Change Log

DLS Method Code: **6107.01**
Procedure Name: **Atrazine-related Metabolites in Urine.**

Date	Changes Made	By	Reviewed By	Date Reviewed
02/18/09	Add Appendix I: Ruggedness Test	Parinya Panuwet	DBarr	2/18/09

Laboratory Procedure Manual

Analytes:	**Diaminochloro-atrazine (DACT), Desethyl-atrazine (DEA), Desisopropyl-atrazine (DIA), Hydroxy-atrazine (AZN-OH), Desethyl-atrazine mercapturate (DEAM), Atrazine mercapturate (ATZ), and Atrazine (AZN)**
Matrix:	**Urine**
Method:	**Atrazine-related Metabolites in Urine**
Method No:	**6107.01**
Revised:	

As performed by:

Organic Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

Contact:

Dr. Dana Barr
Phone: 770-488-7886
Fax: 770-488-7609
Email: DBarr@cdc.gov

Dr. Eric J. Sampson, Director
Division of Laboratory Sciences

Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Division of Laboratory Sciences
Laboratory Protocol

Analyte:	**Diaminochloro-atrazine (DACT), Desethyl-atrazine (DEA), Desisopropyl-atrazine (DIA), Hydroxy-atrazine (AZN-OH), Desethyl-atrazine mercapturate (DEAM), Atrazine mercapturate (ATZ), and Atrazine (AZN)**
Matrix:	**Urine**
Method:	**On-line Solid Phase Extraction (On-line-SPE), High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS), using Atmospheric Pressure Ionization**
Method Code:	**6107.01**
Branch:	**Organic Analytical Toxicology (OAT)**

Prepared By: Parinya Panuwet _____ Signature 11/05/2007 date

Supervisor: Dana B. Barr, Ph.D. _____ Signature 11/07 date

Branch Chief: Larry L. Needham, Ph.D. _____ Signature 11/06/07 date

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Director's Signature Block:

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_____ Signature _____ date

_____ Signature _____ date

_____ Signature _____ date

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1. Clinical Relevance and Summary of Test Principle

Atrazine (6-chloro N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine; CAS Number: 1912-24-9; AZN) is a triazine herbicide that is widely used to kill weeds, primarily on crops such as sugarcane, corn, pineapples, sorghum, and macadamia nuts. It has also been used on evergreen tree farms, evergreen forest re-growth, highway and railroad rights-of-way, and turf application. AZN has been the most used active ingredient in agriculture in the United States for nearly two decades, until it was recently replaced by glyphosate (EPA 2007). Regardless, the amount of atrazine applied annually has remained relatively constant over this time with nearly 80 million pounds applied annually.

With the high volume of its application, AZN enters into the environment, particularly in soil and water. Its primary degradation can occur via soil bacteria and abiotic processes with an environmental half-life of a few weeks to several months (ATSDR 2003 and Mandelbaum et al., 1993). AZN and its degradation products tend to migrate out of the soil into water systems including surface runoff to streams, river, and lakes, or deep ground water systems and aquifers (ATSDR 2003) creating a high potential for human exposure.

The metabolites excreted can also be complicated by the exposure scenario (Barr et al. 2007). Occupational exposures may allow for more exposure to atrazine itself while environmental exposures are likely dominated by exposure to the dealkylated environmental degradates that are still presumed to be biologically active if they retain the chlorine atom. Thus, measurement of multiple potential metabolites is necessary to accurately assess exposures to atrazine and its related degradates.

Qualitative and quantitative analytical methods have been developed to detect atrazine and its metabolites in both human and rat urine (Ikonen et al., 1988; Ademola et al., 1993; Catenacci et al., 1993; Lucas et al., 1993; Bodalbhia et al., 1998; Gilman et al., 1998; Pozzebon et al., 2003; Olsson et al., 2004; Kovivunen et al., 2006; Li et al., 2006; Ross and Filipov, 2006; Nguyen et al., 2007). However, most of these methods have been limited by the number of metabolites that could be detected, the selectivity of the analysis, the sensitivity of the analysis, and/or the complicated pretreatment steps required before detection. To fill this obvious gap in science, we developed a novel, highly sensitive, on-line solid phase extraction-isotope dilution-high performance liquid chromatography-tandem mass spectrometry (on-line SPE-HPLC-MS/MS) method to quantify atrazine and its major metabolites in human urine samples. Our method is capable of detecting seven atrazine metabolites with good sensitivity and precision. In addition, by monitoring multiple precursor-product ion pairs, its selectivity is unparalled. Furthermore, our method eliminates the need for complicated and time consuming sample preparation steps such as derivatization and pre-concentration which typically introduce more human error into the measurements. Our method also provides a cost-effective analysis allowing us to gather information from a large number of samples.

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Reagents that are necessary to perform this procedure are toxic. Avoid inhalation of or dermal exposure to these reagents. Consult the chemical hygiene plan for the pesticide laboratory if any questions about special precautions arise.

b. Radioactive Hazards

None

c. Microbiological Hazards

Although urine is generally regarded as less infectious than serum, the possibility of exposure to various microbiological hazards exists if urine contains visible blood. Take appropriate measures to avoid contact with the specimen (see "Protective equipment" below. A hepatitis B vaccination series is recommended for laboratorians who are exposed to human fluids and tissues. Observe universal precautions.

d. Mechanical Hazards

Follow standard safety practice while performing this procedure to minimize the risk for mechanical hazard. Avoid any direct contact with the electronic components of the mass spectrometer unless all power to the instrument has been shut off. Only qualified technicians perform electronic maintenance and repair.

e. Protective Equipment

Use standard personal protective equipment when performing this procedure. Wear a lab coat; safety glasses; and appropriate gloves. Use a chemical fume hood when preparing the reagents.

f. Training

Anyone performing this procedure should be trained and experienced in the use of a triple-quadrupole mass spectrometer. Although formal training is not necessary, personnel are appropriately trained by an experienced operator of the instrument or untrained personnel must work under the supervision of a trained person. All personnel who operate the instrument must also read all operation manuals.

g. Personal Hygiene

Use caution when handling any biological specimen. Be sure to use gloves and wash hands properly.

h. Disposal of Wastes

Always dispose of solvents and reagents in an appropriate container clearly marked for waste products, and temporarily store them in a flame-resistant cabinet (follow CDC's guidelines entitled Hazardous Chemical Waste Management) Use caution when handling containers, glassware, etc., that come in direct contact with the specimens. Decontaminate sample preparation surfaces with 10% bleach. Wash the glassware or dispose of it in an appropriately labeled autoclave pan.

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

A database named CCEHIP_PSTARS was developed on the CDC/NCEH (SQL server 2005). The Front-End is Microsoft Access on a DLS-PC network (Q: drive) named PSTARS.adp. This database is used to store, retrieve, and analyze data from the pesticide-residue analyses. The Statistical Analysis System (SAS) ® software package (or their equivalent) is used to maintain the data-management structure.

b. Sample Information

Sample information can be retrieved from the database (PSTARS). This includes sample-identification (ID) number, the notebook number associated with the sample preparation, the sample type, the standard number, and any other information not associated with the mass-spectral analysis. All sample information will electronically be transferred onto mass spectrometer-related-software upon sample analysis.

c. Data Maintanance

After inputting all sample and analytical data into the database, check for transcription errors and overall validity. Back up the database at least once weekly onto a computer hard drive.

4. Procedures for Collecting, Storing, and Handling Specimens; Criteria for Specimen Rejection

a. Sample Handling

Use standard urine collection cups to collect urine specimens from participants. Samples should be refrigerated as soon as possible. A minimum of 10 mL of urine should be transferred into sterile, 30 mL Qorpak® vials with screw-cap tops. Samples are subject to labeling, and are stored at -20˚C or below. Dry ice should be used for shipping. Carefully pack vials to avoid breakage during shipment. Store all samples at -20˚C or -70˚C until analysis.

b. Sample Rejection

Reject specimens with volumes less than 1 mL because they cannot be reliably processed.

5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides

Not applicable for this procedure.

6. Preparation of Reagents, Calibrants (Standards), Controls, and All Other Materials; Equipment and Instrumentation

a. Reagents and Sources

Table 1
Reagents and their Manufacturers

Reagents	Manufacturers
Atrazine (98%)	Chemservice (West Chester, PA, USA)
Atrazine mercapturate (98%)	Cambridge Isotope laboratory (Andover, MA, USA)
Diamino chloro atrazine (98%)	EQ Laboratories (Augsberg, Germany)
Desethyl atrazine (99%)	Chemservice (West Chester, PA, USA)
Desethyl atrazine mercapurate (98%)	Cambridge Isotope laboratory (Andover, MA, USA)
Desisopropyl atrazine (99%)	Chemservice (West Chester, PA, USA)
Hydroxy atrazine (97%)	EQ Laboratories (Augsberg, Germany)
(Ethylamine-D_5)-atrazine (98%)	Cambridge Isotope laboratory (Andover, MA, USA)
(Cysteine $^{13}C_3$)-atrazine mercapturate (98%)	Cambridge Isotope laboratory (Andover, MA, USA)
(Cysteine $^{13}C_3$)-diamino chloro atrazine (99%)	Cambridge Isotope laboratory (Andover, MA, USA)
(D_6)-desethyl atrazine (99%)	EQ Laboratories (Augsberg, Germany)
(Cysteine $^{13}C_3$)-desethyl atrazine mercapurate (98%)	Cambridge Isotope laboratory (Andover, MA, USA)
(D_5)-desisopropyl atrazine (99%)	EQ Laboratories (Augsberg, Germany)
(D_5)-hydroxy atrazine (98%)	EQ Laboratories Augsberg, Germany
Methanol	Tedia Company Inc.
Formic Acid	JT Baker
Deionized water	NANOpure Infinity ultrapure water system
Nitrogen Gas (99.99%)	Airgas, Inc.
Argon Gas (99.99%)	Airgas, Inc.

b. Reagent Preparation

1) Liquid Chromatography Mobile Phases

Pump 1: *Mobile Phase A* = 0.3% formic acid in methanol. Prepared by adding 6 mL of formic acid into 1994 mL of methanol and mixing well. *Mobile Phase B* = 0.3% formic acid in deionized water. Prepared by adding 6 mL of formic acid into 1994 mL of deionized water and mixing well.

Pump 2: *Mobile Phase A* = 0.1% formic acid in deionized water. Prepared by adding 2 mL of formic acid into 1998 mL of deionized water and mixing well. *Mobile Phase B* = 0.1% formic acid in methanol. Prepared by adding 2 mL of formic acid into 1998 mL of methanol and mixing well.

2) Sample Dilution Reagent

0.1 % formic acid in aqueous solution. The preparation is done by adding 500 µL of formic acid into 499.5 mL of deionized water.

c. Standards Preparation

1) Stock Solutions of Analytes

Individually weigh ≈4 mg of ATZ, AZN, DIA, DEA and DEAM in 10 mL volumetric flasks. Add a few milliliters of methanol to the flask and the swirl the flask. Once the analytes are dissolved, dilute the contents of the flask to volume with methanol and mix. Store the stock solutions in freezer safe vials below 0˚C.

DACT, AZN-OH and ATZ stock solutions are commercially available at concentrations of 100 µg/mL.

2) Stock Solutions of Labeled Isotope

Prepare individual stock solutions of isotopically labeled analogs of ATZ, AZN, DACT, and DEAM by adding 1 mg of the analyte in 4 mL methanol into separate vials. Shake to dissolve and store at < 0˚C.

AZN-OH, DIA and DEA stock solutions are commercially available at concentrations of 100 µg/mL

3) Labeled Isotope (ISTD) Spiking Solution

The isotope-labeled standard spiking solution was also prepared in methanol, giving an approximate concentration of the individual labeled compounds of 625 ng/mL. Once spiked into 1 mL of sample, the concentration yield is 12.5 ng/mL for ATZ, AZN, DIA, DEA, DEAM, and AZN-OH.

The exception is made for DACT, the concentration is required for 1,250 ng/mL (25 ng/mL in 1 mL of sample)

4) Native Spiking Standard Solutions

Nine standard spiking solutions containing AZN-OH, DACT, DIA, DEA, and DEAM are prepared by serial dilution with methanol. The initial stock solutions cover the concentration range of 0.5-200 ng/mL equivalent in 1 mL of urine (i.e., neat concentrations ranging 0.025-10 µg/mL). For AZN and ATZ, the range of 0.2-80 ng/mL equivalent in 1 mL of urine (i.e., neat concentrations ranging 0.01-4 µg/mL), were prepared.

5) Calibration Verification Materials

CLIA defines calibration materials as "a solution which has a known amount of analyte weighed in or has a value determined by repetitive testing using a reference or definitive test method." According to this definition, our quality control (QC) materials qualify as calibration verification materials.

6) Proficiency Testing Materials

Proficiency testing materials are matrix-based samples (typically spiked samples) with a known or characterized concentration. These samples may be spiked or have endogenous levels of the target analytes

d. Equipment/Supplies

1) Sartorius Ultramicro® Microbalance (Westbury, NY)
2) EDP2® pipettes (Rainin Instrument Co. Woburn, MA)
3) Presterilized filter pipette tips (Rainin)
4) Vortex Genie® vortex mixer (Scientific Industries Inc., Springfield, MA)
5) 1.5-mL amber vial (Agilent Tech., Waldbronn, Germany)
6) Strata-X 25 micron On-line cartridge (Phenomenex, Torrance, CA)
7) Gemini C6-Phenyl, 3 micron, analytical column (4.6x100 mm) (Phenomenex, Torrance, CA)
8) Gemini C6-Phenyl, 3 micron, analytical column (4.6x50 mm) (Phenomenex, Torrance, CA)

e. Instrumentation

The on-line solid phase extraction-high performance liquid chromatography (On-Line SPE-HPLC) is performed on an Agilent 1100 system (Agilent Tech., Waldbronn, Germany) consisting of two quaternary pumps, two degassers, an auto sampler with a 900-µL injection loop, and a temperature-stable column compartment. An external 10-port switching values (Agilent Tech., Waldbronn, Germany) is inserted to facilitate the column switching performance. All the HPLC modules are programmed and controlled using the ChemStation B. 01.03 software (Agilent Tech., Waldbronn, Germany). The SPE column is a polymeric Strata-X (20x2.0 mm, 25 µm particle size, 60-Å pore size, Phenomenex, Torrance, CA, USA) and the HPLC column is Gemini C6-Phenyl (100x4.6 mm, 3 µm particle size, 110-^{0}A pore size, Phenomenex, Torrance, CA, USA) for an analysis of ATZ, AZN, DEA, DIA, DEAM, and AZN-OH. A Gemini C6-Phenyl

(50x4.6 mm, 3 µm particle size, 110-^{0}A pore size, Phenomenex, Torrance, CA, USA) is used separately for an analysis of DACT.

Mass spectrometry analysis is performed on a TSQ Quantum Ultra mass spectrometer (ThermoFisher, San Jose, CA) equipped with an atmospheric pressure chemical ionization (APCI) interface to generate gas phase ions of the target analytes. The mass spectrometer was programmed and controlled using Xcalibur software (ThermoFisher, San Jose, CA). The APCI was set in the positive ion mode with the multiple reaction monitoring (MRM) setup.

7. Calibration and Calibration Verification Procedures

a. Calibration Plot

1) Construct a 9-point calibration plot (exclusion may happen) by performing a linear regression analysis of relative response ratio factor (i.e., area native/area label) versus standard concentration.

2) The lowest point on the calibration curve is above the calculated limit of detection (LOD) and the highest point is above the expected range of results.

3) Determination of the slope and intercept of this curve is by linear least squares fit using SAS® software.

4) R-squared values for the curve must be greater than 0.990. Linearity of standard curves should extend over the entire standard range. Intercepts, calculated from the least squares fit of the data, should not be significantly different from 0; if they are, identify the source of this bias.

5) Periodically or when needed, recalculate the standard curve and re-establish the standard curve to incorporate the newest data points.

b. Calibration Verification

Calibration verification for the overall test system is performed by analyzing three concentrations of calibration verification materials (which are included in each run as QC materials) in the same manner as unknown samples. The concentrations span the useable range of the method but are weighted toward the expected unknown concentrations. The overall system is considered calibrated if the calculated concentrations are within ±3 standard deviations of the characterized mean values for each of the analytes measured in the method. Calibration verification of the overall test system is performed with every analytical run. No data are reported unless the overall test system passes calibration verification. Calibration results and any significant corrective actions are documented either in the PSTARS database or other ancillary documents

c. Proficiency Testing

Proficiency testing should be performed a minimum of once every 6 months. Because no formal PT testing program exists for the target analytes of this method, an in-house program is used. This in-house program currently includes pools prepared in-house but may also include independently prepared materials whose preparation was contracted out to an external laboratory. Where applicable, NIST matrix-based certified reference materials may be included as PT materials. Five randomly selected PT materials will be analyzed in the same manner as unknown samples. These PT materials will be selected from among three different concentration ranges spanning the linear range of the method. The concentration range for each sample will be blinded to all analysts. The analytical results are evaluated by an auditor (e.g, branch statistician) who is independent of the laboratory performing the analyses. A passing score is obtained if at least four of the five samples fall within the prescribed limits established by the auditor. The auditor will notify our laboratory of its PT status (i.e. pass/fail). If a PT challenge is failed, a second attempt to demonstrate proficiency by analyzing a second set of PT samples is undertaken. If the second attempt fails, laboratory operations will cease until an appropriate corrective action is taken. After correction action is taken,

8. Operating Procedures; Calculations; Interpretation of Results

a. Analytical Runs

Each analytical run consists of 36 unknown urine samples, QCL, QCM, QCH, blank urine sample, and nine standard samples. Remove unknown samples and blank urine material from storage freezer and allow them to come to room temperature prior to preparation.

b. Sample Preparation

1) For ATZ, AZN, DEA, DIA, DEAM, and AZN-OH analysis
200 µL of urine is pipetted into 1.5 round-bottom amber vial and spiked with 20 µL of internal standard solution (ISTD). Samples are diluted with 0.1% formic acid to bring the volume to 1 mL. Samples are well mixed prior to analysis. Blank sample is also prepared using the same procedure.

2) For DACT analysis
500 µL of urine is pipetted into 1.5 round-bottom amber vial and spiked with 20 µL of internal standard solution (ISTD). Samples are diluted with 0.1% formic acid to bring the volume to 1 mL. Samples are well mixed prior to analysis. Blank sample is also prepared using the same procedure.

c. On-Line Extraction Conditions

The operational timetable of the switching program to extract the target analytes from urine is given in Tables 6 and 7.

Table 6
Column Switching Program for On-line SPE-HPLC
For ATZ, AZN, DEA, DIA, DEAM, and AZN-OH analyses

Time (min.)	Description	Pump 1	Pump 2	Valve Position	End
0.00	Sample Loading	Off	Isocratic: 100% of 0.1% formic acid (flow rate 1 mL/min)	1	To waste
1.50	Sample Cleaning	Off	Isocratic: 90% of 0.1% formic acid and 10% of 0.1% methanolic formic acid (flow rate 1mL/min)	1	To waste
3.00	HPLC Separation	Gradient: 0.3% formic acid and 0.3% methanolic formic acid	Off	2	To MS/MS
23.00	On-line SPE Regeneration	Off	Isocratic: 100% of 0.1% formic acid (flow rate 1mL/min)	2	To waste
25.00	Switch to Sample Loading Position	Off	Off	1	To waste

Table 7
Column Switching Program for On-line SPE-HPLC
For DACT analysis

Time (min.)	Description	Pump 1	Pump 2	Valve Position	End
0.00	Sample Loading	Off	Isocratic: 100% of 0.1% formic Acid (flow rate 1 mL/min)	1	To waste
1.50	Sample Cleaning	Off	Isocratic: 90% of 0.1% formic acid and 10% of 0.1% methanolic formic acid (flow rate 1mL/min)	1	To waste
3.00	HPLC Separation	Isocratic: 80% of 0.3% formic acid and 20% of 0.3% methanolic formic acid	Off	2	To MS/MS
15.00	On-line SPE Regeneration	Off	Isocratic: 100% of 0.1% formic acid (flow rate 1mL/min)	2	To waste
17.00	Switch to Sample Loading Position	Off	Off	1	To waste

d. Liquid Chromatography Conditions

The HPLC separation uses either a stepwise-gradient elution to maximize the resolution, or isocratic elution, depending on target compound analysis. HPLC pump 1 is designated for performing this task. The combination of 0.3% formic acid and 0.3% methanolic formic acid at a given time according to the procedure is shown in Tables 8 and 9.

Table 8
HPLC Separation
For ATZ, AZN, DEA, DIA, DEAM, and AZN-OH analysis

Time	Flow Rate (mL/min.)	Composition	
		0.3% Formic Acid	0.3% Methanolic Formic Acid
0.00	0	80	20
3.00	0	80	20
3.01	0.5	80	20
5.00	0.5	50	50
9.00	0.5	50	50
10.00	0.5	20	80
14.00	0.5	20	80
15.00	1.0	0	100
19.00	1.0	0	100
20.00	1.0	80	20
23.00	1.0	80	20
23.01	0	80	20
25.00	0	80	20

Table 9
HPLC Separation
For DACT analysis

Time	Flow Rate (mL/min.)	Composition	
		0.3% Formic Acid	0.3% Methanolic Formic Acid
0.00	0	80	20
3.00	0	80	20
3.01	0.5	80	20
9.00	0.5	80	20
10.00	1.0	0	100
12.00	1.0	0	100
13.00	0.5	80	20
15.00	0.5	80	20
15.01	0	80	20
17.00	0	80	20

1) Sequence Setup

Open Chemstation; Instrument 1 (Online)

- To create a sequence: Choose **Sequence > Sequence Table**
- Manually define the following necessary parameters;
  Vial Position→ Enter each individual sample vial position
  Method Name → Enter the specific method associated to target compound analysis
  Injection per Location → Enter 1
  Injection Volume → Enter 500 µL
- To Save Sequence: Choose **Sequence > Save Sequence,** then identify the sequence name

2) Run the sequence

- To open a sequence: Click **Sequence > Load Sequence**
- Choose specific sequence and then click **OK**
- Then click **Sequence > Sequence Table**
- On the bottom of the **Sequence Table**, Click **Run Sequence**
- Click the "**OK**" button when ready to proceed

e. Mass Spectrometry Conditions

Configuration parameters for this instrument are found in Table 10 and 11. The optimized precursor/product ion pairs, as well as the collision off-set energy and the retention time for the target compounds, are summarized in Table 12.

Table 10
MS Configuration
For ATZ, AZN, DEA, DIA, DEAM, and AZN-OH analysis

MS Parameter	Setting
Ionization Type	APCI
Ion Polarity Mode	positive ion
Corona Discharge Current	4.5 µA
Vaporizer Temperature	350˚C
Collision Gas Pressure	1.5 torr
Sheath Gas Pressure	25 psi
Auxiliary gas	5
Ion Sweep Gas Pressure	1 psi
Capillary Temperature	260˚C

Table 11
MS Configuration
For DACT analysis

MS Parameter	Setting
Ionization Type	APCI
Ion Polarity Mode	positive ion
Corona Discharge Current	5 µA
Vaporizer Temperature	200˚C
Collision Gas Pressure	1.5 torr
Sheath Gas Pressure	15 psi
Auxiliary gas	5
Ion Sweep Gas Pressure	1 psi
Capillary Temperature	260˚C

Table 12
Analyte Masses

Analyte	Precursor Ion [M+1]	Product Ions			Collision Energy (V) (Q, C1, C2)	RT* (min.)
		Q Ion	C1 Ion	C2 Ion		
Native						
AZN-OH	198	86	156	69	23, 16, 35	8.08
DACT	146	79	68	62	15, 26, 37	6.50
DEAM	315	185	144	102	12, 23, 37	8.95
DIA	174	68	132	104	31, 17, 31	10.22
DEA	188	146	104	110	21, 25, 22	11.95
ATZ	343	214	102	172	21, 39, 27	11.55
AZN	216	174	104	68	18, 24, 52	14.62
Labeled						
AZN-OH (D_5)	203	161	NA	NA	19	8.08
DACT (D_3)	149	113	NA	NA	20	6.50
DEAM (Cysteine-$^{13}C_3$; ^{15}N)	319	186	NA	NA	20	8.95
DIA (D_5)	179	137	NA	NA	21	10.22
DEA (D_6)	194	144	NA	NA	21	11.95
ATZ (Ring-$^{13}C_3$)	346	217	NA	NA	19	11.55
AZN (D_5)	221	179	NA	NA	19	14.62

Q = Quantitative ion, C = Confirmative ion-, RT=retention time, NA = not applicable
**RT can shift within an acceptable range*

1) Sequence Setup

Open Xcalibur; Xcalibur allows importing data that has been created by PSTAR database

- To import a sequence: Choose **File > Import Sequence**
- Use the Browse button to select the file for import.
- Select all sequence columns to be included in the sequence file.
- Click on **OK** to import the sequence: Xcalibur displays the imported file in Sequence Setup
- Manually define the following parameters;
  File name→ Enter each individual sample file name
  Path → Enter the directory path where the sample's raw file will be stored;
  Inst. Meth → Enter the path and filename of the instrument method file
  Position
  Comments (If needed)
- For Save File: Choose **File > Save AS**

2) Run the sequence

- Click on the Run Sequence Icon on the tool bar.
- Check to make sure that the selected rows are displayed in the Run Sequence Dialogue Box.
- Click Instrument Setting to "Standby" after the sequence has been run at the bottom of the Run Sequence Dialogue Box.
- Click the "OK" button when ready to proceed.

f. Processing data

To process a batch of samples:

- Save all selected raw files and sequence file onto a removable drive.
- Transfer all data onto desktop PC used for data processing
- Specific sequence associated with target raw files has to be modified by identifying **"Level" "Path" and "Processing Method"** prior data processing
- Open **Quan® browser** and select the modified sequence
- Click **Process** button

g. Quantification

- After processing the sample batch, manually evaluate for correct peak detection and baseline selection in the Quan® browser
- To save the settings in a Quan Browser file *(*.XQN)*. Choose **File>Save As**
- Export data files to EXCEL® using the long report format
- To export data files to EXCEL® → **File > Export Excel > Long Report**
- Quan Browser allows the operator to view quantitative results, to evaluate standard curve, QCs and unknowns samples, to integrate chromatogram peaks manually, and to analyze detailed quantification information.

h. Rearrangement of Data Files

Data is automatically rearranged into a multiple worksheet (Excel format)® that is compatible with our existing database using an Excel® macro. This macro also allows for analyst evaluation of quantification and confirmation ions.

i. Transfer of Data

Data is transferred using USB removable drive, floppy disk, or CD-RW disk

j. Importation of Data into Database

Select "Import new data" option in database. A password is required to import the data.

k. Statistical Analysis and Interpretation of Data

See manual procedure 6112.01.

l. Routine and Periodic Maintenance of Key Components

1) Routine Maintenance

1. Mass Spectrometer

- TSQ Quantum Ultra mass spectrometer undergoes routine maintenance by Thermo Scientific Inc. service technicians on a semi-annual basis. All records for this maintenance are kept in a bench side notebook.
- The daily, weekly or monthly maintenances are under the responsibility of operator, which includes cleaning the corona needle, ion transfer tube, skimmer, tube lens and Q00.

2. Agilent 1100 HPLC-System

- System undergoes routine maintenance by service technicians. All records for this maintenance are kept in a bench side notebook.
- The weekly maintenance is under the responsibility of operator, which includes flushing the system with methanol.

2) Periodic Maintenance

- In general, these maintenance procedures are performed by instrument operator if there is a decrease in the system performance (sensitivity or S/N ratio) without any other apparent technical reasons.

9. Reportable Range of Results

The linear range of the standard calibration curve determines the highest and lowest analytical values of an analyte that are reportable. The calibration verification of the method encompasses this reportable range.

a. Linear Limits

Analytical standard curves are linear for all analytes through the range of concentrations evaluated. The linear range for all analytes is from LOD to 200 ppb.

b. Analytical Sensitivity

The detection limits for all analytes are calculated as $3S_0$, where S_0 is the estimated standard deviation (SD) at zero concentration and is determined by linear regression analysis of the absolute standard deviation (SD) versus concentration. The detection limits vary based on the current operating precision and the cleanliness of the analytical system. The range of detection limits determined to date for the analytes are presented in Table 13.

Table 13
Analyte Detection Limits

Analyte	$3S_0$ (ng/ml)
AZN-OH	0.2
DACT	2.8
DEAM	0.4
DIA	0.3
DEA	0.5
ATZ	0.1
AZN	0.1

c. Accuracy

The accuracy of this method is defined as the degree of agreement between the means of measured concentrations of samples and their nominal values. The number of samples tested at each concentration is N=5. Accuracy in this method is measured in terms of percent deviation, the percent average deviation from spiked concentration. Accuracy is shown in Table 14.

Table 14
Accuracy of the Method

Analyte	Accuracy Mean ± SD (% of expected level)			
	5 ng/mL	25 ng/mL	50 ng/mL	100 ng/mL
AZN-OH	5.0±0.4 (100.8)	26.5±1.6 (105.8)	47.3±1.6 (94.5)	96.5±8.9 (96.5)
ACT	5.6±0.5 (112.7)	25.1±1.2 (100.4)	47.8±1.7 (95.7)	94.1±6.4 (94.1)
DEAM	4.3±0.4 (86.6)	25.3±1.3 (101.1)	48.3±2.0 (96.7)	98.2±6.6 (98.2)
DIA	4.9±0.1 (98.3)	25.0±1.6 (100.1)	48.8±1.9 (97.5)	94.6±7.8 (94.6)
DEA	5.0±0.2 (99.5)	25.3±1.3 (101.1)	47.8±1.8 (95.7)	97.3±9.3 (97.3)
ATZ	2 ng/mL	10 ng/mL	20 ng/mL	40 ng/mL
	2.0±0.0) (100.8.)	10.0±0.3 (99.7)	19.0±0.7 (95.0)	39.0±2.1 (97.5)
AZN	2.0±0.0 (98.7)	9.9±0.5 (98.6)	19.2±0.8 (96.1)	38.2±2.4 (95.6)

d. Precision

The total precision of this method is determined by calculating the relative standard deviation (RSD) of repeat measurements (n=30) of quality control materials at three concentrations and includes all sources of variability. Precision of the method is shown in Table 15.

Table 15
Precision (RSD) of the Method

Analyte	QC Low		QC Medium		QC High	
	Mean (ng/mL)	RSD%	Mean (ng/mL)	RSD%	Mean (ng/mL)	RSD%
AZN-OH	4.9±0.7	14.1	10.4±1.3	12.4	57.7±6.5	11.2
DACT	5.5±1.1	20.5	12.2±1.4	11.8	64.8±9.1	11.1
DEAM	5.3±0.8	14.7	11.5±0.9	7.9	57.7±4.8	8.2
DIA	5.3±0.6	12.1	10.5±0.6	5.3	51.2±4.2	8.3
DEA	4.7±0.5	10.2	10.0±0.6	5.6	50.6±4.8	9.4
ATZ	5.1±0.5	9.9	10.5±0.4	4.1	51.8±4.5	8.6
AZN	5.2±0.5	10.4	11.0±0.7	6.5	55.4±5.2	9.4

***RSD**= Relative Standard Deviation*

e. Analytical Specificity

This is a highly selective method that requires the following of each analyte detected: 1) that it elutes at a specific retention time, 2) that it has one precursor ion at specific masses, 3) that it has three specific product ions formed from each precursor ion at specific masses, and 4) that the ion ratios of the three product ions are within a predetermined range.

10. Quality Control (QC) Procedures

a. QC Materials

Quality Control materials for each unknown run are freshly made at the same time when samples are prepared.

b. QC Standard Spiking solution

Three QC standard spiking solutions containing AZN-OH, DACT, DIA, DEA, and DEAM is prepared by serial dilution with methanol of the initial stock solutions to yield the concentration of 2.5, 12.5, and 30 ng/mL equivalent in 1 mL of urine (i.e., neat concentrations are 0.125, 0.625, 1.5 µg/mL).

Similarly, QC standard spiking solutions for AZN and ATZ is prepared by serial dilution with methanol of the initial stock solutions to yield the concentration of 1, 5, and 12.5 ng/mL equivalent in urine (i.e., neat concentrations are 0.050, 0.250, 0.625 µg/mL)

c. Preparation of QC samples

For analysis of AZN, ATZ, AZN-OH, DIA, DEA, and DEAM: 200 µL of blank urine is pipetted into a 1.5 amber vial and spiked with 20 µL of internal standard solution (ISTD) including 20 µL of QC spiking standard. Samples are diluted with 0.1% formic acid to bring the volume to 1.0 mL. Samples are well mixed prior to analysis.

For analysis of DACT: 500 µL of blank urine is pipetted into a 1.5 mL amber vial and spiked with 20 µL of internal standard solution (ISTD) including 20 µL of QC spiking standard. Samples are diluted with 0.1% formic acid to bring the volume to 1 mL. Samples are well mixed prior to analysis.

d. Characterization of QC Materials

Characterize the QC pools (including the blank pool) by 20 consecutive runs (2 analytical runs per day for 10 days) of each QC material. Use the data from these runs to establish the mean and upper-and lower- 99^{th} and 95^{th} control limit.

e. Use of QC Samples

During each analytical run, three QC materials (QCL, QCM, QCH) are analyzed together with unknown samples.

f. Final Evaluation of Quality Control Results

QC materials are evaluated using standard Westgard multirule criteria (www.westgard.com). Repeat out-of-control runs if residual sample is available. No data from runs considered out-of-control will be reported.

11. Remedial Action If Calibration or QC Systems Fail to Meet Acceptable Criteria

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until the source or cause of failure is identified and corrected. If the source of failure, for example, is a mass spectrometer or a pipetting error, correct the problem immediately. Otherwise, prepare fresh reagents and clean the mass spectrometer system. Before beginning another analytical run, re-analyze several QC materials (in the case of QC failure) or calibration verification samples (in the case of calibration failure). After re-establishing calibration or QC, resume analytical runs.

12. Limitations of Method; Interfering Substances and Conditions

This method is an isotope-dilution mass spectrometry method, which is widely regarded as the definitive method for the measurement of organic toxicants in human specimens. By using tandem mass spectrometry, most analytical interferences are eliminated. However, because of the urinary matrix used in this procedure, occasional interfering of unknown substances has been encountered.

13. Critical-Call Results ("Panic Values")

It is unlikely that any result would be a "critical call," which would only occur with poisonings. Typically test results in this laboratory support epidemiological studies, rather than clinical assessments. Data is typically used to determine chronic exposures.

14. Specimen Storage and Handling during Testing

All samples and standards must remain under freezing temperature prior to use. Stability studies suggest that the analytes are stable with 48 hours after preparation if samples remain at room temperature.

15. Alternate Methods for Performing Test and Storing Specimens If Test System Fails

The method is designed to run on an LC-MS/MS instrument and is not generally transferable to other instrumentation. If the system has failed, samples are needed to be re-prepared.

16. Test-Result Reporting System; Protocol for Reporting Critical Calls

Once the validity of the data has been established by the Quality Control/Quality Assurance system outlined above and has been verified by a DLS QAO, generate one hard copy and one electronic copy of the data. Route these data, a cover letter, and a table of method specifications and reference range values through the appropriate channels for approval (i.e., supervisor, branch chief, division director). Once division personnel approve the analytical data, lab chief sends to the contact person who requests the analyses.

Report data in support of epidemiological or health survey studies. At this time there is not protocol for reporting critical calls.

17. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

Use standard record-keeping systems (i.e., notebooks, sample logs, data files, creatinine logs, demographic logs) to keep track of all specimens. Transfer of CLIA-specimens is only permitted to certified laboratories. Any transfer of study samples is handled through the DLS special studies coordinator.

18. References

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Appendix I:
Ruggedness Test

19. Appendix I: Ruggedness Test

For the ruggedness test, five factors possibly influencing accuracy of analytical method were chosen for subsequent test. Those factors were (1) no sample dilution, (2) sample with 1:1 dilution using 2% formic acid, (3) sample with 1:2 dilution using 0.1% formic acid, (4) sample with 1:2 dilution using 5% formic acid, and (5) sample washing with 5% formic acid in 50:50/ Water:MeOH during solid phase extraction.

Two replicants of each level of QC materials were used for this experiment. All samples were quantified against a standard curve containing nine calibration plots, ranging from 0.5 to 200 ng/ml including three QC materials (low, medium and high level) that were prepared according to the final method described on this Laboratory Procedure Manual. Average calculated concentrations per each level of QC materials of each analyte with individual tested factor were obtained. The accuracy was then calculated using the following equation;

$$\text{Accuracy} = 100 \times \frac{[\text{Calculated concentration with individual tested factor}]}{[\text{Calculated concentration of final method}]}$$

Results showing relative accuracy of the seven analytes are shown in the following tables.

ANALYTE: Diaminochloro-atrazine (DACT)			
INFLUENTIAL FACTORS	**AVERAGE CONCENTRATION (ng/ml)**		
	Low Level	Medium Level	High Level
Final Method	2.55	12.42	30.49
1. Undiluted sample	2.42	12.09	30.40
Relative Accuracy (%):	95.01	97.38	99.71
2. Sample diluted 1:1 with 2% FA	2.64	12.40	29.43
Relative Accuracy (%):	103.65	99.88	96.53
3. Sample diluted 1:2 with 0.1% FA	2.64	11.66	29.38
Relative Accuracy (%):	103.65	93.92	96.36
4. Sample diluted 1:2 with 5% FA	2.04	13.38	30.24
Relative Accuracy (%):	80.09	107.77	99.18
5. SPE washed with 5% FA (v/v) 50:50 H20:MeOH	2.51	12.14	29.68
Relative Accuracy (%):	98.55	97.78	97.35

ANALYTE: Desethyl-atrazine (DEA)			
INFLUENTIAL FACTORS	**AVERAGE CONCENTRATION (ng/ml)**		
	Low Level	Medium Level	High Level
Final Method	2.43	12.50	29.90
1. Undiluted sample	2.11	10.44	26.32
Relative Accuracy (%):	86.97	83.53	88.01
2. Sample diluted 1:1 with 2% FA	2.44	11.18	27.18
Relative Accuracy (%):	100.58	89.45	90.89
3. Sample diluted 1:2 with 0.1% FA	2.61	11.47	28.44
Relative Accuracy (%):	107.58	91.77	95.10
4. Sample diluted 1:2 with 5% FA	1.98	12.10	30.03
Relative Accuracy (%):	81.62	96.81	100.42
5. SPE washed with 5% FA (v/v) 50:50 H20:MeOH	2.66	11.59	28.45
Relative Accuracy (%):	109.65	92.73	95.14

ANALYTE: Desisopropyl-atrazine (DIA)			
INFLUENTIAL FACTORS	**AVERAGE CONCENTRATION (ng/ml)**		
	Low Level	Medium Level	High Level
Final Method	2.53	12.33	29.37
1. Undiluted sample	2.49	11.34	27.87
Relative Accuracy (%):	98.42	91.99	94.88
2. Sample diluted 1:1 with 2% FA	2.37	11.06	26.69
Relative Accuracy (%):	93.68	89.72	90.87
3. Sample diluted 1:2 with 0.1% FA	2.26	11.05	25.69
Relative Accuracy (%):	89.33	89.64	87.46
4. Sample diluted 1:2 with 5% FA	2.20	12.33	28.33
Relative Accuracy (%):	86.96	100.02	96.45
5. SPE washed with 5% FA (v/v) 50:50 H20:MeOH	2.39	11.20	27.33
Relative Accuracy (%):	94.47	90.86	93.04

ANALYTE: Desethyl-atrazine mercapturate (DEAM)			
INFLUENTIAL FACTORS	**AVERAGE CONCENTRATION (ng/ml)**		
	Low Level	Medium Level	High Level
Final Method	2.43	11.82	29.72
1. Undiluted sample	2.71	12.05	29.23
Relative Accuracy (%):	111.43	101.95	98.34
2. Sample diluted 1:1 with 2% FA	2.62	11.83	29.18
Relative Accuracy (%):	107.73	100.09	98.17
3. Sample diluted 1:2 with 0.1% FA	2.58	11.68	30.81
Relative Accuracy (%):	106.09	98.82	103.66
4. Sample diluted 1:2 with 5% FA	2.54	14.00	31.68
Relative Accuracy (%):	104.44	118.45	106.58
5. SPE washed with 5% FA (v/v) 50:50 H20:MeOH	2.69	12.32	29.81
Relative Accuracy (%):	110.61	104.24	100.29

ANALYTE: Hydroxy-atrazine (AZN-OH)			
INFLUENTIAL FACTORS	**AVERAGE CONCENTRATION (ng/ml)**		
	Low Level	Medium Level	High Level
Final Method	2.43	12.00	28.71
1. Undiluted sample	2.18	8.76	24.04
Relative Accuracy (%):	89.86	73.03	83.73
2. Sample diluted 1:1 with 2% FA	2.51	11.25	25.11
Relative Accuracy (%):	103.46	93.79	87.45
3. Sample diluted 1:2 with 0.1% FA	2.42	9.02	22.94
Relative Accuracy (%):	99.75	75.20	79.90
4. Sample diluted 1:2 with 5% FA	2.05	10.40	27.18
Relative Accuracy (%):	84.50	86.70	94.66
5. SPE washed with 5% FA (v/v) 50:50 H20:MeOH	ND	10.98	22.54
Relative Accuracy (%):	ND	91.54	78.50

ANALYTE: Atrazine mercapturate (ATZ)			
INFLUENTIAL FACTORS	**AVERAGE CONCENTRATION (ng/ml)**		
	Low Level	Medium Level	High Level
Final Method	0.99	4.90	12.40
1. Undiluted sample	0.82	4.15	9.46
Relative Accuracy (%):	83.25	84.71	76.30
2. Sample diluted 1:1 with 2% FA	0.91	3.94	9.83
Relative Accuracy (%):	92.39	80.42	79.28
3. Sample diluted 1:2 with 0.1% FA	0.88	3.88	9.62
Relative Accuracy (%):	89.34	79.20	77.59
4. Sample diluted 1:2 with 5% FA	0.86	4.29	10.13
Relative Accuracy (%):	87.31	87.57	81.70
5. SPE washed with 5% FA (v/v) 50:50 H20:MeOH	0.92	4.02	9.91
Relative Accuracy (%):	93.40	82.06	79.93

ANALYTE: Atrazine (AZN)			
INFLUENTIAL FACTORS	**AVERAGE CONCENTRATION (ng/ml)**		
	Low Level	Medium Level	High Level
Final Method	1.01	4.95	12.09
1. Undiluted sample	0.88	4.02	10.93
Relative Accuracy (%):	87.22	81.28	90.42
2. Sample diluted 1:1 with 2% FA	0.95	4.22	10.50
Relative Accuracy (%):	94.15	85.32	86.86
3. Sample diluted 1:2 with 0.1% FA	0.99	4.39	10.87
Relative Accuracy (%):	98.12	88.76	89.92
4. Sample diluted 1:2 with 5% FA	1.04	5.24	12.27
Relative Accuracy (%):	103.07	105.94	101.51
5. SPE washed with 5% FA (v/v) 50:50 H20:MeOH	1.02	4.47	11.19
Relative Accuracy (%):	101.09	90.38	92.57