMINES IN URINE AS BIOMARKERS OF EXPOSURE TO 1,6-HEXAMETHYLENE DIISOCYANATE AND ISOCYANURATE

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ABSTRACT

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DETERMINATION OF ALIPHATIC AMINES IN URINE AS BIOMARKERS OF EXPOSURE TO 1,6-HEXAMETHYLENE DIISOCYANATE AND ISOCYANURATE
(Under the direction of Dr. Leena A. Nylander-French)

Biological monitoring of 1,6-hexamethylene diisocyanate (HDI) exposure has been limited to the hydrolysis product of HDI monomer. The determination of acetylated 1,6-diaminohexane (HDA) and isotriamine as biomarkers of HDI and isocyanurate exposure, respectively, can provide important insight into the metabolism and dose-response relationships of these compounds. The objective of this study was to develop a liquid chromatography-mass spectrometry (LC-MS) method to detect HDA, monoacetyl-HDA, diacetyl-HDA, and isotriamine in urine. The LC-MS method was developed using the four standards spiked into urine at concentrations between 0.019-10.144 μg/L. Quantification of serial dilutions of the standards in urine was performed using selected reaction monitoring on a triple quadrupole mass spectrometer. The limit of detection and the limit of quantification in spiked urine were 0.01 and 0.02 μg/L for monoacetyl-HDA, respectively, and 0.16 and 0.31 μg/L for diacetyl-HDA, respectively. HDA and isotriamine were not detectable in urine at concentrations below 10 μg/L because of high background level of other unspecified compounds. The developed LC-MS method is sensitive and specific for the quantification of acetylated metabolites of HDI. However, modifications to the method are necessary to improve the specificity and sensitivity of HDA and isotriamine analyses.

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LIST OF ABBREVIATIONS

Amu	Atomic mass unit
ACS	American Chemical Society
Conc.	Concentration
Da	Daltons
DI-H_2O	De-ionized water
Diacetyl-HDA	*N,N'*-diacetyl-1,6-diaminohexane
Diacetyl-HpDA	*N,N'*-diacetyl-1,7-diaminoheptane
ESI^+	Electrospray ionization in positive-ion mode
GC-MS	Gas chromatography – mass spectrometry
^1H NMR	Proton nuclear magnetic resonance
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HDA	1,6-diaminohexane
HDI	1,6-hexamethylene diisocyanate
$HESI^+$	Heated electrospray ionization in positive-ion mode
HFBA	Heptafluorobutyric anhydride
HpDA	1,7-diaminoheptane
HPLC	High-performance liquid chromatography
ICIS	Interactive chemical information system
LC	Liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
LOD	Limit of detection

LOQ	Limit of quantitation
MDI	Methylene diphenyl diisocyanate
$MgSO_4$	Magnesium sulfate
Monoacetyl-HDA	Monoacetyl-1,6-diaminohexane
Monoacetyl-HpDA	Monoacetyl-1,7-diaminoheptane
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
NAT	N-acetyltransferase
NH_4OH	Ammonium hydroxide
NIOSH	National Institute for Occupational Safety and Health
PFPA	Pentafluoroproprionic anhydride
RT	Retention time
SIM	Selected ion monitoring
SN	Signal-to-noise
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
TDI	Toluene diisocyanate
UPLC	Ultra performance liquid chromatography

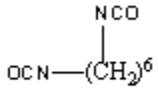
CHAPTER I

INTRODUCTION

Background

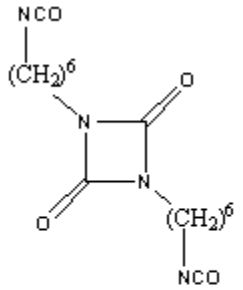
Isocyanates are low molecular weight aromatic or aliphatic compounds characterized by a highly reactive –N=C=O group. They are used in the production of polyurethane products such as spray paints, sealants, polyurethane foams, insulation, adhesives, resins, and surface coatings (NIOSH 1996; Streicher et al. 1998; Weber 2004). It is estimated that over 3 billion pounds of isocyanates are produced yearly worldwide (Woods 1987). In 1995 the National Institute for Occupational Safety and Health (NIOSH) estimated that approximately 280,000 U.S. workers were exposed to isocyanates (NIOSH 1996). The most commonly used diisocyanates are hexamethylene diisocyanate (HDI), toluene diisocyanate (TDI), and methylene diphenyl diisocyanate (MDI). Diisocyanates are characterized by having two isocyanate groups (–N=C=O) and react readily with water, nucleophiles, or with themselves to form dimers and trimers (Liu et al. 2000; NIOSH 1996).

In the automotive refinishing industry, most frequently used paints and clear coats contain small amounts of HDI (<1%) and much higher amounts of HDI oligomers uretdione, biuret, and isocyanurate. Figure 1 shows the chemical structure of the four isocyanates commonly present in HDI-based paints and clear coats. The most abundant oligomer in HDI-based paints and clear coats is isocyanurate (Rosenberg et al. 1984; Pronk et al. 2006; Fent et al. 2008; Fent et al. 2009a; Fent et al. 2009b).



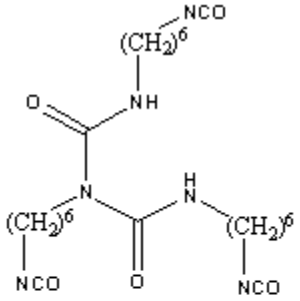
1,6-Hexamethylene Diisocyanate

MW = 168.2 g/mol



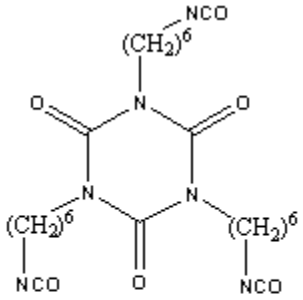
Uretdione

MW = 336.4 g/mol



Biuret

MW = 478.7 g/mol



Isocyanurate

MW = 504.7 g/mol

Figure 1. Chemical structures of the four isocyanates (HDI, uretdione, biuret, and isocyanurate) present in HDI-based paints and clear coats.

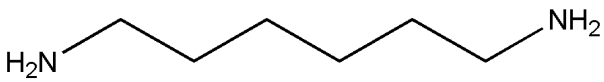
Health Effects

Isocyanate exposure poses acute and chronic health risks to the respiratory system and to the skin (Bernstein 1996). Health effects of isocyanate exposure include rhinitis, irritation of the skin, eyes, and mucous membranes, as well as allergic contact dermatitis and hypersentivity pneumonitis (NIOSH 1978; NIOSH 2005; Goossens et al. 2002). Isocyanates are the leading cause of occupational asthma in workers exposed through the inhalation and dermal routes (reviewed in Bello et al. 2007). Isocyanate-induced asthma is typically a result

of repeated exposure during which sensitization occurs. After sensitization, even a low level of isocyanate inhalation exposure can induce asthmatic response (reviewed in Bello et al. 2007). More recently, it has been postulated that an asthma attack can be induced through dermal exposure of isocyanates (Rattray et al. 1994; reviewed in Bello et al. 2007).

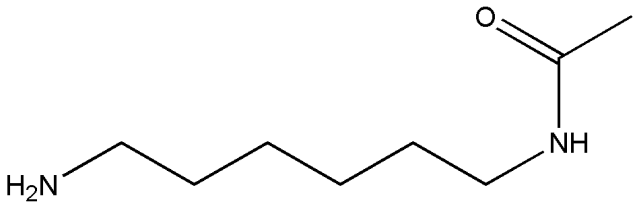
Biological Monitoring of HDI Exposure

Exposure assessment of HDI concentrations in spray painters' breathing zones and skin has been previously conducted and shown measurable levels of HDI (Maitre et al. 1996; Pronk et al. 2006; Fent et al. 2008; Fent et al. 2009a; Fent et al. 2009b). However, biological monitoring of HDI exposure in occupationally exposed workers has been limited to 1,6-diaminohexane (HDA), the hydrolysis product of HDI (Tinnerberg et al. 1995; Maitre et al. 1996; Pronk et al. 2006). The known HDI metabolites in blood and urine are aliphatic carbon-chain amines, which include HDA, monoacetyl-HDA, and diacetyl-HDA (Flack et al. 2010b). The chemical structures of these compounds are presented in Figure 2.



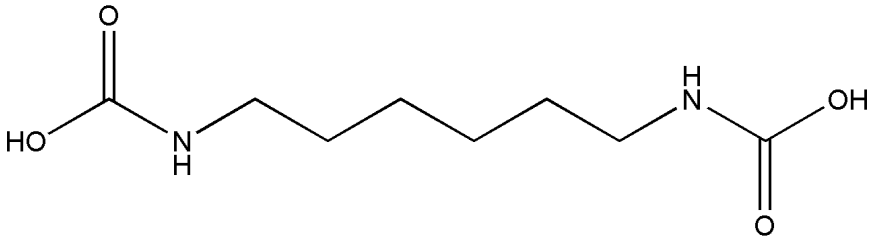
1,6-diaminohexane

MW = 116.21 g/mol



N-acetyl-1,6-diaminohexane

MW = 158.23 g/mol



N,N'-diacetyl-1,6-diaminohexane

MW = 200.28

Figure 2. The chemical structures of HDI metabolites.

Historically, HDA is the only metabolite used as a biomarker of HDI exposure in urine and blood (Tinnerberg et al. 1995; Maitre et al. 1996). Acetylated forms of HDA have not been monitored because acid hydrolysis, the most commonly used sample work-up method, converts monoacetyl-HDA and diacetyl-HDA back to HDA. HDA detected in urine resulting from HDI exposure could actually represent a mixture of the three metabolites (i.e., HDA, monoacetyl-HDA, and diacetyl-HDA). Any variability in individual metabolite formation is masked by acid hydrolysis. This leaves an important gap in characterizing the

individual metabolic differences that may be relevant in the development of hypersensitivity and asthma.

As shown in the proposed metabolic pathway for HDI exposure below in Figure 4 (Flack et al. 2010b), HDI can be hydrolysed to HDA (**5**) or it can form intermediate products that break down to HDA. HDA (**5**) can form protein adducts via CYP450/FMO (**8, 9, 3A**) or monoacetyl-HDA through *N*-acetyltransferase (*NAT*) activity (**6**) (Maitre et al. 1996; Wikman et al. 2002). Monoacetyl-HDA can form diacetyl-HDA through *NAT* (**7**) which is eliminated in urine, or oxidize via CYP450/FMO (**8, 9, 3A**) to form protein adducts. Genetic variations of the *NAT1* enzyme can result in slow or fast *N*-acetylation (Wikman et al. 2002). Fast acetylation of HDA would result in higher levels of diacetyl-HDA eliminated in urine while slow acetylation would result in higher amount of free or protein-bound monoacetyl-HDA (**3A**). The differences in acetylator phenotype can be monitored via alkaline hydrolysis through the variable levels of free diacetyl-HDA (**7**) and adducted monoacetyl-HDA compared to total HDA concentration via acid hydrolysis. Individuals with a slow acetylator phenotype would likely form more HDA protein adducts. Previous studies have shown that exposed workers with the *NAT1* slow acetylator status had a 2.5-fold risk of developing diisocyanate-induced asthma (Wikman et al. 2002) as well as having lower amounts of monoacetyl-HDA in urine (Brorson et al. 1990a).

Studies have focused on biological monitoring of HDI exposure through HDA measurements in blood and urine. However, it has been shown that isocyanurate (HDI oligomer) is the most abundant isocyanate in HDI-based paints and clear coats (Rosenberg et al. 1984; Pronk et al. 2006; Fent et al. 2009a; Fent et al. 2009b). HDI, uretdione, and biuret are present in much smaller amounts (Rosenberg et al. 1984; Pronk et al. 2006; Fent et al.

2009a; Fent et al. 2009b). Although HDI exposure has been studied, biological monitoring of isocyanurate exposure has not been previously addressed. Isotriamine, as shown in Figure 3, is the hydrolysis product of isocyanurate and could prove to be a suitable biomarker for isocyanurate exposure.

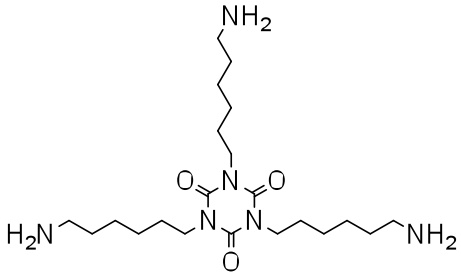


Figure 3. Chemical structure of isotriamine. MW = 426.5 amu.

Therefore, we should expand current efforts to characterize isocyanurate exposure and to determine its metabolite(s) that can be used for biomarker analysis. Liu *et al.* (2004) demonstrated that the correlation between exposure to the oligomer biuret and urinary HDA was weak. This is an indication that isocyanurate exposure may not correlate with urinary HDA levels. New biological monitoring techniques should be developed to determine levels of isocyanurate amines in the body in order to clarify the pathways for isocyanurate metabolism and to investigate the relationship between inhalation and dermal exposure and internal dose received.

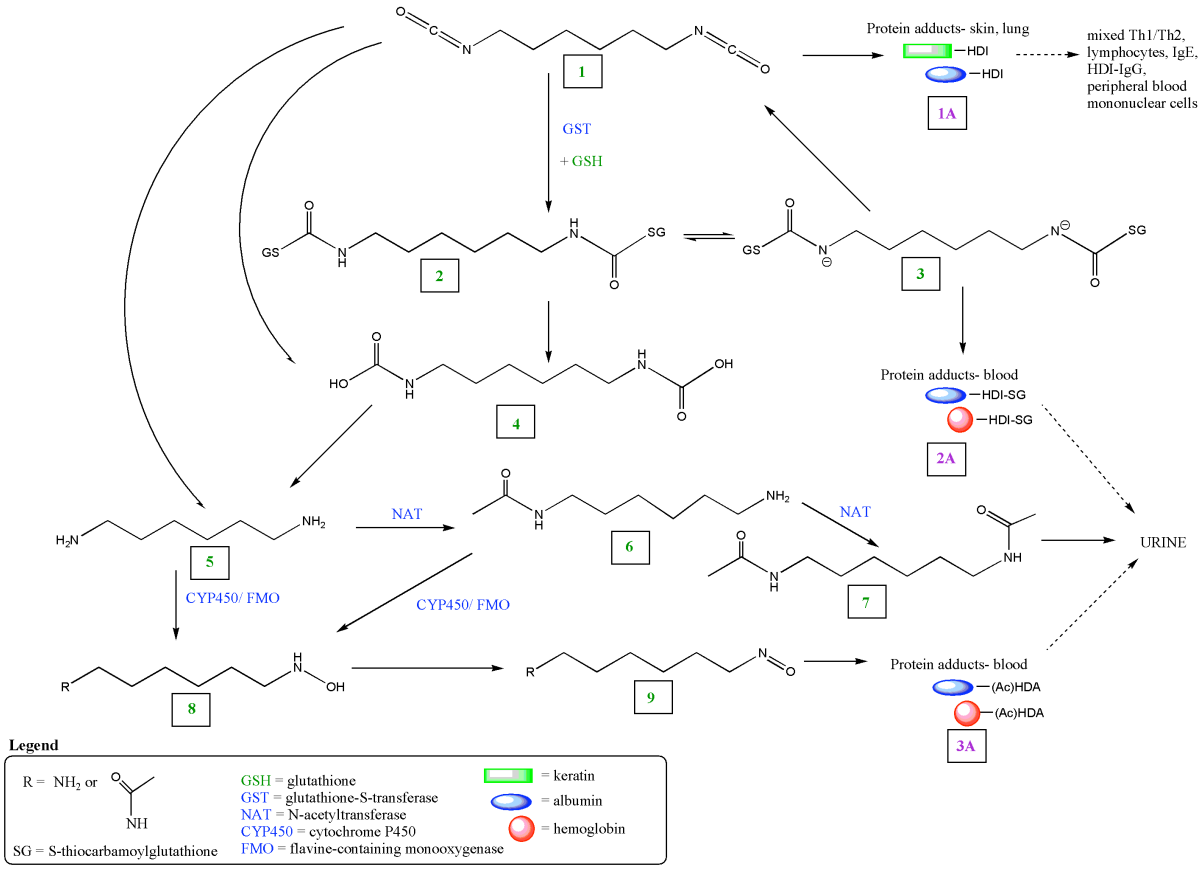


Figure 4. Proposed HDI metabolic pathways. Diagram shows the enzymatic (GST, NAT, CYP450) and non-enzymatic directed HDI metabolic pathways leading to formation of HDI-protein adducts, potential immune response, and elimination (Flack et al. 2010b).

Analysis of Isocyanate Biomarkers

In the past few decades, many analytical methods have been developed for the analysis of free amines of diisocyanates in urine and blood samples collected from exposed workers. The majority of these methods utilize gas chromatography-mass spectrometry (GC-MS). Rosenberg *et al.* (1986) developed a method for a measurement of HDA in urine that

has since been widely used and modified. Determination of urinary HDA typically involves acidic hydrolysis and derivatization with a fluorinated anhydride and analysis of the fluorinated derivatives using GC-MS (Rosenberg et al. 1986; Brorson et al. 1990a; Dalene et al. 1994; Tinnerberg et al. 1995). The procedure calls for acid hydrolysis of urine with sulfuric acid with heat for 4-16 h, followed by adjustment to basic pH with sodium hydroxide and sodium chloride. HDA is extracted from urine using an organic solvent such as toluene, ethyl acetate, diethyl ether, or dichloromethane. The organic extract is dried over anhydrous sodium sulfate. The sample is derivatized using a fluorinated anhydride such as heptafluorobutyric anhydride (HFBA) or pentafluoroproprionic anhydride (PFPA) with heat. Sample is then dried under nitrogen and reconstituted in ethyl acetate or toluene. The reconstituted sample is analyzed by GC-MS. Similar methods have been developed for the work-up and GC-MS analysis of TDA derivatives (Rosenberg et al. 2002; Sennbro et al. 2003; Marand et al. 2004) and MDA derivatives (Rosenberg et al. 2002; Sennbro et al. 2003; Sennbro et al. 2006).

Biomarkers of HDI exposure in urine and blood samples of occupationally exposed workers have been characterized in previous studies using acid hydrolysis and GC-MS analysis (Maitre et al. 1996, Flack et al. 2010ab; Gaines et al. 2010). Total HDA has been characterized in acid-hydrolyzed urine and blood plasma as a biomarker for short-term (Tinnerberg et al. 1995; Maitre et al. 1996; Gaines et al. 2010) and cumulative (Flack et al. 2010b) exposure to HDI monomer. The key constraint of acid hydrolysis of urine and plasma is that measured HDA can represent a combination of free HDA and HDA conjugated to proteins or macromolecules, free monoacetyl-HDA and monoacetyl-HDA conjugated to proteins or macromolecules, and free diacetyl-HDA. Acid hydrolysis converts acetyl groups

on monoacetyl-HDA and diacetyl-HDA back to HDA and also releases bound HDA and monoacetyl-HDA from proteins or other macromolecules. It is not possible to differentiate between the different forms of HDA present and whether they are free or bound to macromolecules. Thus, acid hydrolysis can mask the individual variability associated with HDA metabolism and protein adduct formation.

Alkaline hydrolysis has been used for MDA biomarker analysis in urine, plasma, and hemoglobin (Sepai et al. 1995a; Sepai et al. 1995b; Sennbro et al. 2003), TDA biomarkers in plasma (Sennbro et al. 2003), and more recently for HDA analysis in urine (Dalene et al. 1994; Flack et al. 2010b). This method releases acetylated amines from proteins without converting the acetyl functional groups back to the original amine form. The ability to determine levels of HDA and its acetylated forms allows for better understanding of metabolism and elimination of HDI. However, in only a few studies has alkaline hydrolysis been utilized to analyze acetylated biomarkers in urine and/or blood.

HDA has primarily been analyzed as a fluorinated derivative using GC-MS (Brorson et al. 1990a; Tinnerberg et al. 1995; Flack et al. 2010ab; Gaines et al. 2010). An advantage of GC-MS analysis is high sensitivity with a low limit of detection (LOD) for HDA-HFBA derivatives (Liu et al. 2004; Flack et al. 2010ab; Gaines et al. 2010). However, the extraction and derivatization process introduces sample loss through inefficiency of extraction and volatility of HFBA derivatives. Sakai *et al.* (2002) reported extraction efficiencies for common solvents used in studies investigating TDA isomers. The highest reported efficiency was 76.2% for dichloromethane while toluene and ethyl acetate, more commonly used solvents, had extraction efficiencies of 27.1% and 52.0%, respectively (Sakai et al. 2002). Solvent extraction and the additional steps of derivatization and drying under nitrogen allow

for significant sample loss that is not desirable for analysis of trace amounts of compounds in a biological media.

Liquid chromatography-mass spectrometry (LC-MS) has been used less frequently for biomarker analysis of isocyanate exposure compared to GC-MS analysis. LC-MS analysis has been used previously for HDA derivatives (Skarping et al. 1994a; Tinnerberg et al. 1995) and for derivatives for MDA (Skarping et al. 1994b), IPDA (Tinnerberg et al. 1995), and TDA (Marand et al. 2004). As mentioned previously, extraction and derivatization of samples can result in significant sample loss. It would be advantageous to reduce sample loss by eliminating the need to extract and derivatize a biological sample. This could be achieved by analyzing the free amine form using LC-MS. Sakai *et al.* (2002) successfully monitored TDA isomers using LC-MS with positive electrospray ionization. To date, there is no published method for analysis of HDA, monoacetyl-HDA, diacetyl-HDA, or isotriamine by LC-MS without the requirement of derivatization. Analysis of isotriamine necessitates the use of LC-MS because isotriamine derivatives are too large for GC-MS analysis (>1000 amu). Also, the elimination of extraction and derivatization steps would increase our ability to detect the metabolites in workers with low exposures to HDI and isocyanurate. An LC-MS method could be more sensitive than a GC-MS method for detection of HDI metabolites in biological media while simultaneously allowing for the detection of metabolites of isocyanurate.

I report here an LC-MS method for the determination of monoacetyl-HDA and diacetyl-HDA in urine that does not require any extraction or derivatization. This method is sensitive and specific for these compounds and expands upon previously published methods (Tinnerberg et al. 1995; Maitre et al. 1996, Flack et al. 2010ab; Gaines et al. 2010) to include

the analysis of HDA, monoacetyl-HDA, diacetyl-HDA, and isotriamine. Additionally, this method significantly reduces time for sample work-up and analysis as well as sample loss because extraction and derivatization steps have been eliminated in sample processing.

CHAPTER II

MATERIALS & METHODS

Chemicals

Ethyl acetate (>99.5%), glacial acetic acid, sodium sulfate anhydrous, and sodium hydroxide (ACS grade) were purchased from Fisher Scientific (Hampton, NH) and were used as received. Acetic anhydride (>99%), 1,6-hexanediamine (HDA, 99.5+%) and 1,7-diaminoheptane (HpDA; 98%) were purchased from Acros Organics (Geel, Belgium). Dichloromethane (HPLC grade >99.9%), hydrochloric acid (37%), ether (ACS grade), triethylamine, formic acid (98% and 85%), magnesium sulfate ($MgSO_4$), and *N,N'*-hexamethylene-bisacetamide (diacetyl-HDA; 98%) were obtained from Sigma-Aldrich (St. Louis, MO). Ammonium hydroxide was obtained from E. Merck Industries (Darmstadt, Germany). Acetonitrile (HPLC grade, >99.8%) and methanol (HPLC grade, >99.9%) were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Desmodur 3300 (HDI monomer and oligomers) was received from Bayer AG (Leverkusen, Germany).

Instrumental Analysis

Nuclear magnetic resonance and mass spectrometric analysis (NMR) was performed on a Varian Inova NMR spectrometer (Agilent Technologies, (Santa Clara, CA) operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR.

Initial LC-MS method development was performed with a Thermo Finnigan Surveyor high-performance liquid chromatograph (HPLC); coupled to a Thermo Finnigan LCQ DECA ion-trap mass spectrometer (Waltham, MA). Reversed phase separations were carried out using a Hypersil GOLD aQ^{TM} 3 µm, 2.1 x 150 mm column (Thermo Scientific, Waltham, MA). Samples (15 µL) were injected and eluted at a flow rate of 250 µL/min using the gradient program shown below (Table 1), where solvent A is 0.1% formic acid in DI-H_2O and solvent B is 50:50 acetonitrile:methanol.

Table 1. Gradient program for the HPLC ion-trap method.

Time (min)	% A	% B	Flow Rate (µL/min)
0.00	95	5	250
2.00	95	5	250
9.00	10	90	250
14.00	10	90	250
16.00	95	5	250
20.00	95	5	250

The same gradient program was used for discovery and method development for all analytes. Analytes were ionized in an electrospray ionization source operated in the positive-ion mode (ESI^+) and detected by full-scan (50-600 *m/z*) mode and selected ion monitoring (SIM) mode by monitoring the $[M+H]^+$ ions. The major ions monitored $[M+H]^+$ were *m/z*=117 (for HDA), *m/z*=131 (for HpDA), *m/z*=159 (for monoacetyl-HDA), *m/z*=173 (for monoacetyl-HpDA), *m/z*=201 (for diacetyl-HDA), *m/z*=215 (for diacetyl-HpDA), and *m/z*=427 (for isotriamine).

LC-MS analysis for calibration curves was performed on a Thermo Scientific Accela ultra-performance liquid chromatography (UPLC); coupled to a Thermo Finnigan Quantum triple quadrupole mass spectrometer (Waltham, MA). Reversed phase separations were carried out using a Hypersil GOLD aQ^{TM} 1.9 µm, 2.1 x 100 mm column (Thermo Scientific, Waltham, MA). Samples (25 µL) were injected and at a flow rate of 250 µL/min using the following gradient program shown below (Table 2), where solvent A is 0.1% formic acid in DI-H_2O and solvent B is 50:50 acetonitrile:methanol.

Table 2. Gradient program for the UPLC-triple quadrupole method.

Time (min)	% A	% B	Flow Rate (µL/min)
0.00	98	2	250
2.00	98	2	250
9.00	10	90	250
12.00	10	90	250
13.00	98	2	250
16.00	98	2	250

Analytes were ionized using an electrospray ionization operated in the positive-ion mode (ESI^+) and detected in the SRM mode by monitoring $[M+H]^+ \rightarrow [M + H - NH_3]^+$ for HDA and HpDA, $[M+H]^+ \rightarrow [M + H - \text{acetate}]^+$ for monoacetyl-HDA and monoacetyl-HpDA, $[M+H]^+ \rightarrow [M + H - \text{ketene}]^+$ for diacetyl-HDA and diacetyl-HpDA and $[M+H]^+ \rightarrow$ isocyanuric acid for isotriamine. Parameters for each analyte were optimized by loop injection. Table 3 shows the major parent ions $[M+H]^+$ and corresponding product ions with the optimal tube lens setting and collision energy for each product ion.

Table 3. Selected reaction monitoring method for the UPLC triple quadrupole. Data was obtained with direct injections into the mass spectrometer. Only the second most abundant product ions for monoacetyl-HDA and diacetyl-HDA are listed because they were used in the method for obtaining calibration curves.

	Parent Ion (*m/z*)	Product Ion (most abundant) (*m/z*)	Collision Energy (v)	Product Ion (2^{nd} most abundant) (*m/z*)	Collision Energy (v)	Tube Lens
HDA	117.10	100.20	10			121.88
HpDA	131.10	114.20	11			119.88
Monoacetyl-HDA	159.00	100.20	15	142.19	13	125.39
Monoacetyl-HpDA	173.10	114.21	16			131.39
Diacetyl-HDA	201.10	159.18	14	100.18	20	148.16
Diacetyl-HpDA	215.20	173.19	16			132.89
Isotriamine	427.50	130.02	35			129.14

Thermo Finnigan Xcalibur software generated the chromatograms and mass spectra for the samples analyzed with the ion trap and the triple quadrupole mass spectrometers. Peaks were integrated using the interactive chemical information system (ICIS) application within Xcalibur and manual adjustments were performed as needed. Calibration curves were generated using Microsoft Excel as a ratio of standard peak areas to internal standard peak areas (HDA/HpDA; monoacetyl-HDA/monoacetyl-HpDA; diacetyl-HDA/diacetyl-HpDA). The standard curve for isotriamine was calculated using the peak area because we did not have an internal standard.

Preparation of Diacetyl-HpDA

HpDA (2.6 g, 20 mmol) was dissolved in ethyl acetate (50 mL), and pyridine (10 mL) added followed by acetic anhydride (6 mL) at 0°C dropwise. The mixture was brought to room temperature and stirred for 1 h. The precipitate was filtered and washed with ethyl acetate to obtain diacetyl-HpDA as a white solid.

Preparation of Monoacetyl-HDA and Monoacetyl-HpDA

HDA or HpDA (20 mmol) was dissolved in acetic acid (50 mL) and heated to 60°C to get homogenous solution. After cooling to room temperature, a solution of acetic anhydride (1 mL) in 10 mL of acetic acid was added dropwise and the mixture was stirred at ambient temperature overnight. After removing the bulk of the solvent using a rotary evaporator, the residue was dissolved in water (50 mL) and HCl (37% 2 mL) and washed with ethyl ether to remove any diacetylated side product. The aqueous layer was basified with aqueous NaOH and extracted with ether. The organic layer was dried over $MgSO_4$ and concentrated. The residue was purified by chromatography (dichloromethane/methanol/ ammonium hydroxide) to obtain the desired product as viscous liquid at room temperature.

Preparation of Isotriamine

Desmodur 3300 (1 g) was added to 6N HCl (20 mL) and the mixture was heated to reflux for 30 min. After cooling to room temperature, the reaction mixture was extracted with ethyl ether (2 x 20 mL) and the aqueous layer was concentrated on a rotary evaporator to obtain the salt as white solid. To purify the product the salt was dissolved in water and made basic with the addition of 100 mL ammonium hydroxide. Isotriamine was extracted from the

solution using dichloromethane (3 x 2.5 mL), then centrifuged and freeze/thawed for better separation. The extract was dried under nitrogen, weighed, and dissolved in water to get a 1 mg/mL solution which was stored as a stock solution at 4°C.

Development of Standard Curves in Spiked Urine

Stock solutions of commercially available HDA, HpDA, and diacetyl-HDA as well as synthesized monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HpDA, and isotriamine (each 5 mg/mL) were made by dissolving 12.5 mg of the amine in 5 mL water (2.5 mg/mL) and the solutions were acidified by the addition of 5 µL formic acid (0.1%). The stock solutions were then diluted to 100 µg/mL by adding 80 µL of stock solution to 1.92 mL water. Solutions were diluted further to 10 µg/mL by adding 100 µL of 100 µg/mL solution of each standard to 1.9 mL water. The standards HDA, monoacetyl-HDA, diacetyl-HDA, and isotriamine were diluted to 1 µg/mL by adding 100 µL of 10 µg/mL solution to 1.9 mL water. The internal standards HpDA, monoacetyl-HpDA, and diacetyl-HpDA were diluted to 500 µg/L by adding 50 µL of 10 µg/mL solution of each internal standard to 1.9 mL water. These 500 µg/L internal standard solutions were further diluted to 125 µg/L by adding 500 µL of 500 µg/L solution of each internal standard to 1.5 mL water.

The spiked urine work-up procedure was modified from the published methodfor the analysis of HDA in urine (Sepai et al. 1995; Flack et al. 2010b). Control urine samples (1 mL) collected from two individuals with no known HDI exposure were used for the serial dilutions. Serial dilutions of spiked the urine samples were performed to obtain the following HDA, monoacetyl-HDA, diacetyl-HDA, and isotriamine concentrations: 41.322, 20.492, 10.144, 5.038, 2.507, 1.247, 0.621, 0.309, 0.154, 0.076, 0.038, 0.019, and 0.0094 µg/L.

Initially, 50 µL of each 1 µg/mL standard was added to 800 µL of urine. Formic acid (5 µL) was added to ensure the solution (49.5 µg/L) was acidic and then solution was vortexed. The solution (500 µL) was diluted 1:1 by adding 500 µL of the solution to 500 µL of control urine and 5 µL of formic acid. After the 500 µL was removed from the initial 49.5 µg/L solution, 50 µL of each 125 µg/L internal standard was added to obtain a concentration of 41.322 µg/L. The concentration for each internal standard was 8.26 µg/L. This dilution was performed 13 times to obtain concentrations: 41.322, 20.492, 10.144, 5.038, 2.507, 1.247, 0.621, 0.309, 0.154, 0.076, 0.038, 0.019, and 0.0094 µg/L for HDA, monoacetyl-HDA, diacetyl-HDA, and isotriamine. The samples were centrifuged for 45 min at 14000 G using an Amicon centrifugal mass filter (3000 Da; Amicon, (Houston, TX). This filter collects compounds with a mass greater than 3000 Da, which removes most urinary proteins and other macromolecules. The filtrate (<3000 Da) was transferred to a vial for LC-MS analysis.

CHAPTER III

RESULTS

Chemical Synthesis

The standards monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HpDA, and isotriamine were not commercially available. They were synthesized and characterized by proton and carbon nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR). The NMR spectra of the synthesized standards monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HpDA, and isotriamine are presented in Figures 5 – 8. The ^{1}H NMR spectra confirm the purity of the synthesized compounds. LC-MS analysis was performed to obtain the mass spectra of the synthesized standards monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HpDA, and isotriamine. Figure 9 indicates a base peak of a protonated standard $[M+H]^{+}$ with a minor base peak of the sodium adduct $[M+Na]^{+}$.

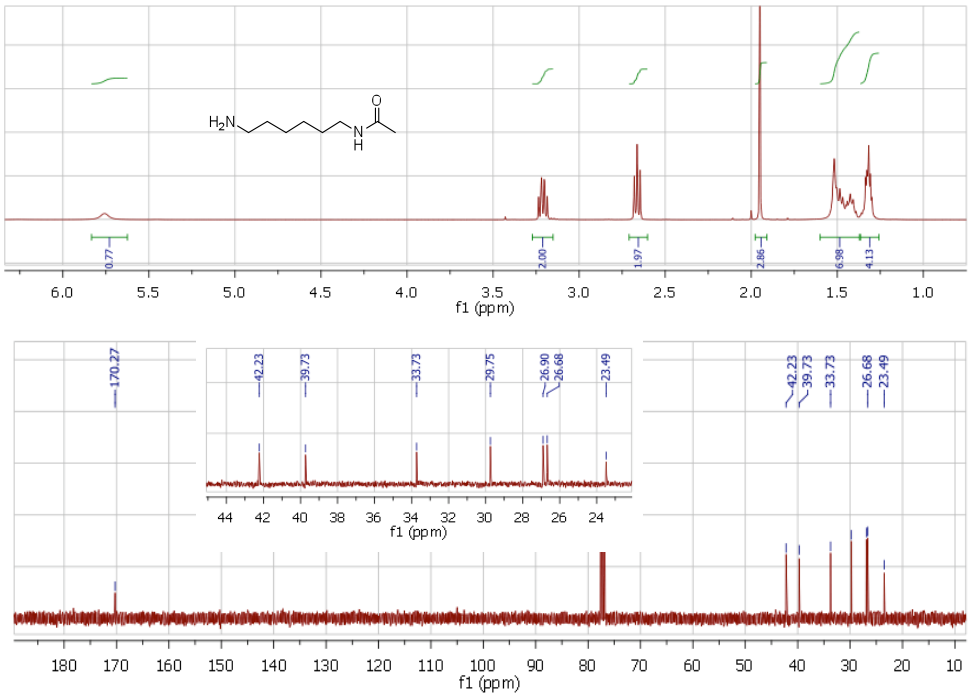


Figure 5. ^{1}H NMR and ^{13}C NMR spectra for monoacetyl-HDA.

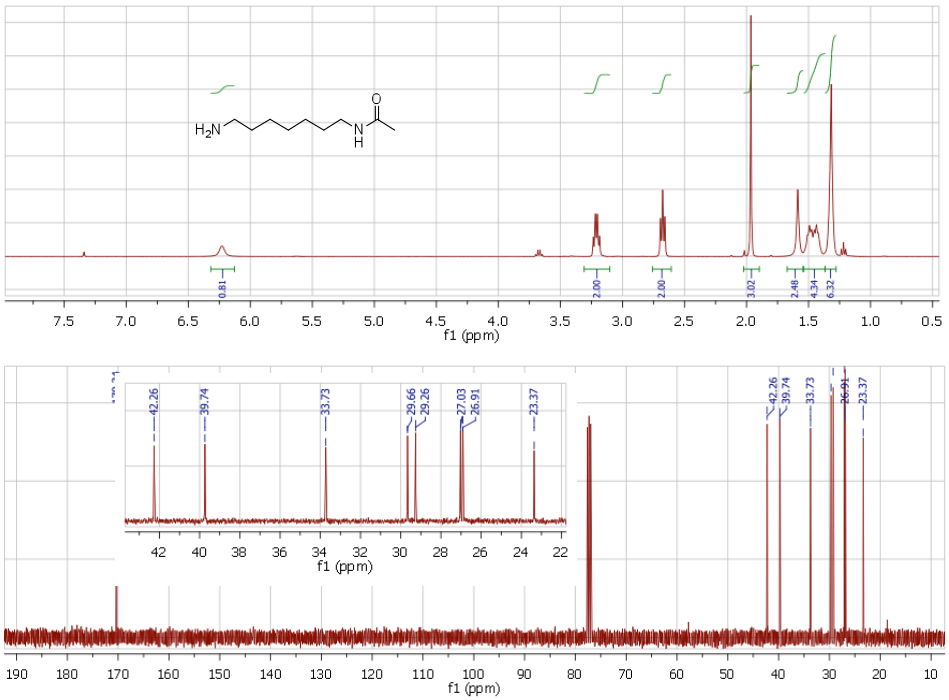


Figure 6. ^{1}H NMR and ^{13}C NMR spectra for monoacetyl-HpDA.

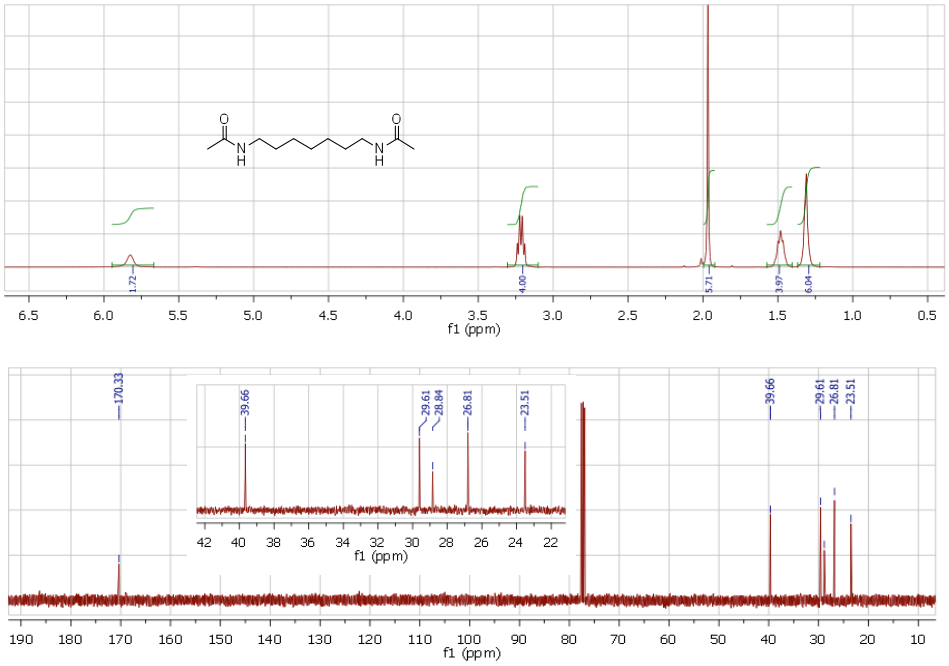


Figure 7. ^{1}H NMR and ^{13}C NMR spectra for diacetyl-HpDA.

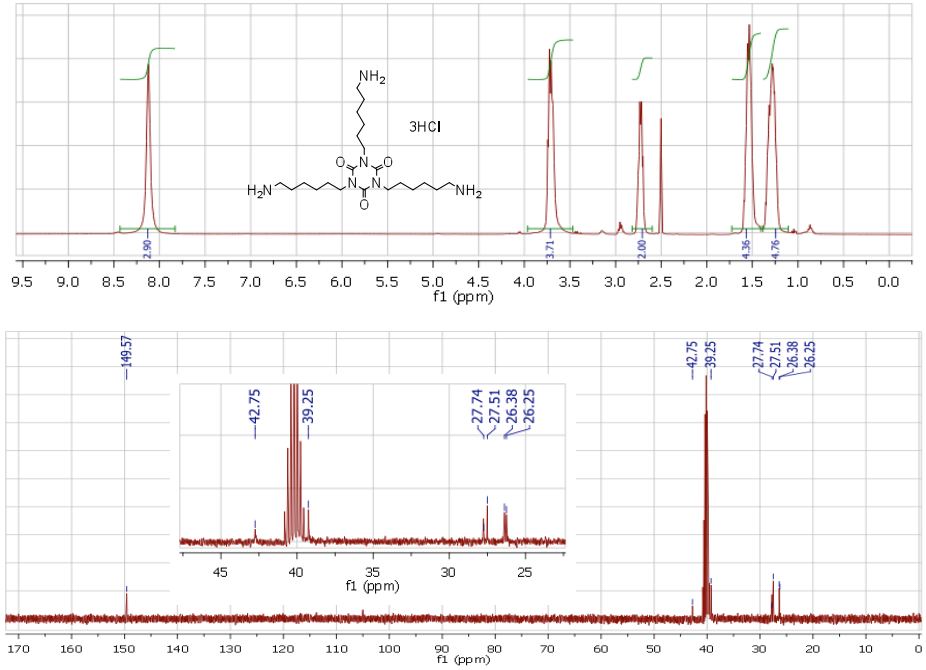


Figure 8. ^{1}H NMR and ^{13}C NMR spectra for isotriamine.

LC-MS Analysis of Standards

The four analytes (HDA, monoacetyl-HDA, diacetyl-HDA, isotriamine) and three internal standards (HpDA, monoacetyl-HpDA, diacetyl-HpDA) were mixed together in urine to obtain a concentration of 10 µg/mL for each compound in the mixture. This sample was analyzed using the procedure, described above for the HPLC-ion trap protocol, to develop the optimal solvent mixture and chromatographic parameters for the analytes HDA, monoacetyl-HDA, diacetyl-HDA, and isotriamine. Figures 9 and 10 display the mass spectrum and respective chromatogram for each compound.

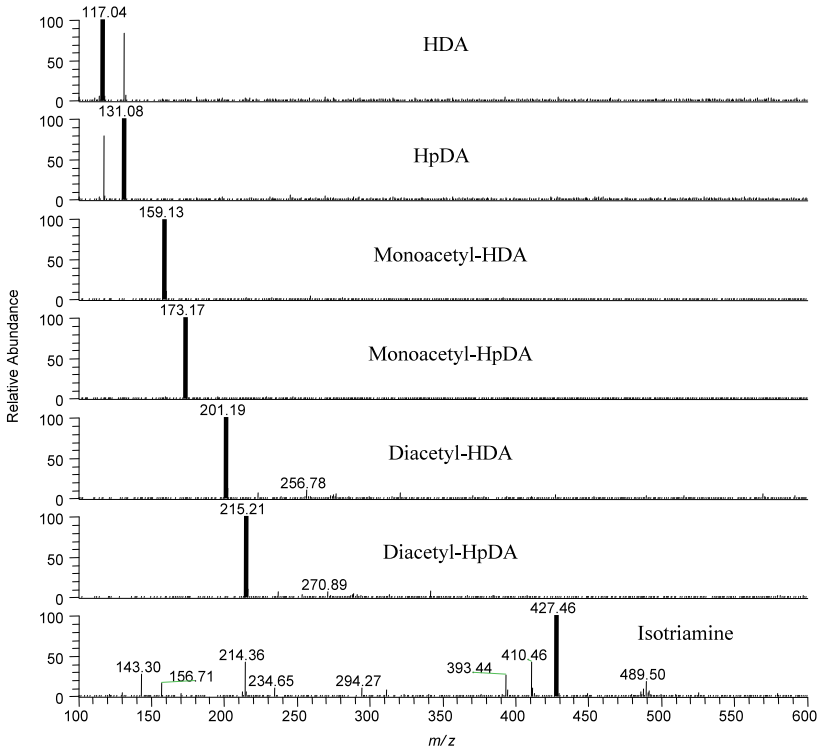


Figure 9. Mass spectra from LC-MS analysis of the target analytes and internal standards (HDA, HpDA, monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HDA, diacetyl-HpDA, isotriamine) in a urine sample. The concentration of each compound was 10 µg/mL.

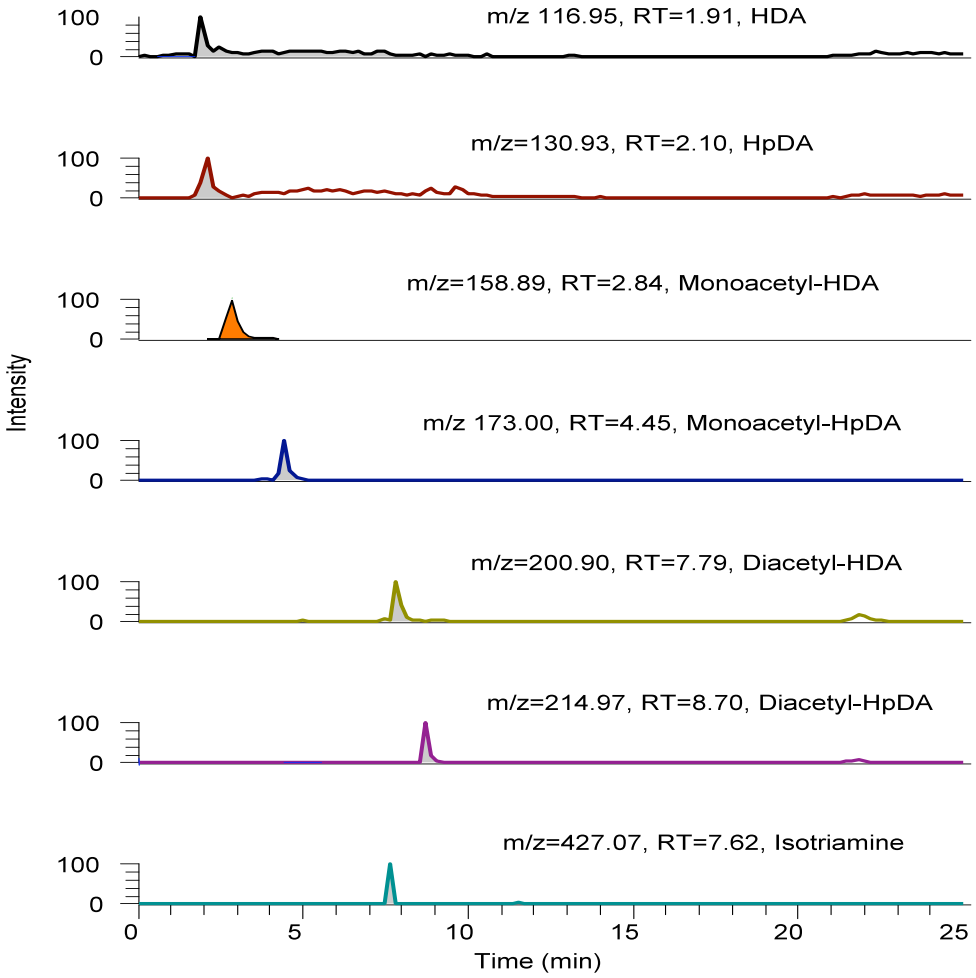


Figure 10. Chromatograms of parent ions of the target analytes and internal standards (HDA, HpDA, monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HDA, diacetyl-HpDA, isotriamine) in a urine sample using HPLC-ion trap analysis in SIM mode. The concentration of each compound was 10 µg/mL.

Standard Curves Using LCQ DECA Ion Trap Mass Spectrometer

Standard curves were generated to quantify the concentrations of HDA, monoacetyl-HDA, diacetyl-HDA, and isotriamine in urine. The standard curves were obtained by analyzing a set of serial dilutions. In the early stages of the method development, the first calibration curves were run with the series of 16 dilutions of the analytes using HPLC coupled with the LCQ DECA ion trap mass spectrometer. Points on the curve represented a ratio of the analyte peak area to the internal standard peak area (i.e., HDA/HpDA; monoacetyl-HDA/monoacetyl-HpDA; diacetyl-HDA/diacetyl-HpDA) with respect to analyte concentration. Peak area of isotriamine was used at this time because an internal standard had not yet been synthesized. Monoacetyl-HDA and diacetyl-HDA demonstrated linearity but the linearity of the standards only included the highest concentrations. Below 10 μg/L, the area ratios were scattered around the lines, which may have resulted from pipetting errors or a lack of sensitivity in the ion trap. The correlation coefficient (R^2) for monoacetyl-HDA and diacetyl-HDA ranged from 0.85-0.95 depending on how many concentration points were excluded.

HDA and isotriamine could be detected at only the 3 highest concentrations using either the available Xcalibur integration software or by manual integration. Therefore, a calibration curve could not be generated. Due to the lack of sensitivity for HDA and isotriamine and an inability to generate linear calibration curves with $R^2 > 0.99$ for monoacetyl-HDA and diacetyl-HDA by ion-trap analysis, the protocol was adapted for use with a UPLC-triple quadrupole mass spectrometer.

Standard Curves Using TSQ Quantum Triple Quadrupole Mass Spectrometer

The serial dilutions discussed in the previous section were repeated for analysis on the Thermo Finnigan Accela UPLC coupled to a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer with an electrospray ionization source operated in the positive ion mode. The triple quadrupole allowed for increased sensitivity due to the ability of the first quadrupole to filter a specific mass and the third quadrupole to detect fragments of the filtered masses. The second quadrupole is the collision cell where parent ions are fragmented. The mass spectrometer was run in selective reaction monitoring mode (SRM), to monitor fragmentation of the protonated parent ion to the most abundant product ion for each of the standards and internal standards.

Initially test samples containing high concentrations of all seven analytes (i.e., HDA, HpDA, monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HDA, diacetyl-HpDA, isotriamine) were injected in order to optimize the chromatographic separation and also to investigate sensitivity of the triple quadrupole mass spectrometer to HDA and isotriamine. The standards were added to urine (2 µg/mL each) and analyzed with LC-MS. Figure 11 displays the chromatogram of all seven analytes in urine. The acetylated compounds were easily distinguishable but, under these conditions, there was no prominent peak present for HDA and isotriamine. These test samples were repeated multiple times, but only high concentrations in individual samples of HDA and isotriamine were detected using the triple quadrupole mass spectrometer. At this point, the LC-MS method does not detect ions of HDA and isotriamine at concentrations below 10 µg/L.

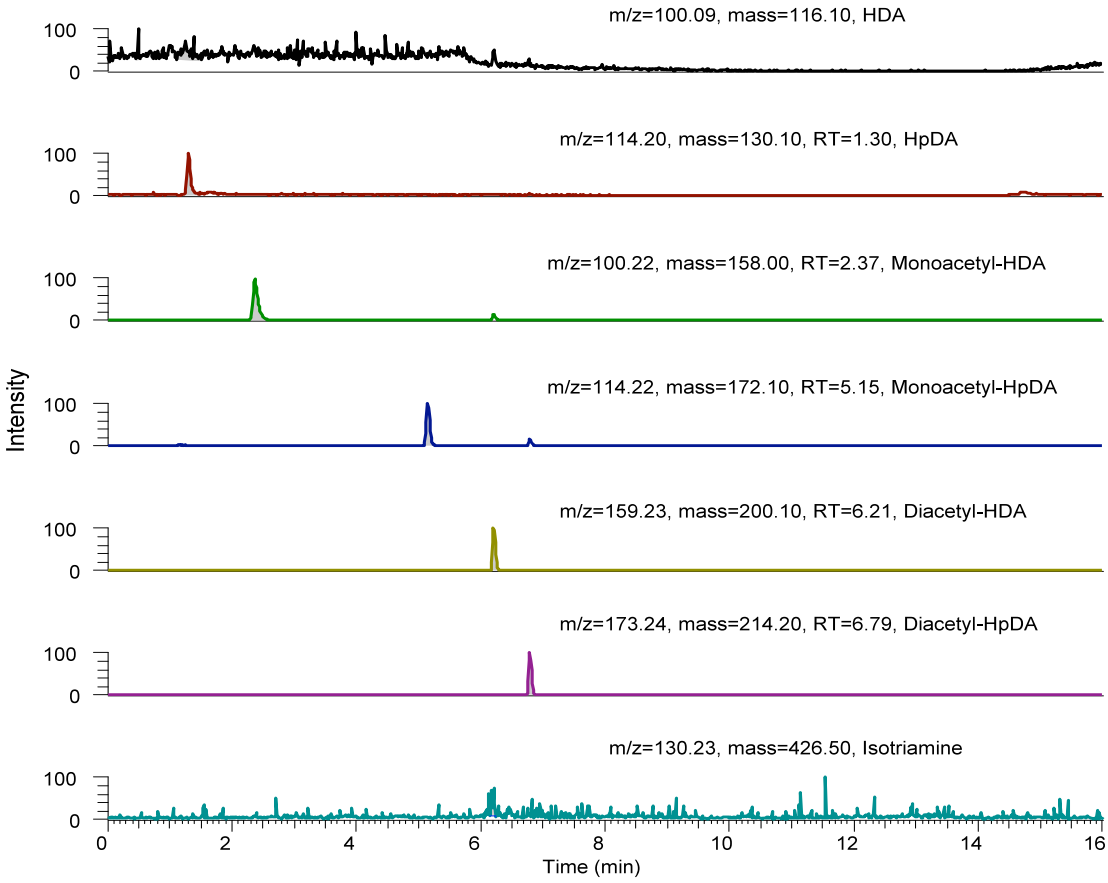


Figure 11. Chromatograms of the product ions of all the target analytes and internal standards (HDA, HpDA, monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HDA, diacetyl-HpDA, isotriamine) in a urine sample. Chromatogram was obtained with the UPLC-triple quadrupole mass spectrometer. The injection volume was 25 µL and the concentration of each sample was 2 µg/mL.

The rest of the analysis focused on creating calibration curves for monoacetyl-HDA and diacetyl-HDA in spiked urine. Figure 12 displays a chromatogram of the monoacetyl-HDA and diacetyl-HDA standards with the SRM method. Monoacetyl-HDA and diacetyl-

HDA were analyzed for the two most abundant product ions to enhance the ability to detect these compounds at low concentrations.

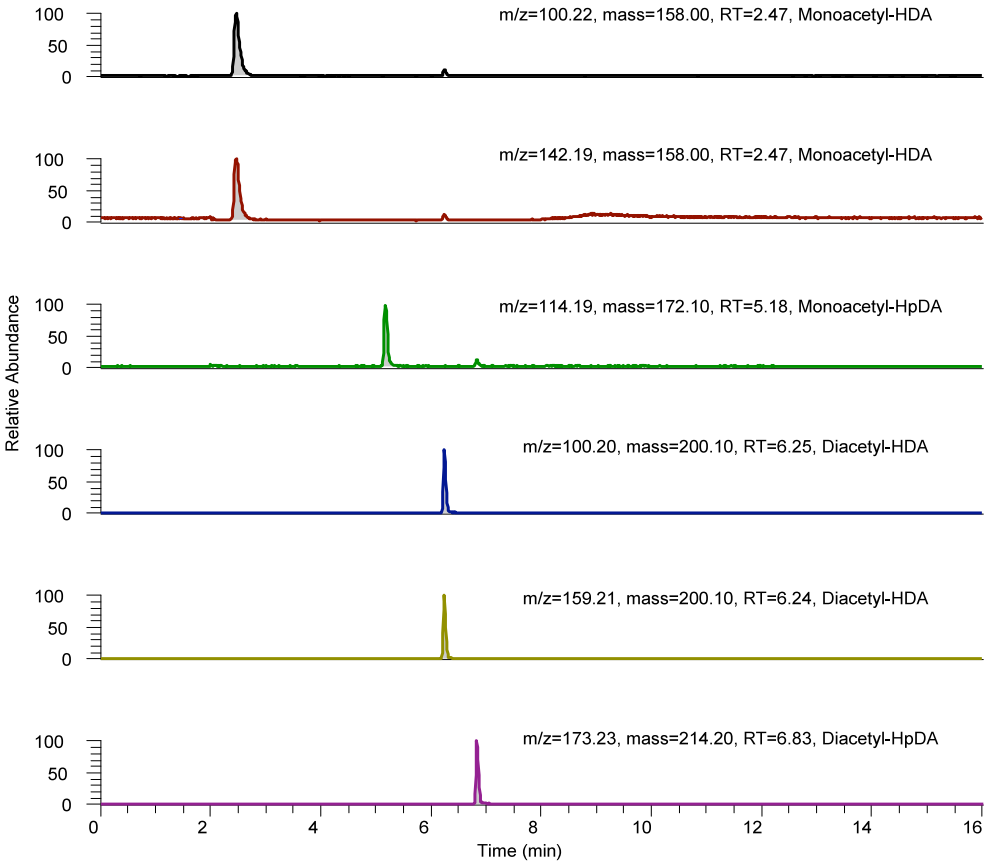


Figure 12. Chromatograms obtained in the SRM mode for monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HDA, and diacetyl-HpDA in a urine sample using the UPLC-triple quadrupole mass spectrometer. The reactions are indicated on the chromatograms. The injection volume was 25 µL. The monoacetyl-HDA and diacetyl-HDA concentrations were 25 µg/L and monoacetyl-HpDA and diacetyl-HpDA were 12.5 µg/L.

A series dilution was performed in urine with monoacetyl-HDA and diacetyl-HDA. Ten samples were used from this dilution series for the analysis (10.144, 5.039, 2.507, 1.247, 0.621, 0.309, 0.154, 0.076, 0.038, and 0.019 µg/L). All samples were analyzed with the integration program or manual integration using the QuanBrowser in Xcalibur.

Standard Curve for Monoacetyl-HDA

The calibration curve for monoacetyl-HDA included 9 of the 10 points. The calibration point corresponding to concentration of 0.038 µg/L was excluded because the percent difference of the calculated amount using the linear regression curve exceeded 10%. The correlation coefficient for the linear regression of the 9 concentrations was $R^2 = 0.9998$. The lowest concentration included in the calibration curve was 0.019 µg/L, which had a signal-to-noise ratio of 7.99. Therefore, the limit of quantitation (LOQ) for this method was 0.02 µg/L (0.13 fmol/µL; 3.15 fmol per 25 µL injection). Since no points below 0.019 µg/L were analyzed, the limit of detection (LOD) for this method was estimated to be half of the LOQ, i.e., 0.01 µg/L (0.06 fmol/µL; 1.6 fmol per 25 µL injection). The results for monoacetyl-HDA analysis of all 10 samples are presented in Table 4. The calibration curve for monoacetyl-HDA is presented in Figure 13.

Table 4. Results for monoacetyl-HDA analysis.

Expected Amount (µg/L)	Calculated Amount (µg/L)	% Difference	Area	IS Area	S/N	Excluded Points	Area Ratio
0.019	0.020	4.93	132223.19	1495026.97	7.99		0.088
0.038	0.232	511.51	152789.17	1384955.52	12.84	excluded	0.110
0.076	0.073	-3.90	121064.24	1288523.32	5.31		0.094
0.154	0.145	-5.42	134902.30	1331098.14	12.17		0.101
0.309	0.319	3.48	145539.02	1220242.30	8.26		0.119
0.620	0.647	4.30	186871.24	1221441.87	7.71		0.153
1.247	1.215	-2.56	251386.66	1188896.55	10.44		0.211
2.507	2.585	3.10	394277.87	1118995.36	11.37		0.352
5.039	4.920	-2.36	680037.50	1147428.15	31.64		0.593
10.144	10.183	0.38	1370738.34	1208526.71	51.02		1.134

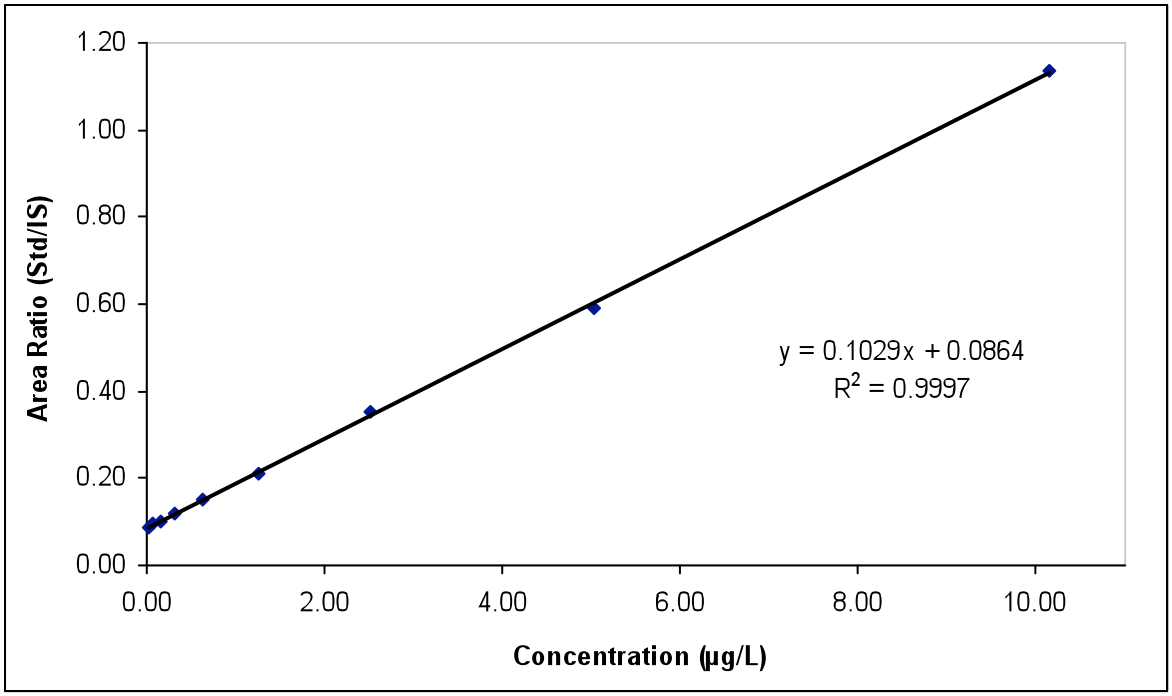


Figure 13. Calibration curve for monoacetyl-HDA with monoacetyl-HpDA as the internal standard. Nine standard concentrations with a range of 0.019-10.144 µg/L were used for this curve.

Standard Curve for Diacetyl-HDA

The calibration curve for diacetyl-HDA included 6 of the 10 points, excluded were the standard concentrations of 0.019, 0.038, 0.076, and 1.247 µg/L. The points 0.019 and 0.038 µg/L were excluded because the S/N ratio was below 3. The points 0.076 and 1.4247 µg/L were excluded because the percent difference of the calculated amount using the linear regression curve exceeded 10%. The correlation coefficient for the linear regression was R^2 = 0.9997. The lowest concentration included in the calibration curve was 0.154 µg/L, which had a signal-to-noise ratio of 3.10. Therefore the LOD for this method was 0.16 µg/L (0.80 fmol/µL; 19.89 fmol per 25 µL injection). The LOQ for this method was 0.31 µg/L (1.54 fmol/µL; 38.54 fmol per 25 µL injection) with a S/N ratio of 6.30. The results for diacetyl-HDA analysis of all 10 samples are presented in Table 5. The calibration curve for diacetyl-HDA is presented in Figure 14. The LOD and LOQ for monoacetyl-HDA and diacetyl-HDA are presented in Table 6.

Table 5. Results for diacetyl-HDA analysis.

Expected Amount (µg/L)	Calculated Amount (µg/L)	% Difference	Area	IS Area	S/N	Excluded Points	Area Ratio
0.019	0.044	130.89	14021.79	2448703.66	1.15	excluded	0.006
0.038	0.048	27.38	13750.47	2230583.24	1.42	excluded	0.006
0.076	0.107	40.26	24328.49	2100644.04	4.32	excluded	0.012
0.154	0.156	1.67	36182.67	2247790.01	3.10		0.016
0.309	0.333	7.80	63865.93	1972161.49	6.30		0.032
0.620	0.629	1.37	123367.72	2066700.33	10.82		0.060
1.247	2.803	124.74	497325.37	1911802.42	39.13	excluded	0.260
2.507	2.405	-4.08	431460.40	1931340.93	30.48		0.223
5.039	5.126	1.73	913473.87	1925919.85	110.75		0.474
10.144	10.129	0.15	1760267.69	1881457.53	167.48		0.936

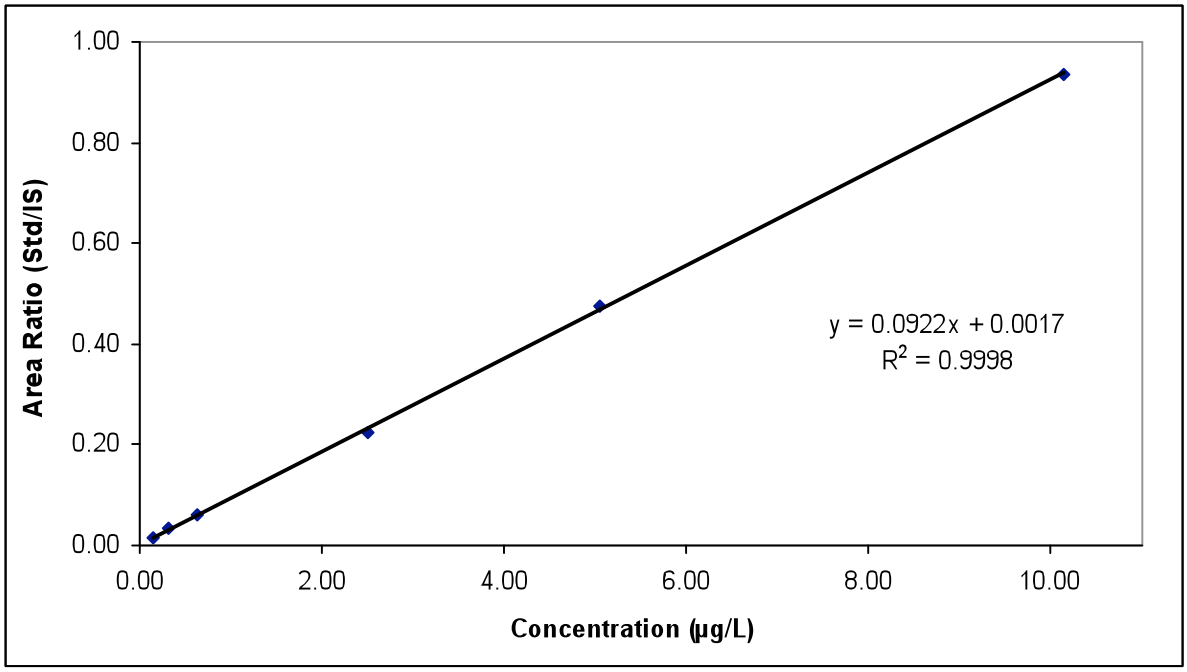


Figure 14. Calibration curve for diacetyl-HDA with diacetyl-HpDA as the internal standard. Six concentrations with a range of 0.154-10.144 µg/L were used for this curve.

Table 6. LOD and LOQ for monoacetyl-HDA and diacetyl-HDA.

	Monoacetyl- HDA		Diacetyl-HDA	
	LOD	LOQ	LOD	LOQ
µg/L	0.01	0.02	0.16	0.31
fmol/µL	0.06	0.13	0.80	1.54
fmol per 25 µL injection	1.57	3.15	19.89	38.54

CHAPTER IV

DISCUSSION

In previous scientific studies (Tinnerberg et al. 1995; Maitre et al. 1996; Flack et al. 2010ab; Gaines et al. 2010), HDA, after derivatization, had been detected in hydrolysed urine and plasma samples collected from occupationally exposed workers using GC-MS or LC-MS methods. Here, we report a development of an LC-MS method to detect HDI monomer metabolites HDA, monoacetyl-HDA, and diacetyl-HDA, as well as isotriamine, a hydrolysis product of HDI oligomer isocyanurate. The method is distinguished by the direct determination of HDI monomer amines and isocyanurate amine without derivatization.

LC-MS analysis was performed with positive electrospray ionization because the amine and acetyl functional groups present on HDI metabolites ionize efficiently. Early efforts included the use of several test columns with poor resolution and separation of the analytes. The analytes with the lowest masses (HDA, HpDA, monoacetyl-HDA, monoacetyl-HpDA) eluted too quickly and, thus, satisfactory separation and detection were not achieved. The aqueous phase had to be increased to 95+% to achieve some separation because of the high polarity and solubility of the analytes in both water and organic solvents. Separation was initially achieved with a C18 Hypurity column (50 x 4.6 mm, 3 μm) that had been previously used for analysis of HDI in air and paint samples after derivatization. Better separation was achieved with the C18 Hypersil Gold column (150 x 2.1 mm, 3 μm) at a

slower flow rate (250 µL/min). This observation led to the development and optimization of the LC-MS method described earlier in the Instrumentation section.

The use of multiple aqueous and organic phases were explored including methanol, acetonitrile:methanol, and acetonitrile for the organic phase and formic acid, ammonium formate, acetic acid, and ammonium acetate in water for the aqueous phase. The best separation, sensitivity, and peak shape was achieved with the combination of 50:50 acetonitrile:methanol as the organic phase and 0.1% formic acid in water as the aqueous phase. Initially, this method was developed using the HPLC ion trap mass spectrometer. At high concentrations all analytes (i.e., HDA, HpDA, monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HDA, diacetyl-HpDA, and isotriamine) could be detected and separated in both water and urine. However, after the analysis of the serial dilutions with all analytes in urine using the ion trap, it was clear that the lower concentrations of HDA and isotriamine could not be detected in urine. Since the ion trap mass spectrometer was not sensitive enough for the analysis of HDA and isotriamine, analysis with a UPLC triple quadrupole mass spectrometer was attempted.

Using the UPLC-triple quadrupole mass spectrometer, the method developed on the HPLC-ion trap mass spectrometer was modified to perform analysis for all analytes (i.e., HDA, HpDA, monoacetyl-HDA, monoacetyl-HpDA, diacetyl-HDA, diacetyl-HpDA, and isotriamine) using a UPLC column. The starting aqueous gradient was raised from 95% to 98% and the overall time for sample analysis was shortened from 20 to 16 min. After some samples of spiked urine were run, it was apparent that the current method or equipment was not suitable for analyzing HDA and isotriamine at concentrations lower than 10 µg/L. The analysis of HDA and isotriamine proved to be challenging with the current method.

Therefore, we focused on obtaining calibration curves for monoacetyl-HDA and diacetyl-HDA.

Our results indicate that the developed LC-MS method is sensitive and specific for determination of monoacetyl-HDA and diacetyl-HDA in urine. This method is ready to be tested for biological monitoring of workers exposed to HDI. The advantage of this new method is the reduced sample preparation time due to elimination of sample extraction and derivatization.

Advantages and Limitations

An improvement of the LC-MS method over the GC-MS method is the reduction in time of sample work-up. Our previous research of HDA in urine and blood samples of occupationally exposed workers utilizes alkaline hydrolysis and solvent extraction. The hydrolysis and extraction of a sample batch (n=40) typically takes 2-3 h. After extraction, the samples are derivatized using HFBA or related compounds. Derivation with HFBA normally requires 1 h after which samples are dried. Drying times vary by solvent. Dichloromethane, the most volatile extraction solvent used, typically takes 45 min to evaporate under nitrogen for 6 mL total volume while toluene can take up to 4 hr. The sample work-up procedure can take 7-8 h before GC-MS analysis. With the newly developed LC-MS method, the only step following alkaline hydrolysis is centrifugal mass filtration. Including hydrolysis, this process takes approximately 2 h, hence reducing sample work-up by roughly 6 h. For a high sample throughput, the new method could reduce analysis time by days or weeks.

Eliminating solvent extraction provides a benefit because the potential loss is minimized. In the previous reported GC-MS and LC-MS methods, toluene was primarily

used as the extraction solvent. As reported by Sakai *et al.* (2002), toluene has low extraction efficiency for TDA isomers, which may be analogous to the efficiency of toluene extraction of HDA. In preliminary experiments, toluene extracted <30% of HDA, monoacetyl-HDA, and diacetyl-HDA in water. Ethyl acetate and dichloromethane were also tested for their extraction efficiencies. Ethyl acetate was similar to toluene and extracted <30% of HDA, monoacetyl-HDA, and diacetyl-HDA. Dichloromethane performed much better but only extracted ~60% of monoacetyl-HDA and <40% of both HDA and diacetyl-HDA. Our new method eliminates solvent extraction, which will significantly reduce sample loss.

In addition to eliminating extraction, the derivatization step is no longer needed for analysis. Derivatization of diacetyl-HDA is more difficult because the substitution of two acetyl functional groups for the two amine groups makes it less likely to react completely with HFBA and other anhydrides. Derivatization of isotriamine with HFBA and GC-MS analysis of the product has not been reported in the published literature. The molecular weight of the isotriamine-HFBA derivative is 1014 amu, which is beyond the upper mass limit (1000 amu) of most GC-MS equipment. Preliminary tests for analysis of isotriamine-HFBA using GC-MS were unsuccessful. It seems unlikely that an isotriamine derivative can be analyzed using GC-MS and, thus, elimination of derivatization and use of LC-MS may prove to be the method of choice.

Another advantage of the LC-MS method is the reduction in chromatographic separation time. The GC-MS methods previously described (Rosenberg et al. 1986; Brorson et al. 1990a; Dalene et al. 1994; Tinnerberg et al. 1995; Creely et al. 2006; Pronk et al. 2006; Flack et al. 2010a; Gaines et al. 2010) require 30-40 min for analysis of each sample. With our LC-MS method, all desired analytes eluted before 7 min. A full analytic cycle for one

sample with this method was 16 min. Thus, this new LC-MS method is more efficient with faster sample work-up and analysis procedures.

This study provides the basis for analysis of biological samples (urine, plasma, hemoglobin) from workers exposed to HDI and the oligomer isocyanurate in automotive spray painting operations. The LC-MS method will greatly reduce sample work-up time and chromatographic separation time as well as expand the sample analysis to include HDI acetylated biomarkers. Further improvements can be made to the LC-MS method. There is greater separation in the chromatographic elution of monoacetyl-HDA and its internal standard monoacetyl-HpDA (~3 min) than diacetyl-HDA and diacetyl-HpDA (~0.5 min). In LC-MS analysis, it is desirable that the internal standard elutes as closely as possible to the analyte. An alternative internal standard for monoacetyl-HpDA could be $^{13}C_2$-labeled monoacetyl-HDA that would have the same structure and differ in mass by 2 amu. Commercially available $^{13}C_4$-labeled acetic anhydride is available and could be used to derivatize HDA.

Currently, we do not have an internal standard that can be utilized for analysis of isotriamine. For the other compounds, hepta-aliphatic chains have been used because of structure and physico-chemical properties similar to the hexane-carbon chains that characterize the analytes. The difference between the analytes and internal standards is one CH_2 group with a mass difference of 14 amu. A hepta-version of isotriamine would have a mass difference of 42 amu. This standard could be synthesized starting with commercially available compounds 7-bromoheptan-1-ol and isocyanuric acid. The procedure is outlined in Figure 15 and is based on published methods (Ghosh et al. 1994; Nilsen et al. 2007; Kunishima et al. 2009).

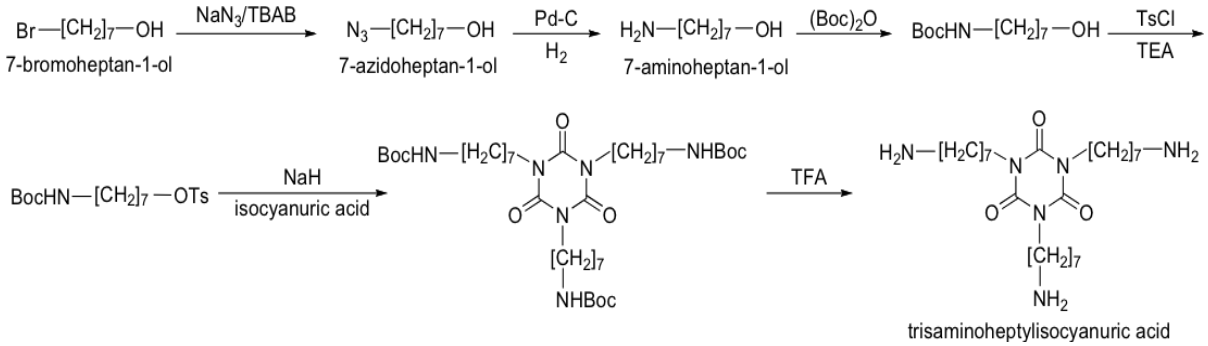


Figure 15. Scheme for synthesis of 1,3,5-tris(6-aminoheptyl)-1,3,5-triazinane-2,4,6-trione.

The method demonstrates high sensitivity for both monoacetyl-HDA and diacetyl-HDA with the limit of detection 0.01 and 0.16 µg/L, respectively. In comparison with a recently published GC-MS method for biomarker analysis from HDI monomer exposure, the LOD for monoacetyl-HDA is lower with LC-MS (0.01 µg/L) than it is with the GC-MS (0.02 µg/L) (Flack et al. 2010b). However, the LOD for diacetyl-HDA (0.16 µg/L) is approximately ten times higher using LC-MS than it is using GC-MS (0.015 µg/L) (Flack et al. 2010b). Table 7 lists the LOD and LOQ for monoacetyl-HDA and diacetyl-HDA using the LC-MS method developed in this study and using GC-MS method reported in Flack *et al.* (2010b). The LOD for diacetyl-HDA with the LC-MS can likely be improved by achieving better separation for diacetyl-HDA from an unknown urine metabolite peak, which elutes within 0.05 min under our analytical conditions. There was little separation achieved of the unknown urine metabolite peak from the diacetyl-HDA peak with the set of samples used to create the calibration curve. At lower concentrations, the peak appeared as a small shoulder on the tailing side of the urine peak and was not identified as an individual peak by the

computer software. Manual integration of the peaks was necessary and resulted in an unacceptable S/N ratio <3. One option to improve this chromatography is to modify the gradient program to hold the initial eluant composition for 1-2 min, around the retention time for diacetyl-HDA. Initial attempts at decreasing the rate of the gradient were promising. At the 5.5-min mark the program was held isocratic for 2 min and the percent of organic and aqueous phases was modified slightly each time. Figure 16 displays the chromatographic separation of the urine metabolite peak and diacetyl-HDA with the current method. Figure 17 displays the improved chromatographic separation of the urine metabolite peak and diacetyl-HDA peak with the gradient held isocratic for 2 min.

Table 7. Comparison of LOD and LOQ (µg/L) between the LC-MS method developed in this study[a] and the GC-MS method reported in Flack *et al.* (2010b)[b]. Flack *et al.* (2010b) used derivatization after base hydrolysis or no treatment.

	LOD[a]	LOQ[a]	LOD[b]	LOQ[b]	LOD[b]	LOQ[b]
			Base Hydrolysis		No Treatment	
Monoacetyl-HDA	0.01	0.02	0.022	0.080	0.020	0.070
Diacetyl-HDA	0.16	0.31	0.050	0.20	0.015	0.050

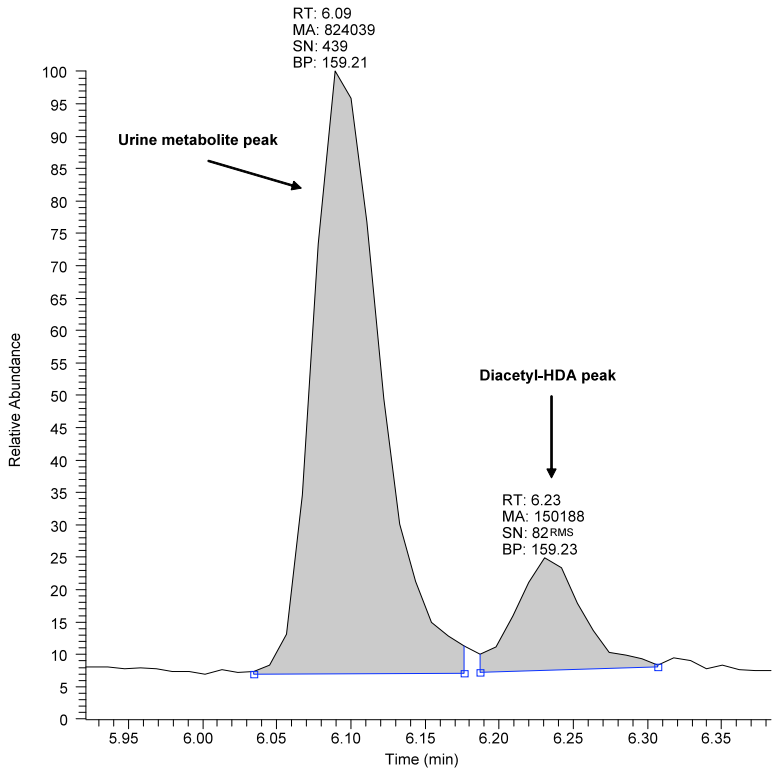


Figure 16. Chromatogram of diacetyl-HDA with the current LC-MS method. The gradient was increased from 2% organic to 90% organic from 2-9 min. Separation of the two peaks is approximately 0.04 min.

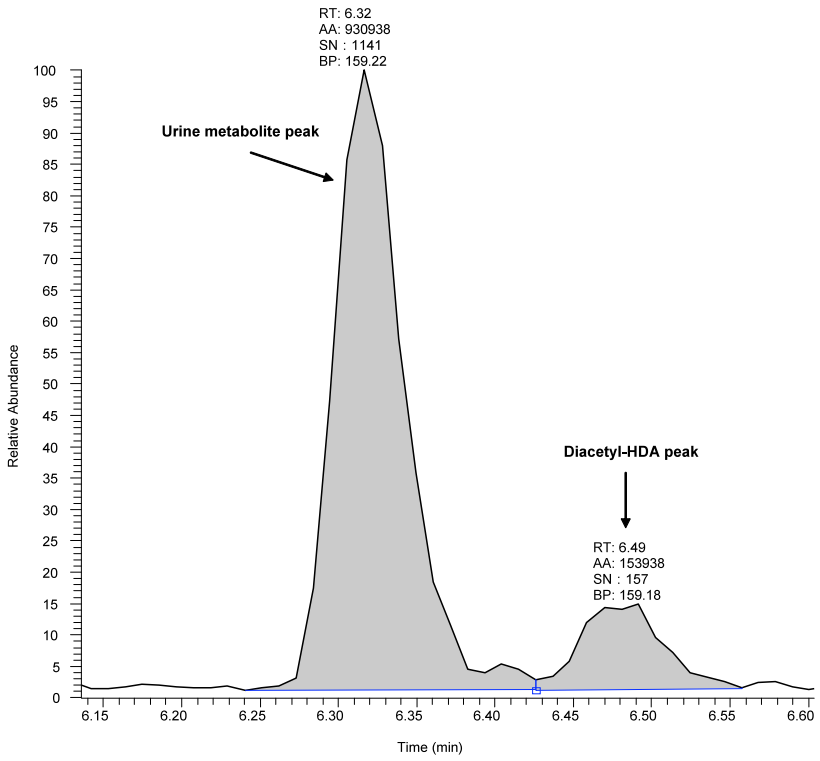


Figure 17. Chromatogram of diacetyl-HDA with an isocratic gradient. The gradient was held isocratic at 60% aqueous, 40% organic for 2 min (5.5-7.5 min range). Separation of the two peaks by approximately 0.08 min was achieved using this modification of the LC-MS method gradient.

Another limitation of this method is that it is only specific for monoacetyl-HDA and diacetyl-HDA. The goal of developing this method is to have fast, efficient means of analyzing all four desired compounds (i.e., HDA, monoacetyl-HDA, diacetyl-HDA, and isotriamine) in a single analytical run., It is particularly important to develop a method for analyzing isotriamine since isocyanurate is the most abundant isocyanate in the automotive spray painting environment and no method for analysis of isocyanurate metabolites has been published.

Published methods exist for analysis of HDA in urine using GC-MS (Tinnerberg et al. 1995; Maitre et al. 1996, Gaines et al. 2010). GC-MS analysis for HDA can be performed using extraction and derivatization if the LC-MS analysis of HDA cannot be developed. However, recent trials using a triple quadrupole mass spectrometer with heated electrospray ionization ($HESI^+$) have shown improved sensitivity for HDA and isotriamine. The $HESI^+$ source is held at 300°C and can be far more sensitive than ESI^+ because high heat nebulizes liquid more efficiently. Thus, more particles are transferred to the mass spectrometer giving the machine better sensitivity. This could also improve LODs for monoacetyl-HDA and diacetyl-HDA.

Extraction of the sample is a possibility if it is deemed that the urine matrix does not allow for specific analysis of HDA and isotriamine. The GC-MS methods cited in the Introduction section include a simple extraction procedure using a solvent such as toluene or dichloromethane. This extraction removes the compound from the urine and the extract can be easily concentrated drying under nitrogen. However, some extraction experiments were performed with all seven analytes in urine using dichloromethane. HDA and isotriamine were not detected. Another possibility is a solid-phase extraction (SPE), which is commonly used in drug testing for amines. SPE could be more efficient than solvent extractions in a glass vial and would effectively remove most urine metabolites. There are no published methods regarding an SPE work-up for isocyanates and isocyanate metabolites. A SPE method would also be beneficial to monoacetyl-HDA and diacetyl-HDA because it would eliminate almost all urine metabolites and reduce background and interference in the mass spectrometer. It would also extend the life of the guard column and the UPLC column by reducing the levels of water-soluble metabolites, which build up in the columns over time.

This would reduce cost and require less maintenance and cleaning of the LC and MS components. The LC-MS method developed for analysis of monoacetyl-HDA and diacetyl-HDA analytes will make it possible to analyze large quantities of urine samples more efficiently with faster sample work-up procedures and a rapid LC gradient program. More improvement is needed to include the analysis of HDA and isotriamine.

CHAPTER V

CONCLUSION

Biomonitoring of HDI exposure is important because of its widespread use in the automotive refinishing industry where exposure may cause occupational asthma and other debilitating respiratory and skin effects. The HDI hydrolysis product, HDA, has been monitored extensively in urine and blood as a biomarker for HDI exposure. Scientific studies have concentrated on biomarkers of exposure to HDI, which comprises less than 1% of isocyanates in spray paints and clear coats. Previous studies have indicated that isocyanurate comprises the largest component of worker exposure in occupational spray-painting settings. Expanding biomonitoring of HDI exposure to include metabolites of isocyanurate can increase the array of biomarkers related to overall exposure and contribute to understanding the association between exposure and susceptibility to disease. Additionally, most studies have not included the acetylated products of HDA, monoacetyl-HDA and diacetyl-HDA. These compounds could prove to be important biomarkers of HDI exposure and, are potential biomarkers of susceptibility to HDI-induced asthma. The variability in the concentration of HDI and isocyanurate biomarkers in urine and blood is an important component in defining the role individual genetic differences in metabolism.

The LC-MS method developed here is able to detect acetylated metabolites of HDI in urine. This method employs SRM monitoring specific for the target analytes monoacetyl-HDA and diacetyl-HDA using LC-MS with positive ion electrospray ionization. The work-

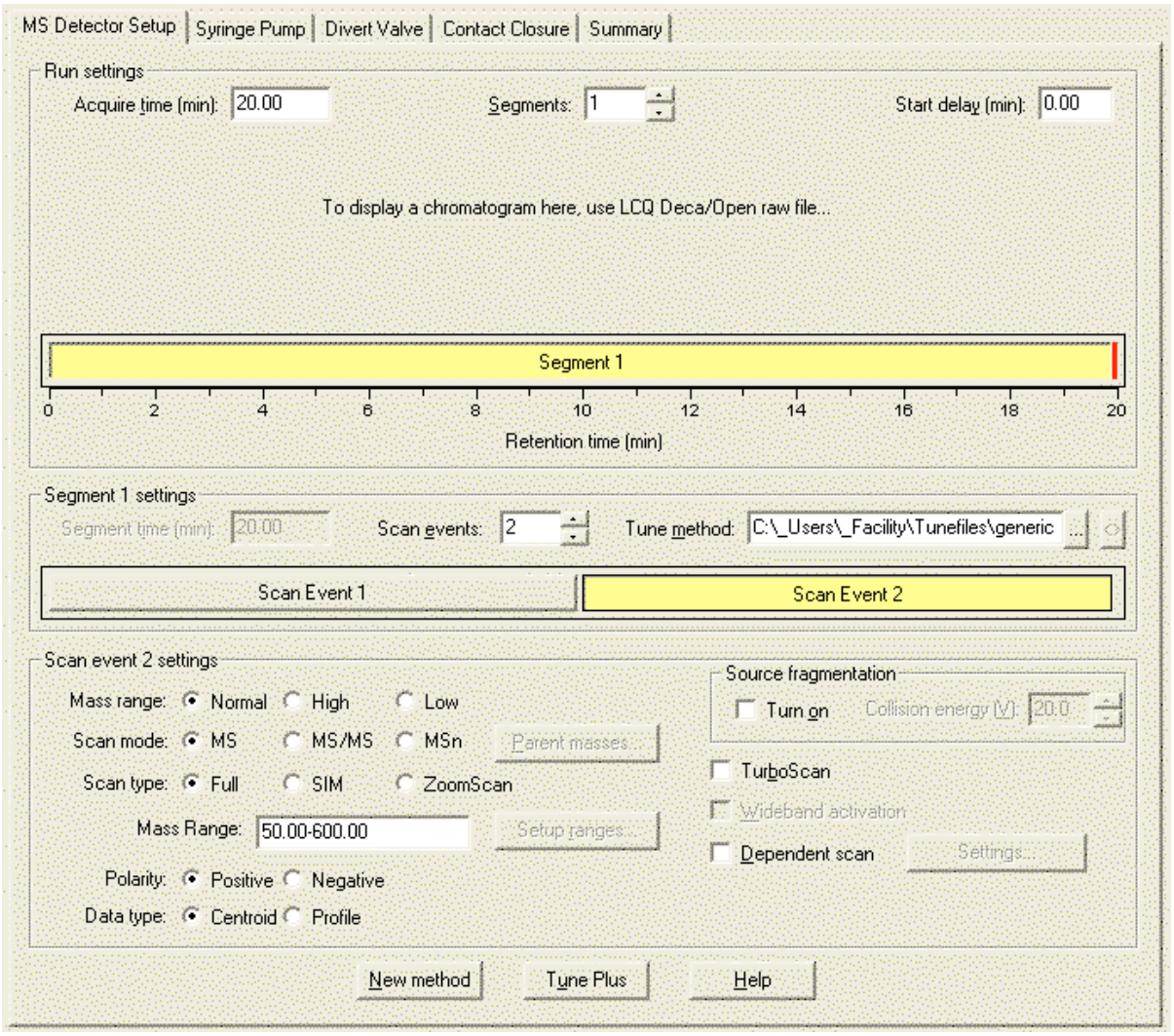
up procedure for urine is simple and results in little or no sample loss. The division of urine filtrate and protein concentrate will allow us to determine the fraction of free and conjugated analytes in future studies. Currently, our method is not specific and sensitive for analysis of HDA and isotriamine at concentrations below 10 µg/L. Recent trials have shown promising results for the analysis of HDA and isotriamine using a heated electrospray ionization source ($HESI^+$), which nebulizes the liquid particles more efficiently than ambient temperature ESI^+. Efforts will be directed toward modification of this method to include the analysis of HDA and isotriamine in urine.

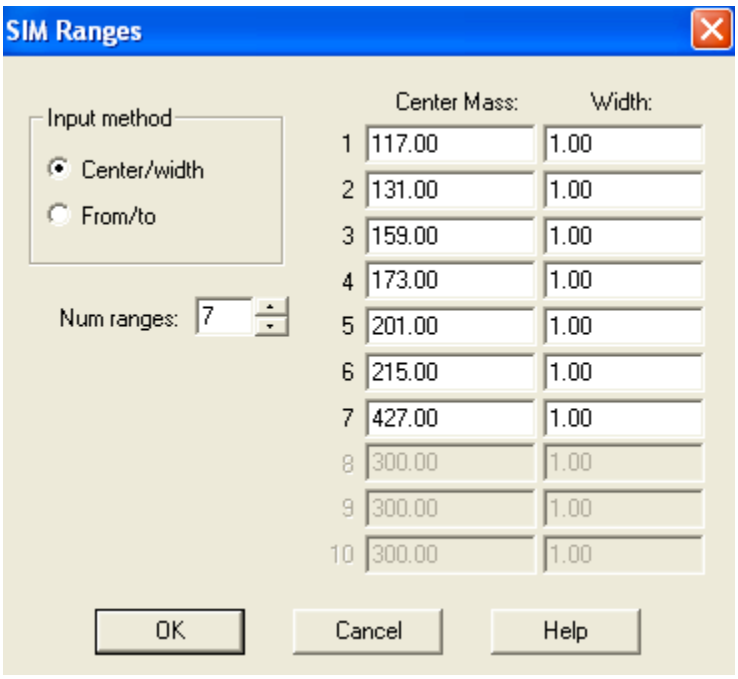
Our new method will allow researchers to quickly and efficiently determine levels of acetylated urinary biomarkers in relation to HDI monomer exposure with suitable modification may potentially be applied to determine HDA and isotriamine levels in urine. Additionally, this method can be developed further to analyze HDI and isocyanurate metabolites in blood. When validated, these biomarkers have a potential to allow for better evaluation of HDI and isocyanurate exposure and investigation of individual susceptibility to adverse health effects due to exposure to these compounds.

Future research will focus on the improvement of the current method to analyze HDA and isotriamine at low concentrations in urine and blood. Our goal will be to apply the LC-MS method for the analysis of these metabolites in urine and blood samples collected from workers occupationally exposed to HDI-based paints and clear coats. Further, after validation of these biomarkers (i.e., HDA, monoacetyl-HDA, diacetyl-HDA, and isotriamine) we intend to examine the exposure-dose relationship due to inhalation and dermal exposure to HDI and isocyanurate.

APPENDIX A

LC-MS METHOD FOR HPLC ION TRAP MASS SPECTROMETER

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Input method
Center/width
From/to
Num ranges: 7
Center Mass:
Width:
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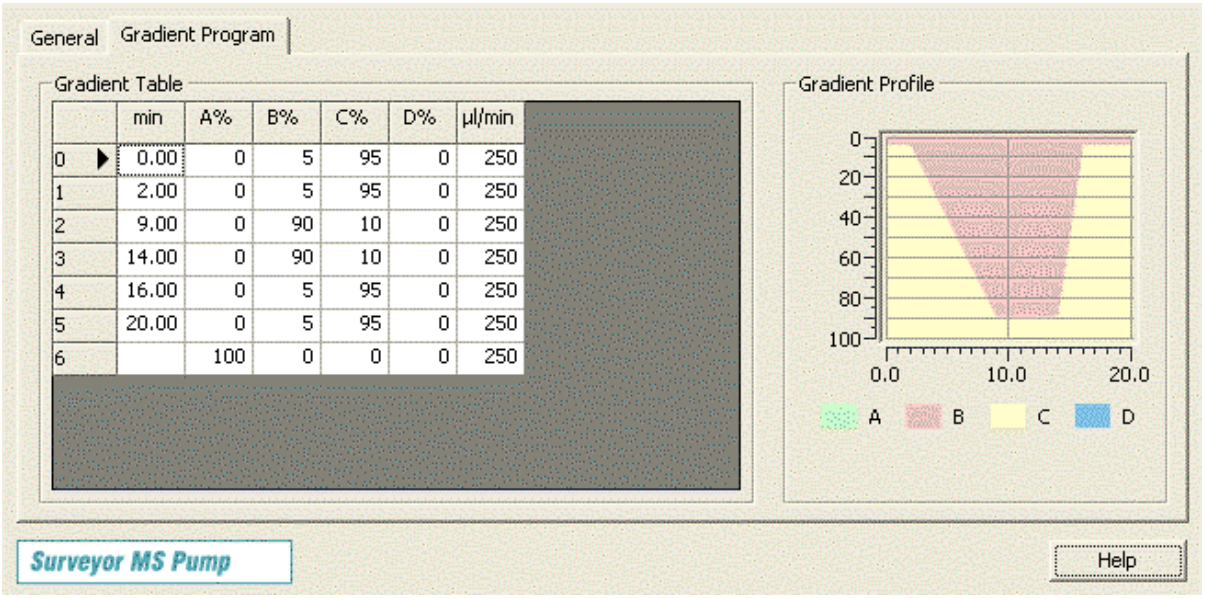
2 131.00 1.00
3 159.00 1.00
4 173.00 1.00
5 201.00 1.00
6 215.00 1.00
7 427.00 1.00
8 300.00 1.00
9 300.00 1.00
10 300.00 1.00
OK
Cancel
Help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Gradient Program
Gradient Table
min A% B% C% D% µl/min
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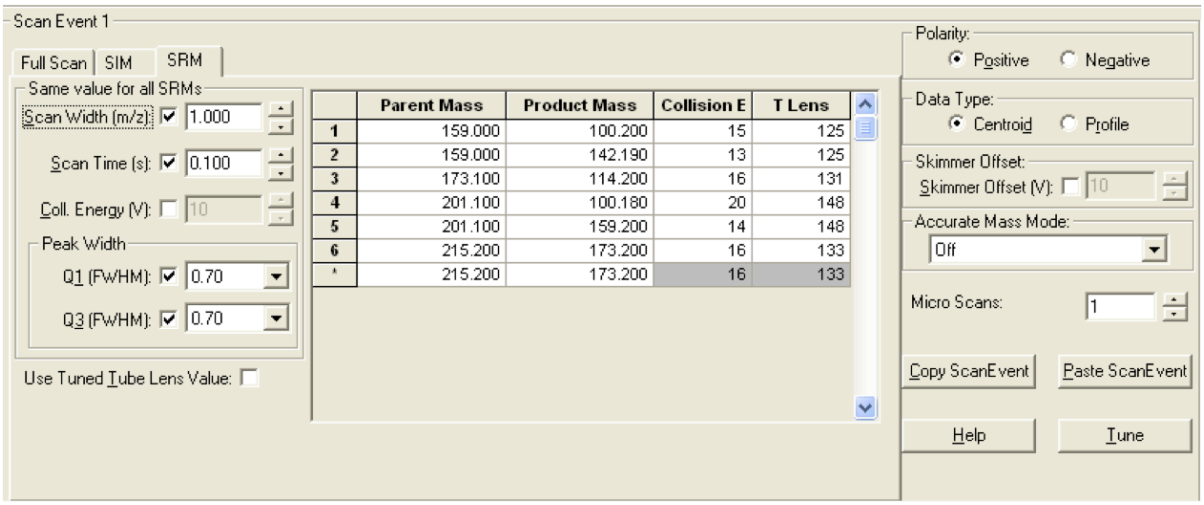
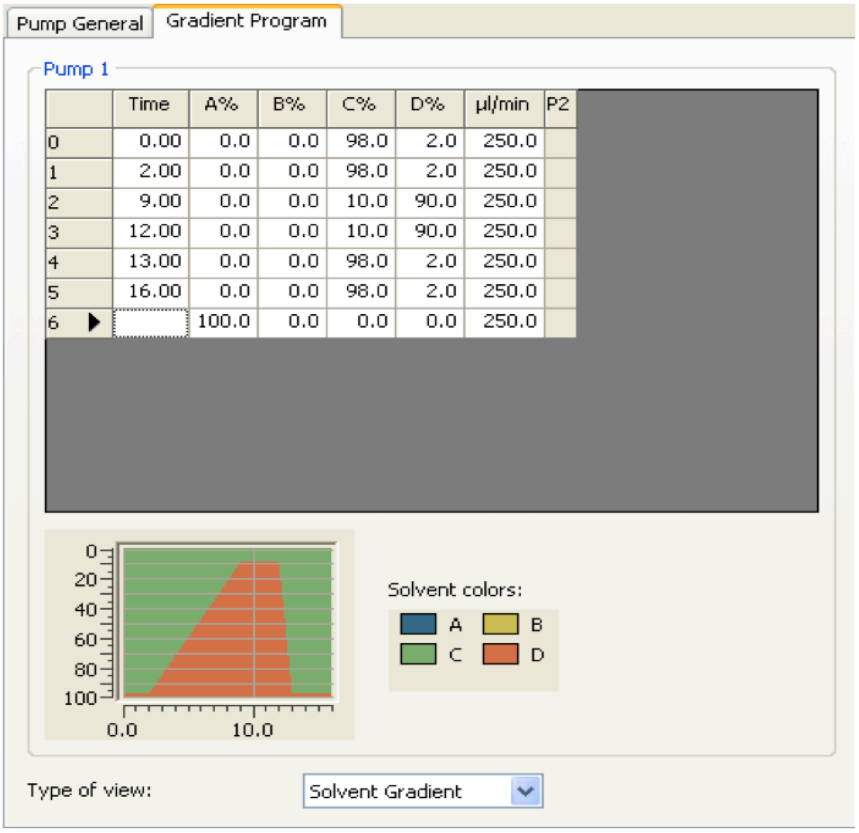
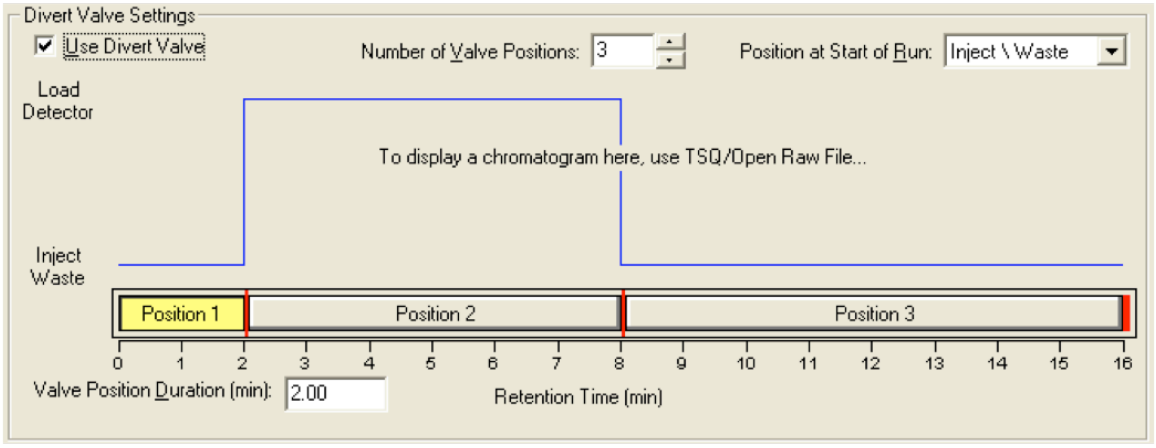
1 2.00 0 5 95 0 250
2 9.00 0 90 10 0 250
3 14.00 0 90 10 0 250
4 16.00 0 5 95 0 250
5 20.00 0 5 95 0 250
6 100 0 0 0 250
Gradient Profile
0
20
40
60
80
100
0.0
10.0
20.0
A
B
C
D
Surveyor MS Pump
Help

APPENDIX B

LC-MS METHOD FOR UPLC TRIPLE QUADRUPOLE MASS SPECTROMETER

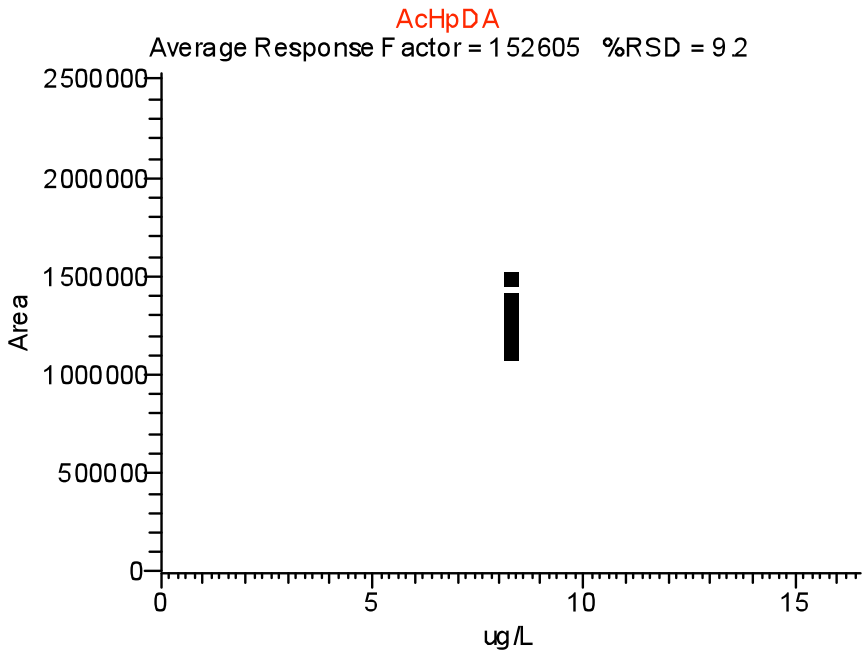


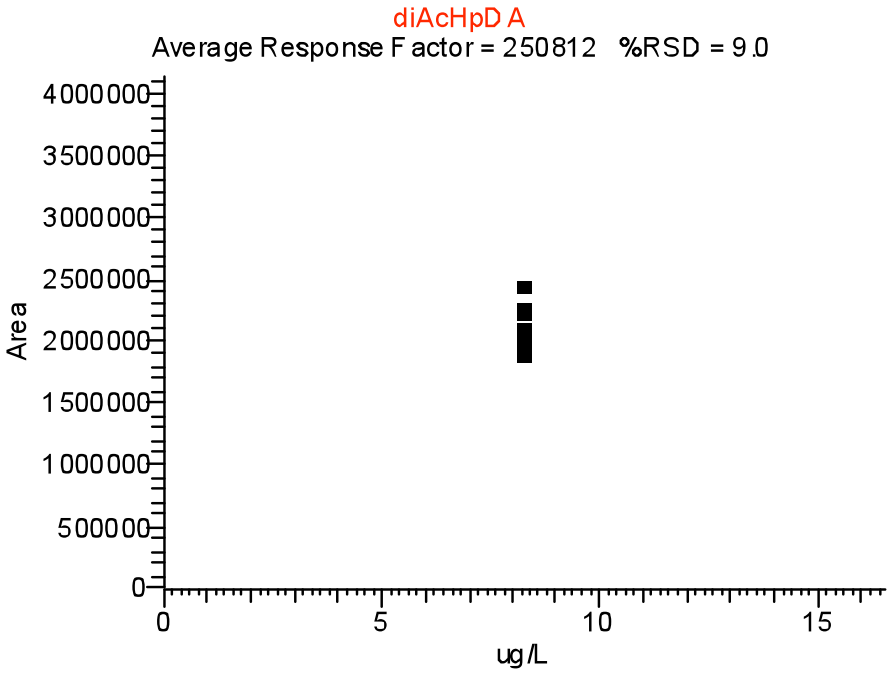



Divert Valve Settings
Use Divert Valve
Number of Valve Positions: 3
Position at Start of Run: Inject \ Waste
Load
Detector
To display a chromatogram here, use TSQ/Open Raw File...
Inject
Waste
Position 1
Position 2
Position 3
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Valve Position Duration (min): 2.00
Retention Time (min)

APPENDIX C

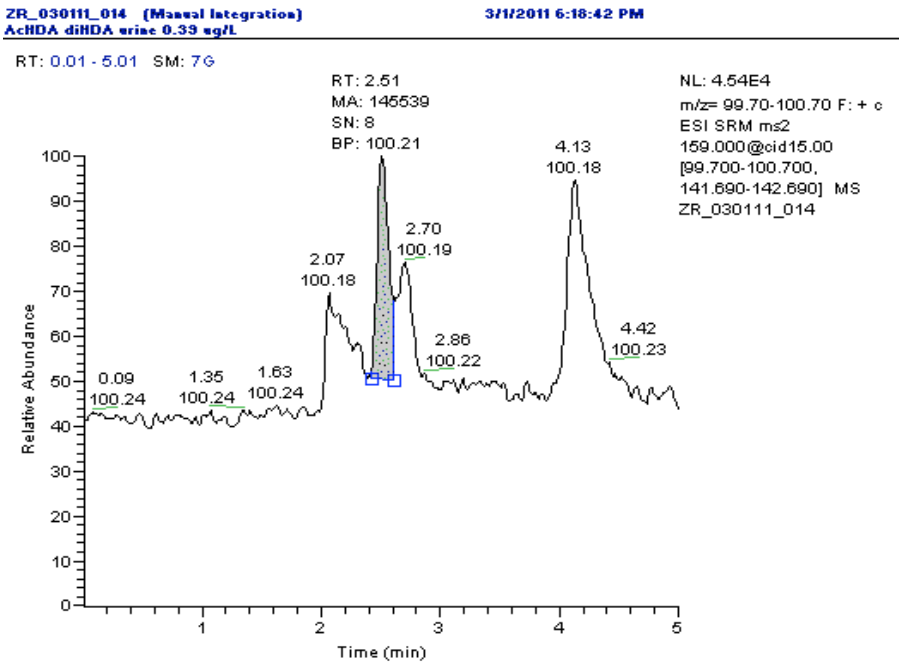
INTERNAL STANDARDS

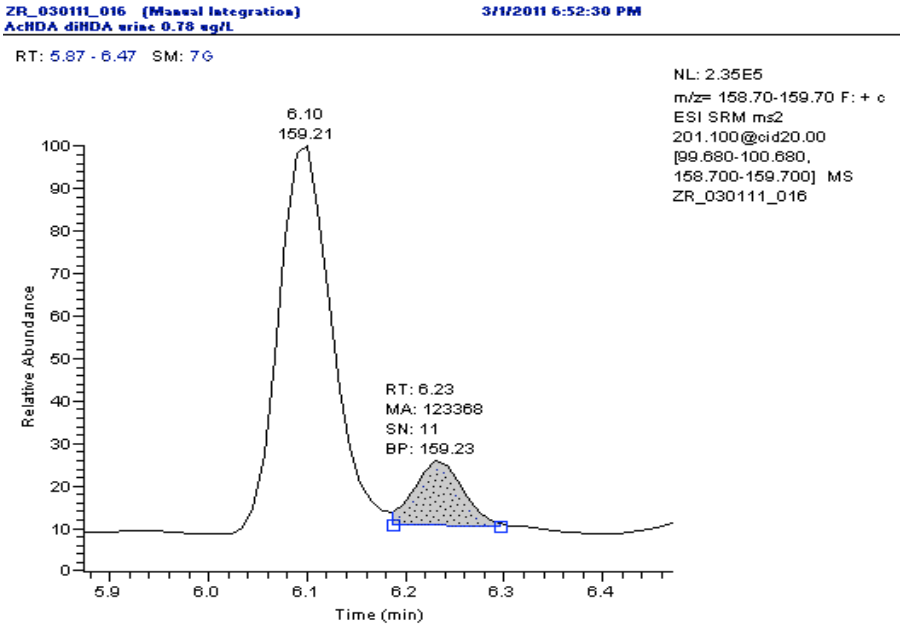




APPENDIX D

MANUAL INTEGRATION OF MONOACETYL-HDA AND DIACETYL-HDA IN THE QUANBROWSER





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