2.0 INTRODUCTION

Independent laboratory validation of enforcement methods is required by the EPA (Draft Guidelines for the Independent Laboratory Validation of Environmental Chemistry Methods). The study was designed to demonstrate the utility, ruggedness, and efficiency of the subject methods and to identify any inherent weakness in the subject methods as written. The analytical methods DuPont-2550, "Analytical Method for the Determination of Hexazinone and Its Metabolites in Water by GC/NPD Analysis", and DuPont-2292 Draft 2 (April 23, 1999), "Enforcement Analytical Method for the Determination of Hexazinone and Metabolites of Interest in Soil and Water Using Electrospray-LC/MS/MS", both applicable for the quantitation of hexazinone, IN-T3937, IN-A3928, IN-T3935, IN-G3453, IN-JS472, and IN-G3170 in both lysimeter and ground water, were validated. Lysimeter and ground water are matrices that may be subject to enforcement testing for DPX-A3674, IN-T3937, IN-A3928, IN-T3935, IN-G3453, IN-JS472, and IN-G3170.

Samples were acidified and concentrated by solid phase extraction (SPE) using disposable graphitized carbon cartridges. The analytes were eluted from the SPE column with acid-acetone solution. The eluate was evaporated to dryness and brought to the water phase for a second column cleanup using a C18 SPE column. The analytes were then eluted from the column with acid-methanol. The eluate was evaporated to about 1 mL, then brought to a volume of 4.0 mL with methanol. The extract was split at this point and 2.0 mL was evaporated, redissolved in 2.0 mL HPLC water, sonicated, then filtered through a 0.2 micron Acro Disc filter. This extract was then analyzed by ESI-LC/MS/MS using positive mode multiple reaction monitoring (MRM) for hexazinone, IN-T3937, IN-A3928, IN-T3935, IN-G3453, IN-JS472, and IN-G3170. Quantitation was based on the integration of a single multiple reaction monitoring (MRM) transition response. The other 2.0 mL was evaporated and reconstituted into acetone, ethyl acetate, and toluene (for a total of 2.0 mL final volume) and analyzed by GC/NPD. Samples which were analyzed with only one instrument were not split, and were brought to a final volume of 4.0 mL in the proper solvents.

3.0 MATERIALS AND METHODS

3.1 Test Substances

DPX-A3674

DuPont Code Number:	DPX-A3674
Common Name:	Hexazinone
Reference Number:	not available

Chemical Name:	S-triazine-2,4/1H,3H/-dione 3-cyclohexyl-1-methyl-6-dimethyl-amino
CAS Registry No:	51235-04-2
Lot/Batch Number:	Dash # 233
Purity:	99.9%
Centre I.D. No.:	99-08-59
Storage Conditions:	Refrigerated

IN-T3937

DuPont Code Number:	IN-T3937
Common Name:	Metabolite A of Hexazinone
Reference Number:	AG0084-154
Chemical Name:	S-triazine-2,4/1H,3H/-dione 6-dimethylamino-3-/4-hydroxycyclo-hexyl/-1-methyl-
Lot/Batch Number:	Dash # 3
Purity:	99.0%
Centre I.D. No.:	99-08-55
Storage Conditions:	Frozen

IN-A3928

DuPont Code Number:	IN-A3928
Common Name:	Metabolite B of Hexazinone
Reference Number:	E76965-35
Chemical Name:	S-triazine-2,4/1H,3H/-dione 3-cyclohexyl-1-methyl-6-methylamino
CAS Registry Number:	56611-54-2
Lot/Batch Number:	Dash # 4
Purity:	96.3%
Centre I.D. No.:	99-08-56
Storage Conditions:	Room Temperature

IN-T3935

DuPont Code Number:	IN-T3935
Common Name:	Metabolite C of Hexazinone
Reference Number:	E5255-9
Chemical Name:	S-triazine-2,4/1H-dione 3-/4-hydroxycycohexyl/-1-methyl-6-methylamino-
CAS Reference Number:	72585-88-7
Lot/Batch Number:	Dash # 3
Purity:	95.6%
Centre I.D. No.:	99-08-57
Storage Conditions:	Frozen

IN-G3453

DuPont Code Number:	IN-G3453
Common Name:	Metabolite A1 of Hexazinone
Reference Number:	E70731-2
Chemical Name:	S-triazine-2,4/1H,3H/-dione 3/trans-2hydroxycyclohexyl/-1-methyl-6-dimethylamino
Lot/Batch Number:	Dash # 2
Purity:	99.4%
Centre I.D. No.:	99-08-58
Storage Conditions:	Room Temperature

IN-JS472

DuPont Code Number:	IN-JS472
Common Name:	Metabolite 1 of Hexazinone
Reference Number:	not available
Chemical Name:	1,3,5-triaxine-2,4(1H,3H)-dione,6-(dimethylamino)-1-methyl-3-(4-oxycyclohexyl)
Lot/Batch Number:	Dash # 2
Purity:	95.5%
Centre I.D. No.:	99-08-54
Storage Conditions:	Room Temperature

IN-G3170

DuPont Code Number:	IN-G3170
Common Name:	Metabolite G3170 of Hexazinone
Reference Number:	not available
Chemical Name:	S-triazine-2,4/1H,3H/-dione-1-methyl-6-dimethylamino-
Lot Number:	Dash # 2
Purity:	92.0%
Centre I.D. No.:	99-08-53
Storage Conditions:	Room Temperature

Characterization and certification records for the analytical standards will be archived by:

E.I. du Pont de Nemours and Company
Wilmington, DE 19898, U.S.A.

3.2 Test System

The analytical methods DuPont-2550, "Analytical Method for the Determination of Hexazinone and Its Metabolites in Water by GC/NPD Analysis", and DuPont-2292 Draft 2 (April 23, 1999), "Enforcement Analytical Method for the Determination of Hexazinone and Metabolites of Interest in Soil and Water Using Electrospray-LC/MS/MS", both applicable for the quantitation of hexazinone, IN-T3937, IN-A3928, IN-T3935, IN-G3453, IN-JS472, and IN-G3170 in water, were validated. In this study, the analytical methods were validated in both lysimeter and ground water.

Selected lysimeter water samples from DuPont Study No. AMR 3202-94 (CAL 9903356-9903364) were shipped to Centre Analytical Laboratories, Inc. The samples were shipped from Quanterra, Inc. in West Sacramento, CA on 4/16/99, received ambient at Centre Analytical Laboratories, Inc. on 4/19/99 and logged in and given unique identification numbers. They were then stored in a walk-in cooler at a temperature of +2°C to +8°C until 4/29/99 when the samples were combined. These samples were then combined forming the composite lysimeter water sample CAL no. 9903427 which was returned to the cooler until extraction.

Selected ground water sample from DuPont Study No. AMR 3202-94 (CAL 9902704-9902712 and 9903307-9903355) were shipped to Centre Analytical Laboratories, Inc. These samples were then combined forming the composite ground water sample CAL no. 9903428.

Samples 9902704-9902712 were shipped from Morse Laboratories in Sacramento, CA on 3/29/99, received frozen on dry ice at Centre Analytical Laboratories, Inc on 3/31/99 and immediately logged in and given unique identification numbers. These samples were then stored in a walk-in freezer at a temperature of $\leq$ -10°C until 4/29/99 when the samples were combined.

Samples 9903307-9903355 were shipped from Quanterra, Inc. in West Sacremento, CA on 4/16/99, received ambient at Centre Analytical Laboratories, Inc. on 4/19/99, and logged in and given unique identification numbers. They were then stored in a walk-in cooler at a temperature of +2°C to +8°C until 4/29/99 when the samples were combined.

The composite ground water sample consisting of samples 9902704-9902712 and 9903307-9903353 was then given the CAL no. 9903428 and stored in a walk-in cooler at a temperature of +2°C to +8°C until extraction.

3.3 Equipment

All equipment used in this study followed the guidelines specified in the method. Equivalent changes in apparatus were made in the following instance:

- The method specifies an HP5890 gas chromatograph and HP6890 autosampler, instead a Varian 3500 Gas Chromatograph with a Varian 8200 autosampler was used.

3.4 Reagents

Reagents used during the validation included the following:

Acetone- J.T. Baker, HPLC grade

Envi-Carb SPE cartridge – Supelco

C18 SPE cartridge - Varian

Dimethyldichlorosilane – Supelco

Ethyl Acetate – J.T. Baker, HPLC grade

Formic Acid - EM Science

Glacial Acetic Acid – EM Science

Hexane- Burdick & Jackson, HPLC grade

Methanol - Burdick & Jackson, HPLC grade

Potassium phosphate – J.T. Baker

Silica Gel - J.T. Baker

Sodium Chloride – J.T. Baker

Toluene - J.T. Baker, HPLC grade

All water was Type I distilled, deionized water (CAL)

3.5 Principles of the Analytical Methods

Samples were acidified and concentrated by solid phase extraction (SPE) using disposable graphitized carbon cartridges. The analytes were eluted from the SPE column with acid-acetone solution. The eluate was evaporated to dryness and brought to the water phase for a second column cleanup using a C18 SPE column. The analytes were then eluted from the column with acid-methanol. The eluate was evaporated to about 1 mL, then brought to a volume of 4.0 mL with methanol. The extract was split at this point and 2.0 mL was evaporated, redissolved in 2.0 mL HPLC water, sonicated, then filtered through a 0.2 micron Acro Disc filter. This extract was then analyzed by ESI-LC/MS/MS using positive mode multiple reaction monitoring (MRM) for hexazinone, IN-T3937, IN-A3928, IN-T3935, IN-G3453, IN-JS472, and

IN-G3170. Quantitation was based on the integration of a single MRM transition response. The other 2.0 mL was evaporated and reconstituted into acetone, ethyl acetate, and toluene (for a total of 2.0 mL final volume) and analyzed by GC/NPD. Samples which were analyzed with only one instrument were not split, and were brought to a final volume of 4.0 mL in the proper solvents.

3.6 Modifications, Interpretations and Critical Steps

These methods were run exactly as written, with the modification described in the protocol appendix. Protocol Deviation number 3 specifies a change made to the re-equilibration solvent from 100% acetonitrile (mobile phase B) to 100% aqueous 0.01M acetic acid (mobile phase A). Consultation with the sponsor verified that this was a typographical error in the original draft of the method. This protocol deviation did have an impact on the validity of the study. Without this deviation the instrument would not have been properly equilibrated between sample injections and the validation would not have passed. There was an additional change in the methods entailing a minor equivalent equipment substitution outlined in Section 3.3

3.7 Instrumentation

DuPont-2550

1. Instrument: Varian 3500
   Gas Chromatograph

2. Detector: Nitrogen Phosphorus Detector

3. Column: Rtx-35, 15m x 0.53mm x
   0.5 µm df

4. Integrator: HP Chemstation

5. Instrument Conditions:

   a. Gas Flow Rates: Hydrogen, 4 mL/min
   Column, 5 mL/min.
   Make-up, 15 mL/min.
   Gas, Helium

   b. Retention Time: ~ 4.8, 6.6, 6.9, 7.5, 7.7, 7.8,
   8.1 min.

   c. Run Time: ~ 9.5 min.

d. Injection Volume: 2 µL

e. Injector Temperature: 290°C

f. Detector Temperature: 285°C

g. Column Temperature: 140°C hold for 1 min., to 290°C at 20°C /min, hold for 1 min.

DuPont-2292 Draft 2 (April 23, 1999)

Instrument: LC/MS/MS
Micromass Quattro LC
Electrospray Ion source
Desolvation Temp.: 400°C
Desolvation N_2 Flow Rate: 850 L/hr
Source Temp.: 100°C
Nebuliser N_2 Flow Rate: 100 L/hr

Computer: Digital 266i Personal Workstation

Software: Microsoft Windows NT
Version 4 Build 1381: Service Pack 3
Micromass Limited
MassLynx 3.1 Build 004

HPLC Equipment: Hewlett Packard (HP) Series 1100
HP Quat Pump
HP Vacuum Degasser
HP Autosampler
HP Column Oven

HPLC Column: Zorbax Rx-C8, 4.6 mm x 25cm

Column Temperature: 35° C

Mobile Phase (A) : Aqueous 0.01 M acetic acid

Mobile Phase (B) : Acetonitrile

Time	% A	% B
0.0	100	0
3.0	90	10
10.0	50	50
15.0	25	75
15.1	5	95
20.0	5	95
20.1	100	0
30.0	STOP	STOP

Total run time: 30 min.
Flow Rate: 1.0 mL/min (Split Flow 5:1)
Injected Volume: 50 µL

Ions monitored :

Analyte	Parent ion	Daughter ion	Approximate Retention Time (min.)
Hexazinone	253.0	171.0	13.1
IN-G3170	171.0	71.1	7.2
IN-T3935	255.0	156.8	8.7
IN-T3937	269.0	171.0	9.3
IN-JS472	267.0	171.0	9.9
IN-G3453	269.0	171.0	10.3
IN-A3928	239.3	156.9	12.2

A full-scan was performed to verify appropriate m/z responses and abundance ratios (See Appendix 1).

3.8 Calculations

The calculations were performed exactly as described in the analytical methods. For DuPont-2550, the ppb found and percent recoveries were determined from calculations using the peak area responses of each analyte in the sample. These data were plotted versus concentration (ng/mL) of the corresponding standard to obtain linear regression standard calibration curves. Standard curves were prepared each analysis day.

An example of the calculation is presented here using an actual sample of IN-G3170 in lysimeter water Spk B, reported in Table 1:

1. $$\mu g/mL \text{ analyte} = \frac{8687.855 - (-50.189)}{65720.231} = 0.133 \ \mu g/mL$$

2. ppb analyte =

$$\frac{0.133\ \mu g/mL \times 1000 \times 2\ mL\ final\ vol. \times 1\ GC\ dil.\ fact.^{*}}{50\ mL\ sample}$$

$$= 5.32\ ppb$$

* Dilution factor is defined as : total volume (mL) ÷ volume extract added (mL)

3. % Recovery =

$$\frac{5.32\ ppb - \left(\frac{(1.64\ ppb\ \ control(1) + 1.90\ ppb\ \ control\ (2))}{2}\right)}{4.0\ ppb\ \ added}$$

$$= 89\%$$

For DuPont-2292 Draft 2 (April 23, 1999), the ppb found and percent recoveries were determined from calculations using the peak area responses of each analyte in the sample and the average response factor from the one preceding standard and the one following standard.

An example of the calculation is presented here using an actual sample of IN-G3170 in lysimeter water Spk B, reported in Table 3:

1. The ppb Found calculated for Run 052799-010:

Response Factor of preceding Std.
3627/5.00 = 725 area/ng/mL

Response Factor of following Std.
7379/10.0 = 738 area/ng/mL

Average RF =732

$$\text{Calc. Amt. (ng/mL)} = \frac{3311}{(732)(1/1)} = 4.53 \text{ ng/mL}$$

$$\text{Amt (ppb)} = \frac{(4.53 \text{ ng/mL})(4 \text{ mL})(20 \text{ HPLC dil. fact.})}{(100 \text{ mL})} = 3.62 \text{ ppb}$$

% recovery = (ppb found/ppb added) x 100
= (3.62 ppb/4.00ppb) x 100 = 89%*

Note: non-rounded values were used for all calculations