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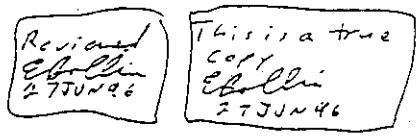

MORSE LABORATORIES, INC. SOP# Meth-93

Revision #4 Date 05/20/96

DETERMINATION OF HEXAZINONE AND ITS METABOLITES IN WATER

Reason for Revision: To incorporate the addition of a sonication step for the final sample extract in mixed solvent to ensure consistent recovery for Hexazinone metabolite G3170 in soil pore (lysimeter) water. This change will be used for all water samples.

1.0 INTRODUCTION

This standard operating procedure (SOP) is intended for the determination of residues of hexazinone and hexazinone metabolites A (IN-T3937), B (IN-A3928), C (IN-T3935), A1 (IN-G3453), 1 (IN-JS472), and G3170 (IN-G3170) in water.

2.0 EQUIVALENCE STATEMENT

During the conduct of this analysis, equivalent apparatus, solvents, glassware, or techniques (such as sample concentration) may be substituted for those specified in this method, except where otherwise noted. In the event an equivalent piece of equipment or an equivalent technique is used, its use will be documented in the study records, when appropriate.

3.0 MATERIALS

3.1 Apparatus and Equipment

Assorted laboratory glassware including: graduated cylinders, short stem glass funnels, pipets, volumetric flasks, evaporating flasks, microliter syringes.

Analytical Balances:	Analytical balance capable of weighing to 0.1 mg for weighing analytical standards
Centrifuge Bottles:	250 mL Nalgene® polyethylene bottles with screw cap closures
Evaporators:	N-Evap, Model 115, attached to a nitrogen source (Organomation Associates, South Berlin, MA)
Filter Paper:	Whatman, #541

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Gas Chromatograph:	Hewlett-Packard 5890E gas chromatograph equipped with an N/P detector, an HP6890 Autosampler, and an HP 3365II ChemStation
GC Column:	15 M × 0.53 mm i.d. fused silica column crossbonded with 0.5 μm film thickness Rtx-35
Mixer:	Vortex Genie 2 (VWR Scientific, Bridgeport, NJ)
EDP Electronic Pipets with suitable tips:	Rainin, Ridgefield, NJ.
Reservoirs (75 mL, 25 mL, or 15 mL) and adapters for use with SPE cartridges:	Varian Analytical Instruments, Sunnyvale, CA
Solid Phase Extraction Apparatus:	Vac Elut Mode SPS 24 (Varian Analytical Instruments, Sunnyvale, CA), or Visiprep™ Solid-Phase Extraction Vacuum Manifold (Supelco, Inc., Bellefonte, PA)
Solid Phase Extraction Columns:	Supelclean™ Envi™-Carb SPE Tubes, Custom, 12 mL Polypropylene Tubes with Teflon Frits packed with 1.5 gram of ENVI-CARB (Supleco, Inc., Bellefonte, PA) C18 Mega Bond Elut, 6 cc/1 gram (Varian Part #1225-6001) (Varian, Inc., Harbor City, CA)
Test Tubes:	16 × 100 mm, borosilicate, silylated, with Teflon®-lined screw caps
Ultrasonic Bath:	Branson Model 3200 Ultrasonic Bath (VWR Scientific)

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3.2 Reagents

Acetone:	Pesticide residue quality
Dimethyldichlorosilane:	Supelco, Catalog No. 3-3009 (Supelco, Inc., Bellefonte, PA)
Ethyl Acetate:	Pesticide residue quality
Glacial Acetic Acid:	Reagent grade
Hexane:	Pesticide residue quality
Methanol:	Pesticide residue quality
Toluene:	Pesticide residue quality
Water:	HPLC grade

3.3 Primary Standards

Analytical grade, available from DuPont Agricultural Products Global Technology Division, E. I. duPont de Nemours and Company, Wilmington, DE

Analyte	Standard Identification
Hexazinone	DPX-A3674
Metabolite A	IN-T3937
Metabolite B	IN-A3928
Metabolite C	IN-T3935
Metabolite A1	IN-G3453
Metabolite 1	IN-JS472
Metabolite G3170	IN-G3170

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3.4 Reagent Preparation

3.4.1 3mM Acetic Acid

Add 173 μL glacial acetic acid per one liter of HPLC grade water. Record the pH of this solution. This solution is stable for approximately two weeks. Discard the solution when it starts showing signs of bacterial contamination.

3.4.2 9:1 Acetone:3mM Acetic Acid

Mix 90 mL acetone with 10 mL 3mM acetic acid solution. Prepare fresh daily.

3.4.3 9:1 Methanol:3mM Acetic Acid

Mix 90 mL methanol with 10 mL 3mM acetic acid solutions. Prepare fresh daily.

3.4.4 Mixed solvent solution (20% acetone/50% ethyl acetate/30% toluene GC Diluting Solution)

In a 50 mL mixing cylinder add 10 mL acetone and 25 mL ethyl acetate. Dilute to volume with toluene. Mix well. Prepare fresh as needed.

3.5 Analytical Standard Preparation

3.5.1 Stock Standard Solutions

12.5 mg (corrected for purity) of each analytical standard is accurately weighed, quantitatively transferred to a 25 mL volumetric flask, and brought to volume with methanol to make individual stock standard solutions having a concentration of 500 μg/mL. These stock standard solutions are to be stored at 1-8 °C when not in use. These solutions are stable for approximately six months when stored under these conditions.

3.5.2 Fortification (Spiking) Solutions

Typically, mixed standard solutions containing all seven analytes per standard solution are prepared by adding the appropriate amounts of standard solutions into a volumetric flask and diluting to volume with methanol.

For preparation of various standard concentrations see form ML 471 in Appendix I.

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Store all spiking solutions in a refrigerator when not in use.

3.5.3 Intermediate Standard Solutions

Typically, intermediate standard solutions containing all analytes are used to prepare the GC standards. The intermediate standard solutions and the GC standards are prepared in volumetric flasks and diluted with mixed solvent solution (20% acetone/ 50% ethyl acetate/30% toluene).

For preparation of various standard concentrations see form ML 472 in Appendix I.

Store these standard solutions in a refrigerator when not in use.

3.5.4 GC (Calibration) Standard Solutions

Typically, GC standards containing all seven analytes are prepared. For preparation of the various standard concentrations, see form ML 472 in Appendix I.

Store GC standards in a refrigerator when not in use.

4.0 ANALYTICAL PROCEDURE

4.1 Principle

A suitable aliquot of the sample is acidified and concentrated by solid phase extraction (SPE) using disposable graphitized carbon cartridges. The analytes are eluted from the SPE column with acid-acetone solution. The eluate is evaporated to dryness and brought to the water phase for a second column cleanup using C18 SPE column. The analytes are eluted from the column with acid-methanol. The eluate is evaporated to dryness and the residue dissolved in a suitable mixed solvent solution for gas chromatographic analysis using NP detection.

4.2 Sample Preparation

Note: Use disposable laboratory equipment whenever possible. All glassware used for the analysis must be meticulously cleaned prior to use.

1. Measure the amount of sample for analysis in a graduated cylinder. Record the volume of sample used for analysis.

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2. Transfer the sample aliquot for analysis to a 250 mL polyethylene bottle. Fortify at this point. Add water to the sample so that the total volume prior to addition of acid is 100 mL. Swirl to mix.

3. Acidify the sample with 20 μL glacial acetic acid. Swirl to mix.

4. Filter the acidified sample into a second 250 mL polyethylene bottle. The sample is now ready for SPE column processing.

4.3 Solid Phase Extraction (SPE)/Concentration and Purification

1. Pack the carbon column by gently pushing the column frit onto the carbon packing. Condition the column with one column volume of 9:1 acetone: 3mM acetic acid. Elute to dry and dry the column for approximately 30 seconds more with vacuum, adjusting the vacuum gauge to minimize loss of small amounts of carbon. Continue conditioning the column with two column volumes of water without allowing the column to go to dry. Discard all elutions.

2. Attach a reservoir to the cartridge and pass the acidified sample through the cartridge. Use vacuum to facilitate sample elution. Do not allow the cartridge to go to dry. Discard this sample load. Remove the reservoir.

3. Rinse the sample container with one column volume (10-11 mL) of water and add rinsing to the column. Elute to dryness and dry for approximately 30 seconds more with vacuum. Adjust the vacuum gauge to minimize the loss of small amounts of carbon. Discard this wash.

4. Rinse the column with one column volume of hexane. Allow the hexane wash to go to dryness and dry for approximately 30 seconds more with vacuum. Adjust the vacuum gauge to minimize the loss of small amounts of carbon. Discard the hexane wash.

5. Elute the analytes into a 16 × 100 mm silanized glass test tube (marked at approximately 1 mL and approximately 5 mL) with 10 mL of 9:1 acetone:3mM acetic acid. Elute by gravity. However, vacuum may be used to start the elution.

Note: See Appendix II for detailed instructions for silylating glassware.

THIS IS A STOPPING POINT. Refrigerate extracts for further processing.

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6. Evaporate the sample extract to approximately 1 mL at 40 °C - 50 °C using the N-Evap.

7. Wash the sides of the sample test tube with approximately 2 mL acetone and evaporate the extract to 0.2 to 0.5 mL.

8. Repeat washing the sides of the test tube with a second 2 mL portion of acetone.

9. Take the extract to dryness.

10. Dissolve the residue in 200 µL acetone. Briefly vortex the extract and sonicate for 5 minutes. Again, vortex briefly.

11. Add approximately 1 mL of water and evaporate to the water phase.

12. Add approximately 4 mL HPLC water and continue the evaporation step to ensure removal of all traces of the acetone.

13. Adjust to approximately 5 mL with HPLC water.

THIS IS A STOPPING POINT. Refrigerate extracts for further processing.

14. Vortex. Sonicate for 5 minutes. Vortex. The extract is now ready for additional SPE column cleanup.

THIS IS A STOPPING POINT. Refrigerate extracts for further processing.

4.4 Additional Column Cleanup with C18 SPE Cartridge:

Note: To ensure uniformity of the column packing in the cartridge, tap the cartridge on the lab bench, then gently push the column frit on to the column packing prior to use.

1. Pass 5 mL 9:1 (Methanol:3mM Acetic Acid) to the column. Elute to dryness and dry for 30 seconds more with vacuum. Continue conditioning with 2 × 5 mL HPLC water without allowing the column to go to dryness.

2. Load the aqueous extract from 4.3.14 on to the column and elute to approximately 1 cm above the column packing.

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3. Rinse the sample test tube with approximately 2 mL HPLC water and add this rinsing to the column. Elute just to dryness. Discard the sample load and wash.

4. Wash the column with 2 × 5 mL hexane. Elute the second 5 mL hexane wash to dryness. Continue drying the column for 30 seconds more with vacuum. Discard the hexane washes.

5. Elute the analytes into a 16 × 100 mm silanized glass test tube calibrated at 4.0 mL with 10 mL 9:1 Methanol:3mM Acetic Acid. A suitable reservoir may be used for this step to facilitate elution. Elute by gravity. However, vacuum may be used to start the elution.

THIS IS A STOPPING POINT. Refrigerate extracts for further processing.

4.5 Concentration and Processing of Sample Eluates for GC Analysis:

1. Evaporate the sample extract to approximately 1 mL at approximately 50 °C using the N-Evap.

2. Wash the sides of the sample test tube with approximately 1 mL methanol and evaporate the extract to 0.2 to 0.5 mL.

3. Repeat washing the sides of the test tube with a second 1 mL portion of methanol.

4. Take the extract to dryness. Determine the final volume of the extract at this point so that the concentration of the sample in the final extract is 25 mL/mL.

5. Dissolve the residue in a volume of acetone equivalent to 20% of the final volume of the extract. Briefly vortex the extract and sonicate for 5 minutes. Again, vortex briefly.

6. Add a volume of ethyl acetate equivalent to 50% of the final volume of the extract and ensure complete solution of the analytes by vigorously vortexing the mixture.

7. Adjust the final volume of the sample extract with toluene. Sonicate for 5 minutes. Mix thoroughly for GC analysis. The final concentration of the sample in the extract is 1 mL = 25 mL.

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4.6 GC Analysis

Note: The column and conditions stated in the method have been satisfactory for the matrix being analyzed. The specific column packing/coating, carrier gas, column temperature and flow rate listed are typical conditions for this analysis. Specific conditions used will be noted on each chromatographic run and will not otherwise be documented.

4.6.1 Operating Conditions

Instrument:	HP5890E gas chromatograph equipped with an N/P detector, electronic pressure controlled inlet (packed/purged), an HP6890 autosampler, and an HP 3365 II ChemStation.		
Column:	15 M × 0.53 mm i.d. fused silica column crossbonded with 0.5 μm film thickness Rtx-35		
Inlet Liner:	2 mm i.d. liner lightly packed with fused silica wool		
Injection Volume:	2 μL		
Carrier Gas:	Helium		
Flow:	Column:	5 mL/min. (constant)	
	Makeup:	15 mL/min.	
Temperature:	Injector:	290 °C	
	Detector:	285 °C	
	Column:	Initial:	150 °C
		Rate 1:	25 °C/min. to 275 °C hold 2.2 minutes,
		Rate 2:	10 °C/min.
		Final:	285 °C hold for 1.00 minute

4.6.2 Sample Analysis

Prepare a four-point standard curve by injecting constant volumes of combined analyte standard solutions prepared in a mixed solvent solution. Use constant volume injections for sample extracts as well. Sample responses not bracketed by the standard curve require dilution and reinjection.

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4.6.3 Calculations

Calculations for instrumental analysis are conducted using a validated software application to create a standard curve based on linear regression. These regression functions are used to calculate a best fit line (from a set of standard concentrations in μg/mL versus peak area response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line.

The equation used for the least squares fit is:

$$y = mx + b$$

where,

y = peak area response

x = μg/mL found for peak of interest

m = slope

b = y-intercept

The calculations for ppb analyte found and percent recovery (for fortified samples) for each of the seven analytes are:

1. ppb analyte =

$$\frac{\mu g/mL\ analyte\ found \times 1000 \times mL\ final\ volume \times GC\ dil.\ fact.}{mL\ sample}$$

where:

μg/mL analyte is calculated by linear regression based on peak area response.

1000 = conversion factor from μg to ng

mL sample = amount of sample analyzed

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mL final volume	=	volume of *final extract submitted* to GC
GC dil. fact.	=	the magnitude of dilution required to bracket the response of the sample within the standard curve responses. When the sample requires no dilution, the GC dilution factor = 1

2. The percent recovery for fortified control samples is calculated as follows:

$$\%\ Recovery = \frac{ppb\ found\ in\ fortified\ control - ppb\ found\ in\ control}{ppb\ added} \times 100$$

5.0 REFERENCES

1. Dupont Report No. AMR 3888-95 (Draft), "Analysis of Hexazinone and Metabolites in Soil and Groundwater using GC/MS," received 11/22/95.

2. Dupont Report No. AMR 2896-94

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Silylation of Glassware

Purpose:

Silylation is a process used to chemically treat glassware in order to prevent or minimize binding of analyte residues to the glass surface.

Caution: DO NOT ALLOW DIMETHYLDICHLOROSILANE TO COME IN CONTACT WITH WATER. CHLORINE GAS AND HYDROGEN CHLORIDE GAS WILL BE PRODUCED.

THIS PROCEDURE MUST BE DONE UNDER A FUME HOOD. THE TECHNICIAN MUST WEAR HEAVY LATEX GLOVES.

Procedure:

1. Prepare 100 mL of a 5% (v/v) solution of dimethyldichlorosilane (DMDCS) in hexane.

   To a glass stoppered glass container (approximately 200 mL volume) add 95 mL hexane. Slowly add 5 mL DMDCS. Stopper and invert to mix.

   Larger volumes can be prepared using the proportions discussed above, however attempt to prepare amounts that will be nearly totally used to avoid disposal of excess solution.

2. Pour a small amount of the DMDCS solution into the glassware to be treated. Rotate the glassware to thoroughly coat the inside surfaces. Pour excess solution into the next piece of glassware to be treated.

3. Allow the treated glassware to dry (approximately 20 minutes). Rinse with deionized water, then acetone. Again allow to dry.

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4. Glassware is now ready for use.

Note:
- Any glassware that is cleaned with a brush after it has been silylanized, must be resilylanized.
- Store pure DMDCS at room temperature.
- 5% solutions of DMDCS in hexane are stable for 5 days when stored well-stoppered at room temperature.

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APPROVED BY:
DATE: 5/20/96

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ANALYTICAL METHOD FOR THE DETERMINATION OF HEXAZINONE AND METABOLITES OF INTEREST IN SOIL AND WATER USING ELECTROSPRAY-LC/MS/MS

F. W. Brill, Tom Gardner

1.0 SUMMARY

A residue method is described for the extraction, purification, and quantitation of hexazinone (DPX-A3674) and metabolites A (IN-T3937), A-1 (IN-A3453), B (IN-A3928), C (IN-T3935), 1 (IN-JS472), and G3170 (IN-G3170) in soil and water.

Water samples (20 mL) were filtered, concentrated, and purified using solid phase extraction cartridges (Envi-Carb and C18). The instrumental analysis for detection and quantitation of the analytes was accomplished by LC/MS/MS with an electrospray interface in multiple reaction monitoring (MRM).

Soil samples (5g) were extracted with 0.1M KH_2PO_4/0.5M NaCl solution, filtered, concentrated, and purified using solid phase extraction cartridges (Envi-Carb and C18). The instrumental analysis for detection and quantitation of the analytes was accomplished by LC/MS/MS with an electrospray interface in multiple reaction monitoring (MRM).

The limit of quantitation (LOQ) for water was 0.100 ppb for hexazinone and all 6 metabolites. The LOQ for soil was 2.0 ppb for hexazinone and all 6 metabolites. Test samples were fortified at the LOQ and 5 x LOQ for each analyte in both soil and water to validate the method

To be completed

2.0 INTRODUCTION

Hexazinone (DPX-A3674) is the active ingredient in Velpar® herbicides registered for postemergence control of many annual and biennial weeds. Analyte structures, chemical names, DuPont code numbers, and *Chemical Abstracts* registry numbers are provided in Figure 1.

In this method, water samples are acidified, filtered, purified and concentrated by SPE. A combination of an Envi-Carb and a C18 SPE step are used to remove most matrix and substances that may interfere with the instrumental analysis.

The purified extracts were analyzed by ESI-LC/MS/MS using positive mode multiple reaction monitoring (MRM) for hexazinone and metabolites A, A-1, B, C, 1, and G3170. Quantitation was based on the integration of a single MRM transition response. This analysis quantitatively detects hexazinone and all 6 metabolites, the LOQ for each determined to be 0.1 ppb in water and 2.0 ppb in soil.

To be completed

3.0 MATERIALS

Equivalent equipment and materials may be substituted unless otherwise specified (see section 5.3); note any specifications in the following descriptions before making substitutions. The equivalency/suitability of any substitution should be verified with acceptable control and fortification recovery data. The materials are listed in order of first appearance in the method.

3.1 Equipment

Standards Preparation:

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- Mettler AE 163 analytical balance (Mettler Instrument Corp., Hightstown, N.J.)
- Pipets/pipettors and tips suitable for preparation of standards and sample fortifications
- Assorted flasks, beakers and volumetric cylinders

Sample Extraction:

- Mettler PM460 top-loading analytical balance (Mettler Instrument Corp.)
- 250-mL high density polypropylene centrifuge tubes (Nalgene)
- RapidVap® Evaporation System (Labconco Corp., Kansas City, Mo.)
- Tissumizer® homogenizer Model FTD-1810 (Tekmar Company, Cincinnati, Ohio)
- 0.45-µm filter unit, P.N. 150-0045 (Nalge Company, Rochester, NY)
- Sorvall® RC5C centrifuge with SS34 rotor (DuPont Company Sorvall® Products, Wilmington, Del.) or IEC HN-SII centrifuge (Damon/IEC Division, Needham, Mass.).
- Buchner Funnel
- Filter Paper, Whatman #5 (Cat. No. 1005 070) to fit Buchner Funnel

Sample Purification:

- C18 Mega Bond Elut®, 20cc/1g, P.N. 1225-6001 (Varian, Harbor City, CA.)
- Supelclean™ Envi-Carb SPE column, 60mL/1.5g, CUSTOM (Supelco Inc., Bellefonte, Pa.)
- 15-mL cap. reservoir, empty, P.N. 1213-1010 (Varian); Luer stopcock, P.N. 1213-1005 (Varian); Bond Elut® adapters, P.N. 1213-1005 (Varian)
- N-Evap Analytical Evaporator, Model 111 (Becton Dickinson & Co., Franklin Lakes, N.J.)
- 2-µm, 13-mm AcroDisc 13CR PTFE syringe filter, P.N. 4422 (Gelman Sciences, Ann Arbor, Mich.)
- 4.5-µm, 25-mm AcroDisc 13CR PTFE syringe filter, P.N. 4419 (Gelman Sciences, Ann Arbor, Mich.)
- Vacuum manifold, P.N. 5-7030 (Supelco Inc., Bellefonte, Pa.)
- 3200R-4 Bransonic® Ultrasonic Cleaner (Bransonic Ultrasonics Corp., Danbury, Conn.).

LC/MS Analysis:

The following HPLC/MS system was used for this method including equipment necessary to divert the flow going into the mass spectrometer to waste and of post-column splitting of the HPLC flow.

- Micro-Mass Quattro II triple sector quadrupole instrument with API source/interface configured for ESI operation.
- MassLynx data acquisition software running under Windows NT.
- Waters Model 1100 HPLC system including pump module, autosampler, degassing module, column compartment module, (Waters Corp., Milford, Mass.)
- Electrically-actuated, high-pressure 6-port switching valve with 1/16 in. fittings, #EC6W, used for effluent diversion from MS (4-port valve would be adequate, #EC4W) (Valco Instrument Co., Inc., Houston, TX)
- High-pressure stainless steel 1/16 in. fitting tee, #ZT1, for use in post-column flow splitting. Split ratio adjusted by altering restriction on waste-side of tee by altering length of capillary tubing. (Valco Instrument Co., Inc.)
- Zorbax® 4.6 mm id × 250 mm Rx-C8, 5-µm particle size, P.N. 880967-901 (MAC-MOD Analytical, Inc., Chadds Ford, Pa.) Substitute columns may give sub-optimal performance.

3.2 *Reagents and Standards*

Reagents:

- OmniSolv® glass distilled acetone, hexane, methanol

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- 'Baker Analyzed Reagent' potassium phosphate, dibasic, crystal, P.N. 3252-01, KH_2PO_4 (J.T. Baker, Phillipsburg, N.J.)
- 'Baker Analyzed Reagent' sodium chloride, crystal, P.N. 3252-01, KH_2PO_4 (J.T. Baker, Phillipsburg, N.J.)
- GR glacial acetic acid (EM Science)
- All water was Milli-Q® distilled, deionized water (Millipore Corp., Bedford, Mass.).

Standards: All standards were synthesized by DuPont Agricultural Products, Wilmington, Del. Standards should be greater than 95% purity

- DPX-A3674-213 (hexazinone)
- IN-T3937-3 (metabolite A)
- IN-G3453-2 (metabolite A-1)
- IN-A3928-4 (metabolite B)
- IN-T3935-3 (metabolite C).
- IN JS472-2 (metabolite 1)
- IN G3170-2 (metabolite G3170)

3.3 *Safety and Health*

No unusually hazardous materials are used in this method. All appropriate material safety data sheets should be read and followed, and proper personal protective equipment should be used.

4.0 METHODS

4.1 *Principles of Analytical Method*

4.1.1 *Sample Extraction*

The 0.1M KH_2PO_4/0.5M NaCl soil extraction solution yielded high recoveries and has been demonstrated as an effective extraction agent for hexazinone and its metabolites. Water is not extracted, it is directly filtered, concentrated, and purified on the SPE columns.

4.1.2 *Extract Purification*

Carbon phase (Envi-Carb) and Reverse phase (C18), Bond Elut® SPE procedures are used to provide extensive extract purification. The water and the soil extracts are passed onto and retained on Envi-Carb columns. The column is then washed with water and hexane to remove most of the matrix material. Elution is with an acetone:3 mM acetic acid (90:10 v:v) solution. The acetone in the eluate is removed and the sample is brought up in water and loaded onto a C18 column. The C18 is then washed with water and hexane, following which it is eluted with methanol. The sample is blown down to dryness through a series of procedures designed to minimize loss of analyte on the container walls and is brought up to a final volume in water, for a final filtration and LC/MS analysis.

4.1.3 *LC/MS Analysis*

The method utilizes electrospray ionization (ESI) and is operated in positive ion mode using Multiple Reaction Monitoring, (MRM) on the Micromass instrumentation for all of the analytes. A single transition was monitored for each analyte: [MH+1] fragmenting to the [M-cyclohexane moiety]$^+$. Selection of these transitions was based upon the mass spectra generated during the method development process with the instrument in scanning mode. The spectra generated by ESI-LC/MS for the analytes are shown in Figure 2. The base ion, $[MH]^+$, for each analyte is selected for quantitation. The sensitivities of each analyte varied, the G3170 showing the least sensitivity in the positive ESI SRM mode, the hexazinone showing the most.

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4.2 Analytical Procedure

4.2.1 Glassware and Equipment Cleaning Procedures

Disposable labware are used whenever possible in this method. Reusable labware, which includes the evaporation vessels and volumetrics for standard solutions, are cleaned by washing with a laboratory grade detergent followed by tap water rinses (3) and distilled water rinses (3). A final acetone rinse may be used to remove the residual water and promote drying. Certain items of glassware must be treated silanized to prevent absorption of analytes onto the glass.

4.2.2 Preparation and Stability of Reagent Solutions

0.1M KH_2PO_4/0.5M NaCl Solution: Add 13.6g KH_2PO_4 and 29.2g NaCl into a 1 liter volumetric flask. *Dissolve the compounds with HPLC or Milli-Q water. Bring to volume and mix well.* Record the pH of the solution (should be ~4). This solution is stable for 1 month, discard earlier if it show signs of turbidity or bacteria growth. Scale volume as necessary.

3 mM Acetic Acid: Add 173 µL of glacial acetic acid per 1 L of HPLC or Milli-Q water. The solution is stable for 1 month. Discard earlier if it shows signs of turbidity or bacteria growth.

1/10 diluted Glacial Acetic Acid. Prepare a 1:10 dilution of glacial acetic acid by placing 9 mL of HPLC or Milli-Q water into a 15 mL plastic centrifuge tube or any other suitable container with a cap. Add 1 mL of glacial acetic acid, mix carefully. Cap when not in use.

Extraction solution for Soil; is prepared by combining acetone and the 0.1M KH_2PO_4/0.5M NaCl Solution in a 90:10 v/v ratio. This solution is prepared daily as needed, however will be stable for 1 month.

90:10 Acetone:3mM Acetic Acid Solution: Mix 90 mL of acetone with 10 mL of 3 mM Acetic Acid solution. This solution is used for SPE column conditioning and elution. Scale volume as needed, stable for 1 month.

90:10 Methanol:3mM Acetic Acid Solution: Mix 90 mL of methanol with 10 mL of 3 mM Acetic Acid solution. This solution is used for SPE column conditioning and elution. Scale volume as needed, stable for 1 month.

HPLC Aqueous Mobile Phase: A 0.01M acetic acid solution for use as mobile phase is prepared by filling a 1 L volumetric flask with distilled, deionized water, adding 600 µL of concentrated acetic acid, gently agitating the mixture, and diluting to volume in distilled, deionized water. This solution should be prepared as needed or sooner if turbidity or bacterial growth is observed.

4.2.3 Stock Solutions Preparation

If possible, standards with a purity greater than 95% are to be used. Individual 100±2-µg/mL stock standards solutions for hexazinone and the 6 listed metabolites were prepared by weighing 5±0.1 mg of each standard in a separate, tared 50-mL volumetric flask and diluting to volume in acetone. Sample weights were determined to 3 significant figures. The analytical balance must provide a weight precision to 3 significant figures, or the amount and volume has to be adjusted to meet this criteria. For example, increase the amount to 10.0±0.2 mg and use a 100-mL volumetric flask. Clearly label each stock solution with date prepared, analyte, and concentration. Stock solutions are stored under refrigeration (4±2 °C) and must be replaced at least every 120 days or when approximately half-volume. Keep stock solutions stoppered to restrict solvent evaporation.

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4.2.4 *Intermediate Standard and Fortification Solution Preparation*

Water/Soil Intermediate Standard: An Intermediate 1.0 µg/mL mixed standard solution is made from the 7 individual 100 µg/mL stock solutions. Place 0.5 mL of each of the 7 individual 100 µg/mL stock solutions into a 50 mL volumetric flask using a pipet. Place the volumetric onto the N-Evap and pass nitrogen through the flask to blow off all of the acetone, remove immediately. Add HPLC or Milli-Q water and fill to the 50 mL line. Vortex, sonicate for 5 min.

Water Fortification and Standard Solution: A 50 ng/mL water fortification and standard solution is prepared from the mixed 1.0-µg/mL Intermediate Standard. Remove the Intermediate Standard from the refrigerator and allow it to reach room temperature. Then shake by hand to insure standard consistency before making dilutions. Place 2.5 mL of the 1.0 µg/mL intermediate stock solution into a 50 mL volumetric flask. Fill up to the line with water and mix. Label the solution with the date prepared, analytes, and concentration. Store the solution in the refrigerator. It is stable for 90 days.

Soil Fortification and Standard Solution: A 200 ng/mL water fortification and standard solution is prepared from the mixed 1.0-µg/mL Intermediate Standard. Remove the Intermediate Standard from the refrigerator and allow it to reach room temperature. Then shake by hand to insure standard consistency before making dilutions. Place 10.0 mL of the 1.0 µg/mL intermediate stock solution into a 50 mL volumetric flask. Fill up to the line with water and mix. Label the solution with the date prepared, analytes, and concentration. Store the solution in the refrigerator. It is stable for 90 days.

4.2.5 *LC Calibration Standard Solution Set Preparation*

Water: The calibration standard set is made from the 50 ng/mL Water Fortification and Standard solution. Remove the Water Fortification and Standard solution from the refrigerator and allow it to reach room temperature. Then shake by hand to insure standard consistency before making dilutions. Refer to the table below. Place the specified aliquot of the 50.0 µg/mL Water Fortification and Standard solution into a 10 mL volumetric flask. Fill to the line with HPLC or Milli-Q® water. Label as calibration standard solutions with date prepared, analytes, and concentration. Store these solutions in the refrigerator. They are stable for 90days.

Identifier	Aliquot of 50 ng/mL Std.(uL)	Final Vol.	Standard Concentration (ng/mL)
WS1	2000	10 mL	10
WS2	1000	10 mL	5.0
WS3	400	10 mL	2.0
WS4	134	10 mL	0.67

Clearly label the calibration solutions with the date prepared, analytes, and concentration. Autosampler vials should be filled to approximately 2/3 capacity and capped to minimize solvent evaporation. The 10 mL volumetric flasks of standards will be stable for 2 weeks. Use fresh aliquots from them for each analysis set.

Soil: The calibration standard set is made from the 200 ng/mL Soil Fortification and Standard solution. Remove the Soil Fortification and Standard solution from the refrigerator and allow it to reach room temperature. Then shake by hand to insure standard consistency before making dilutions. Refer to the table below. Place the specified aliquot of the 200.0 µg/mL Soil Fortification and Standard solution into a 10 mL volumetric flask. Fill to the line with HPLC or Milli-Q® water. Label as calibration standard solutions with date prepared, analytes, and concentration. Store these solutions in the refrigerator. They are stable for 90days.

Identifier	Aliquot of 200 ng/mL Std.(uL)	Final Vol.	Standard Concentration (ng/mL)
SS1	2500	10 mL	50.0

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SS2	1250	10 mL	25.0
SS3	500	10 mL	10.0
SS4	167	10 mL	3.33

Clearly label the calibration solutions with the date prepared, analytes, and concentration. Autosampler vials should be filled to approximately 2/3 capacity and capped to minimize solvent evaporation. The 10 mL volumetric flasks of standards will be stable for 2 weeks. Use fresh aliquots from them for each analysis set.

4.2.6 *Source and Characterization of Samples*

4.2.7 *Storage and Preprocessing of Samples*

4.2.8 *Sample Fortification Procedure*

Water: All water test samples are fortified with hexazinone and the 6 listed metabolites from the 50 ng/mL Water Fortification and Standard solutions. The appropriate fortification volume is drawn into a pipet/pipettor and placed into 20 mL of water. The water is then mixed. Refer to the table below for the volumes of Water Fortification and Standard solution to use for making the LOQ and 5 x LOQ fortifications.

Soil: All soil test samples are fortified with hexazinone and the 6 listed metabolites from the 200 ng/mL Soil Fortification and Standard solutions. The appropriate fortification volume is drawn into a pipet/pipettor and placed into 5g of soil. The soil is allowed to stand for 10 minutes. Refer to the table below for the volumes of Soil Fortification and Standard solution to use for making the LOQ and 5 x LOQ fortifications.

Matrix	Amount to Fortify	Volume Fortification Soln. Low Fortification (LOQ)	Volume Fortification Soln. High Fortification (LOQ)	Levels: all analytes LOQ and 5 x LOQ
Water	20 mL	40 µL	200 µL	0.1 ppb,. 0.5 ppb
Soil	5 g	50	250	2.0 ppb,. 10 ppb

4.2.9a *Concentration and Purification Procedures for Water*

Solution Requirements/Sample (extraction + purification):

Milli- Q water	50 mL
methanol	10 mL
hexane	10 mL
90:10 acetone: 3 mM Acetic Acid	10 mL
90:10 methanol:3mM Acetic Acid	10 mL

SAMPLE CONCENTRATION AND PURIFICATION

1. Remove samples from refrigerator or freezer and allow to warm to room temperature.
2. Measure 20 mL of the water sample and place it into a 250 mL centrifuge bottle.
3. Fortify test samples if required.
4. Add 80 mL of Milli-Q water and then acidify by adding 200 µL of the 1/10 diluted glacial acetic acid solution. Cap and briefly vortex mix.

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ENVI-CARB BOND ELUT® CLEANUP

1. Place the 1.5 g Envi-Carb Bond Elut® into the vacuum manifold and condition them by placing 10 mL of 90:10 acetone:3 mM acetic acid solution into the tubes and adjusting the flow to a fast drip (~10 mL/minute). Using a gentle vacuum to pull through all of the liquid, let the column dry for 30 seconds after the last of the liquid has exited. Load and condition the column with two 10 mL volumes of water. Do not let the liquid level drop below the top of the sorbent after the second water load has passed through. Do not allow the tube to dry.
2. Load the water sample onto the column, in several portions if necessary due to column reservoir capacity. Turn on the vacuum and allow the sample to pass through the column at a slow drip. Do not allow the column to go dry. Do not collect the liquid.
3. Place 5 mL of water into the empty 50 mL centrifuge tube, vortex. Place this rinse onto the Bond Elut and allow to pass through at a slow drip. Pull all the water through, allow to dry, passing air through for about 30 seconds after last drop has come off.
4. Place 5 mL of hexane into the Bond Elut and allow to pass through at a slow drip. Allow to dry, passing air through for about 30 seconds after last drop has come off.
5. Open the vacuum manifold and place plastic 15 mL centrifuge tubes into the manifold in order to collect the eluate.
6. Place 10 mL of 90:10 acetone:3 mM acetic acid solution into the Bond Elut tube and allow to pass through at a slow drip. Pull all of the liquid through into the collection tubes. Break the vacuum and remove the tubes containing the eluate.
7. Place sample on an N-Evap and evaporate to approximately 1 mL at 40-50 deg.
8. Wash the sides of the sample test tube with approximately 2 mal of acetone and evaporate the extract to 0.2 to 0.5 mL. Repeat this washing with a second 2 mL volume of acetone. Evaporate the sample to dryness
9. Add 200 µL of acetone to the tube, vortex and sonicate for 5 minutes, vortex again. Add approximately 1 mL of water and evaporate to the water phase. Add approximately 4 mL of HPLC water and continue the evaporation step to ensure removal of all traces of acetone This is a very critical step, the presence of any acetone will prevent the polar analytes from being retained on the C18 column in later steps.)
10. Adjust to approximately 5 mL with HPLC water. Vortex, sonicate for 5 minutes, vortex .
11. Proceed directly to the C18 SPE clean-up. This is a stopping point. Samples can be placed into the refrigerator for storage for up to 1 week.

C18 BOND ELUT CLEANUP/FINAL PREPARATION

1. Place the C18 Bond Elut® tubes onto the vacuum manifold and condition them by placing 5 mL of methanol:3mM Acetic Acid (90:10 v/v) solution into each tube and adjusting the flow to a fast drip. Drain the tube and allow to dry for 30 seconds more under vacuum. In the same manner condition next with 10 mL of water, in two 5 mL increments, waiting until the first 5 mL is completely below the frit before adding the second 5 mL. Stop the flow when the level of the water drops slightly below the top of the sorbent in the bottom tube. Do not let the tubes dry.
2. Place the Envi-Carb eluate solution (step 11 ENVI-CARB cleanup above) into the C18 Bond Elut® tube. Apply vacuum, open the stopcock and load the eluate onto the column with a very slow drip. Stop when the level of the liquid is 1 cm above the top of the sorbent. Discard the aqueous effluent.
3. Backrinse the original Envi-Carb eluate container by placing 2.0 mL of water into it vortex mixing for a few seconds. Pour this rinsate into the C18 cartridge. Allow this (rinse)water wash to pass through the C18 and discard. Allow all of the water to be drawn out of the tube. Immediately shut off vacuum, discard load volume

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4. Place 5.0 mL of hexane into the C18 cartridge. Allow the hexane to pass through at a moderate drip. After the hexane has completely passed through the column, add another 5.0 mL of hexane and allow that to completely pass through the column as well. Allow the vacuum to pull air through the cartridge until there is no more dripping.
5. Break vacuum and place a new 15 mL plastic centrifuge tube into the vacuum manifold under the C18 tubes to collect the eluate.
6. Place 10 mL of methanol:3 mM Acetic Acid (90:10 v/v) solution into the column. Allow the eluate to pass through at a moderate drip and collect. Discard the C18 column.
7. When elution is finished, place the tube containing the extract onto the N-Evap and blow it down to approximately 1 mL.
8. Wash the sides of the tube with approximately 1 mL methanol and blow down to approximately 0.2 t 0.5 mL. Repeat with another 1 mL wash and blow down to dryness. Immediately stop the nitrogen flow and remove the tube from the N-Evap.
9. Add exactly 2.0 mL of HPLC or Milli-Q water to the tube containing the evaporated C18 eluate. Vortex mix for 5 seconds and place into the ultrasonic bath. Allow the sample to remain in the bath (set at 30 to 45 degrees) for 5 to 10 minutes
10. Filter approximately 1.0 mL of this final volume solution through a 0.2 micron AcroDisc filter into an appropriate autosampler vial. Cap.
11. This sample is now ready for *LC/MS/MS* analysis. It can be stored for up to 3 weeks in a refrigerator at 4 °C.

4.2.9b *Concentration and Purification Procedures for Soil*

Solution Requirements/Sample (extraction + purification):	
Milli- Q water	50 mL
Extraction solution	20 mL
methanol	10 mL
hexane	10 mL
90:10 acetone: 3 mM Acetic Acid	*10* mL
90:10 methanol:3mM Acetic Acid	10 mL

SAMPLE CONCENTRATION AND PURIFICATION

SAMPLE PREPARATION AND EXTRACTION

1. Remove samples from refrigerator or freezer and allow to warm to room temperature.
2. Weigh out a 5 g sample of the soil and place it into a 50 mL centrifuge bottle.
3. Fortify samples if required.
4. Add 20 mL of 0.1M KH_2PO_4/0.5M NaCl extraction solution. Manually shake 10 times. Place onto a wrist-action shaker and shake for 10 minutes at maximum speed.
5. Centrifuge the sample for 10 minutes at approximately 2500 rpm.
6. Attach a 20 mL syringe to a 0.45 µm Acrodisc filter. Decant the supernatant into the 2 syringe. Filter into a 50 mL centrifuge tube. Place tube immediately onto an N-Evap at 40 to 50 °C and begin evaporation.
7. Repeat steps 4 through 5. When there is sufficient room in the 50 mL centrifuge tube (on the N-Evap) filter the second supernatant into it. Rinse syringe with 2 x 10 mL volumes of acetone and place acetone into the 50 mL centrifuge tube containing the filtrate.
8. Evaporate the combined extract/rinse to the water phase (approximately 15 mL).
9. Add HPLC water until the volume is approximately 50 mL.
10. Just prior to proceeding to the SPE cleanup sonicate extract for 5 min. and vortex mix.

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ENVI-CARB BOND ELUT® CLEANUP

1. Place the 1.5 g Envi-Carb Bond Elut® into the vacuum manifold and condition them by placing 10 mL of 90:10 acetone:3 mM acetic acid solution into the tubes and adjusting the flow to a fast drip (~10 mL/minute). Using a gentle vacuum to pull through all of the liquid, let the column dry for 30 seconds after the last of the liquid has exited. Load and condition the column with two 10 mL volumes of water. Do not let the liquid level drop below the top of the sorbent after the second water load has passed through. Do not allow the tube to dry.
2. Load the soil extract onto the column, in several portions if necessary due to column reservoir capacity. Turn on the vacuum and allow the sample to pass through the column at a slow drip. Do not allow the column to go dry. Do not collect the liquid.
3. Place 5 mL of water into the empty 50 mL centrifuge tube, vortex. Place this rinse onto the Bond Elut and allow to pass through at a slow drip. Pull all the water through, allow to dry, passing air through for about 30 seconds after last drop has come off.
4. Place 5 mL of hexane into the Bond Elut and allow to pass through at a slow drip. Allow to dry, passing air through for about 30 seconds after last drop has come off.
5. Open the vacuum manifold and place plastic 15 mL centrifuge tubes into the manifold in order to collect the eluate.
10. Place 10 mL of 90:10 acetone:3 mM acetic acid solution into the Bond Elut tube and allow to pass through at a slow drip. Pull all of the liquid through into the collection tubes. Break the vacuum and remove the tubes containing the eluate.
11. Place sample on an N-Evap and evaporate to approximately 1 mL at 40-50 deg.
12. Wash the sides of the sample test tube with approximately 2 mal of acetone and evaporate the extract to 0.2 to 0.5 mL. Repeat this washing with a second 2 mL volume of acetone. Evaporate the sample to dryness
13. Add 200 µL of acetone to the tube, vortex and sonicate for 5 minutes, vortex again. Add approximately 1 mL of water and evaporate to the water phase. Add approximately 4 mL of HPLC water and continue the evaporation step to ensure removal of all traces of acetone This is a very critical step, the presence of any acetone will prevent the polar analytes from being retained on the C18 column in later steps.)
10. Adjust to approximately 5 mL with HPLC water. Vortex, sonicate for 5 minutes, vortex
11. Proceed directly to the C18 SPE clean-up. This is a stopping point. Samples can be placed into the refrigerator for storage for up to 1 week.

C18 BOND ELUT CLEANUP/FINAL PREPARATION

1. Place the C18 Bond Elut® tubes onto the vacuum manifold and condition them by placing 5 mL of methanol:3mM Acetic Acid (90:10 v/v) solution into each tube and adjusting the flow to a fast drip. Drain the tube and allow to dry for 30 seconds more under vacuum. In the same manner condition next with 10 mL of water, in two 5 mL increments, waiting until the first 5 mL is completely below the frit before adding the second 5 mL. Stop the flow when the level of the water drops slightly below the top of the sorbent in the bottom tube. Do not let the tubes dry.
2. Place the Envi-Carb eluate solution (step 11 ENVI-CARB cleanup above) into the C18 Bond Elut® tube. Apply vacuum, open the stopcock and load the eluate onto the column with a very slow drip. Stop when the level of the liquid is 1 cm above the top of the sorbent. Discard the aqueous effluent.
3. Backrinse the original Envi-Carb eluate container by placing 2.0 mL of water into it vortex mixing for a few seconds. Pour this rinsate into the C18 cartridge. Allow this

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(rinse)water wash to pass through the C18 and discard. Allow all of the water to be drawn out of the tube. Immediately shut off vacuum, discard load volume

4. Place 5.0 mL of hexane into the C18 cartridge. Allow the hexane to pass through at a moderate drip. After the hexane has completely passed through the column, add another 5.0 mL of hexane and allow that to completely pass through the column as well. Allow the vacuum to pull air through the cartridge until there is no more dripping.
5. Break vacuum and place a new 15 mL plastic centrifuge tube into the vacuum manifold under the C18 tubes to collect the eluate.
6. Place 10 mL of methanol:3 mM Acetic Acid (90:10 v/v) solution into the column. Allow the eluate to pass through at a moderate drip and collect. Discard the C18 column.
10. When elution is finished, place the tube containing the extract onto the N-Evap and blow it down to approximately 1 mL
11. *Wash the sides of the tube with approximately 1 mL methanol and blow down to* approximately 0.2 t 0.5 mL. Repeat with another 1 mL wash and blow down to dryness. Immediately stop the nitrogen flow and remove the tube from the N-Evap.
12. Add exactly 2.0 mL of HPLC or Milli-Q water to the tube containing the evaporated C18 eluate. Vortex mix for 5 seconds and place into the ultrasonic bath. Allow the sample to remain in the bath (set at 30 to 45 degrees) for 5 to 10 minutes
10. Filter approximately 1.0 mL of this final volume solution through a 0.2 micron AcroDisc filter into an appropriate autosampler vial. Cap.
11. This sample is now ready for LC/MS/MS analysis. It can be stored for up to 3 weeks in a refrigerator at 4 °C.

4.3 *Instrumentation*

4.3.1 *Description*

This method requires an LC/MS/MS instrument equipped with an electrospray interface for the analysis of water and soil. An autosampler is also required for the unattended analysis of multiple samples and calibration solutions. The analytical data in this method was obtained from a Micromass Quattro II LC/MS equipped with the HP Series 1100 Modular HPLC System which includes a dual channel pump, autosampler, vacuum degasser, temperature-controlled column compartment, and a UV-Vis detector (not required for this method). The HPLC and Mass Spectrometer operating parameters for these systems are given in section 4.3.2.

The chromatographic column and mobile phases specified provide good peak shapes and resolution of the analytes. This allows sufficient time to switch mass spectrometric acquisition parameters thereby allowing better optimization of each channel. The HPLC solvent program includes a period of high-organic solvent flow to purge matrix materials from the column and allows sufficient re-equilibration time between runs. Do not alter these periods. In general, instrument response is good to excellent for the analytes on the positive ESI SRM channels. Always use the combination of HPLC conditions and mass spectrometer parameters specified for each matrix.

4.3.2 *Operating Conditions: Micromass Quattro II with HP Series 1100 HPLC*

ANALYTICAL CONDITIONS FOR SOIL AND WATER

High Performance Liquid Chromatography (HPLC)

Mobile Phase A:	Aqueous 0.01 M acetic acid
Mobile Phase B:	Acetonitrile

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Mobile Phase Program:

Time(min)	%A	%B
0	100	0
3	90	10
10	50	50
15.0	25	75
15.1	5	95
20.0	5	95
20.1	0	100
30.0	0	100

On-column Flow Rate: 1.0 mL/min, split ~5:1, Divert 0-6.9, 15.5-30 min
Injection Volume: 50 µL
Column: Zorbax RX-C8, 25 cm × 4.6 mm id, 5 µm; with guard column attached
Column Temperature: 30 °C

Mass Spectrometer (MS)

Ionization Mode: Positive ESI
HV Lens: 0.26 KV
Capillary Voltage: 4.0 KV
Cone Voltage: 32 Volts
Collision Voltage: 18

General parameters: Positive ESI Modes
Collision Gas Pressure: 1.7-2.0x10e-3
MS1 LM/HM Resolution: both 10
MS2 LM/HM Resolution: both 12
Source Temperature: 85 °C

Acquisition Functions: set as shown below:

	Parent	Daughter	Dwell
1 ESP+ Start 7.0min End 9.5 Int Chan. Delay: 0.02 sec	171.1	71.1	0.12 sec
2 ESP+ Start 9.5min End 12.0 Int Chan. Delay: 0.02 sec	255.3	157.1	0.12 sec
	267.2	171.0	0.12
	269.3	171.0	0.12
3 ESP+ Start 12.0min End 17.0 Int Chan. Delay: 0.02 sec	239.2	157.0	0.08
	253.2	171.1	0.08

Approximate Retention Times of the 7 Analytes

Analyte	Approx. Retention Time (min)	Acquisition Function:Transition
metabolite G3170	8.89	1: 171.1 -71.0
metabolite A	10.68	2: 269.3-171.0
metabolite A-1	11.62	2: 269.3-171.0

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metabolite 1	11.27	2: 267.2-171.0
metabolite C	10.12	2: 255.3-157.0
hexazinone	14.46	3: 253.2-171.0
metabolite B	13.62	3: 239.2-157.0

Alternate Mass Spectrometer (MS) Conditions: Confirmation Channels

If co-eluting matrix components interfere with any of the analytes on their primary positive electrospray MS/MS channels, use the following electrospray conditions and alternate channels to both confirm that these co-eluting peaks are in fact interferences and to quantitate the analytes. Alternate channels can be determined from examining the full scan spectra or the daughter ion spectra of each analyte shown in Figure 2. The alternate electrospray parameters (increased collision gas energy) specified will also significantly reduce or eliminate the interfering peaks on the original channels. Re analyze samples using the alternate channels along with the originals for comparison.

Ionization Mode:	Positive ESI
HV Lens	0.1 KV
Capillary Voltage:	3.6 KV
Cone Voltage	*25 Volts*
Collision Voltage	30

General parameters	Positive ESI
Collision Gas Pressure	1.7-2.0x10e-3
MS1 LM/HM Resolution	*both 8*
MS2 LM/HM Resolution	both 12
Source Temperature:	85 °C

Acquisition Functions set for selected alternate MRM transitions:

4.3.3 Calibration Procedures

Instrumentation performance was initially evaluated for calibration, response, linearity by the following procedures:

1. *The LC/MS/MS instrument was tuned in positive electrospray mode to optimize the* (M-cyclohexane moiety)$^+$ peak while still showing the base peak $[MH]^+$ .For the negative electrospray compounds, the instrument was tuned to maximize the $[M-1]^-$ peak..
2. Full-scan, on-column analysis of a ≥ 4-µg/mL calibration solution containing each of the analytes was performed to verify appropriate m/z responses and abundance ratios.
3. Analysis of calibration solutions (see section *4.2.5* for preparation) and construction of a 5-point calibration curve was performed to verify linearity ($R^2 \geq 0.98$) over the analytical range and evaluate detector response (low calibration solution response ≥ 5/1 signal-to-noise).

Routine manufacturer instrument calibration and maintenance procedures should be followed as necessary to optimize performance. It is also necessary to ensure that the electrospray interface components are clean. A dirty source can cause drift in instrument response.

4.3.4 *Sample Analysis*

Initially, at least 1, and preferably 2 runs of a matrix-containing sample should be made to equilibrate the ESI-LC/MS system prior to running a sample set unless instrument performance allows otherwise.

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Calibration solutions and samples should be alternately analyzed through the analysis set so test samples can be quantified using the average response of bracketing standard (i.e., standard, sample, standard, sample, standard, etc.) If instrument performance is stable it is permissible to run 2 samples between each standard, provided a standard precedes the first sample of the set and follows the last sample of the set. At a minimum, a check sample consisting of a control fortified at the LOQ and processed through the method should be run with every sample set.

4.4 *Calculations*

4.4.1 *Methods*

The ppm found and percent of applied recoveries are determined from calculations which compare the peak area responses of each analyte to the response of bracketing analytical standards. Use only the primary MRM channel for quantitation. Do not use the Total Ion Chromatogram. Peak area response values should be entered in an EXCEL® spreadsheet template designed to perform the appropriate calculations.

The response factor (RF) is calculated for each analyte in all calibration standard analyses by dividing the peak area by the analyte concentration. One may use either the average response factor of the entire set of standards if the response has been stable over the period of analysis, or one may use the average of the closest two standards which bracket the sample being quantitated if instrument drift has been noted. In either case, valid recovery data are generated if the percent relative standard deviation (%RSD) for the peak area responses of the standards used is less than or equal to 20%. Values for the amount (ppm) detected in samples are determined by comparing the peak area of each sample analyte detected with the average response factor and correcting for aliquots, weight of sample, and final volume.
The percent of applied recoveries for fortified samples are calculated by dividing ppm found by ppm applied and multiplying by 100.

C (conc. of sample µg/mL) = Area(sample)/RF(avg.)

ppb (sample) = C/W * (1/AF) * FV

W = weight of sample (g, 1 mL = 1g)
AF = aliquot fraction (vol aliquot/total vol.)
FV = total volume of final analysis solution (mL)

Sample Calculations (Bracketing Standards):
For a hypothetical analyte:

20 mL (=20g) water sample fortified at 0.100 ppb, aliquot fraction = 1, Final Volume = 2 mL

Response of 0.67 ng/mL Std = 700 area counts
Response of 2.0 ng/mL Std = 2200 area counts
Response of 0.100 ppb fortification = 1200 area counts

RF(avg.)= [(2200/2.0 ng/mL) + (700/0.67 ng/mL)]/2 = 1072(mL/ng)
Conc. sample = 1200/1072(mL/ng) = 1.119 ng/mL
AF = 20mL/20mL = 1.0

ppb sample = [1.119 (ng/mL)/20g] * (1/1) * 2 mL = 0.1119 ng/g = 0.1119 ppb
recovery = 0.1119/0.1000 * 100% = 112%

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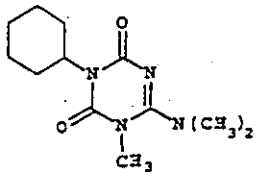
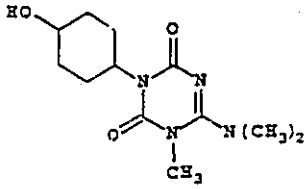
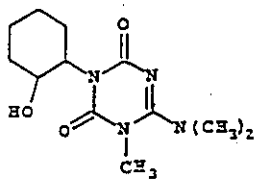
FIGURE 1
CHEMICAL STRUCTURES AND NAMES



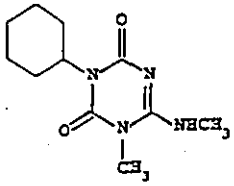
Hexazinone
DuPont Code No.: DPX-A3674
Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 3-cyclohexyl-6-dimethylamino -1-methyl
CAS Registry No.: 51235-04-2



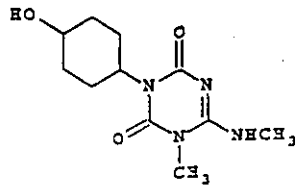
Metabolite A
DuPont Code No.: T3937
Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 6-dimethylamino-3-(4-hydroxycyclo- hexyl)-1-methyl
CAS Registry No.: none



Metabolite A1
DuPont Code No.: G3453
Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 3(trans-2hydroxycyclohexyl)-1-methyl-6-dimethylamino-
CAS Registry No.: none



Metabolite B
DuPont Code No.: IN-A3928
Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 3-cyclohexyl-1-methyl-6-(methylamino)-
CAS Registry No.: 56611-54-2



Metabolite C
DuPont Code No.: IN-T3935
Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 3-(4-hydroxycyclohexyl)-1-methyl-6 (methylamino)-
CAS Registry No.: 72585-88-7

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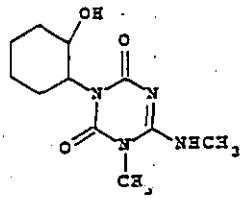
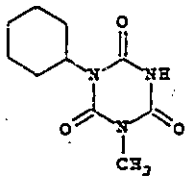
FIGURE 1 (CONTINUED)
CHEMICAL STRUCTURES AND NAMES



Metabolite C:
DuPont Code No.: IN-G3454

Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 3-(2-hydroxycyclohexyl)-1-methyl-6- (methylamino)-

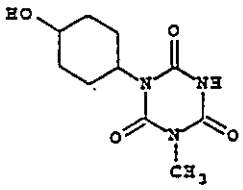
CAS Registry No.: none



Metabolite D
DuPont Code No.: IN-B2838

Chemical Name: S-Triazine-2,4,6-(1H,3H,5H)-trione, 1-cyclohexyl-3-methyl

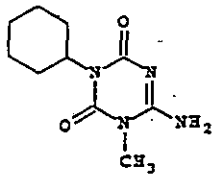
CAS Registry No.: none



Metabolite E
DuPont Code No.: IN-T3936

Chemical Name: S-Triazine-2,4,6-(1H,3H,5H)-trione, 1-(4 hydroxycyclohexyl)-3-methyl

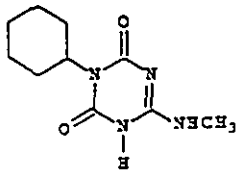
CAS Registry No.: none



Metabolite F
DuPont Code No.: IN-L3221

Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 3-cyclohexyl-6-amino-1-methyl-

CAS Registry No.: none



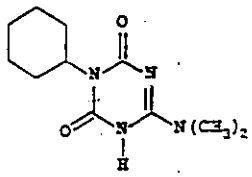
Metabolite G
DuPont Code No.: IN-T4916

Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 3-cyclohexyl-6-methylamino

CAS Registry No.: none

DuPont-2548

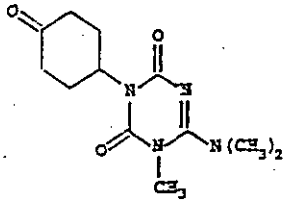
FIGURE 1 (CONTINUED)
CHEMICAL STRUCTURES AND NAMES



Metabolite H
DuPont Code No.: IN-M3111

Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 3 cyclohexyl-6-dimethylamino

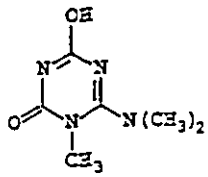
CAS Registry No.: none



Metabolite 1
DuPont Code No.: IN-JS472

Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 6-(dimethylamino) -1-methyl-3-(4-oxocyclohexyl)

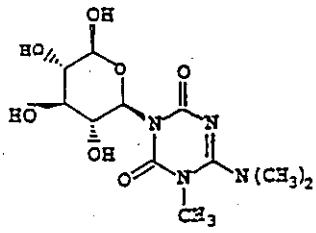
CAS Registry No.: none



Metabolite G3170
DuPont Code No.: IN-G3170

Chemical Name: S-Triazine-2,4-(1H,3H)-dione,1-methyl-6-dimethyl amino

CAS Registry No.: none



Metabolite G3170 n-glucoside
DuPont Code No.: IN-NC633

Chemical Name: S-Triazine-2,4-(1H,3H)-dione, 1-methyl-6-dimethylamino n-glucoside

CAS Registry No.: none

DuPont-2548

Modifications to Dupont-2550 to confirm by LC/MS/MS

If sample splitting is needed to allow confirmation by LC/MS/MS, the 16x100 mm silanized glass tube used in step 4.4.5 must be calibrated at 2.0 mL as well as 4.0 mL.

Follow the method (Met-93, revision 4) through step 4.5.1. At step 4.5.1 insert at end: Adjust to 4.0 mL with methanol, vortex to mix, sonicate 5 minutes. Readjust to 4.0 mL with methanol and vortex. Remove 2 mL for LC/MS/MS if required.

Evaporate to dryness. Continue with method DuPont-2292 (soil and water LC/MS/MS method) final preparation step 4.2.9: C18 Bond Elut Cleanup/Final Preparation, step 8.

Standards for LC/MS/MS analysis may be prepared from standard solutions used for GC/NPD analysis by evaporating the organic solvent and redissolving the residue in water with mixing and sonication.