

Test Material: Hexazinone

MRID: 45132802

Title: Independent Laboratory Validation of a Proposed Environmental Chemistry Analytical Method for the Determination of Hexazinone and Metabolites in Soil by GC/NPD Analysis and Confirmation by LC/MS/MS

EPA PC Code: 107201

OCSPP Guideline: 850.6100

For CDM Smith

Primary Reviewer: Lynne Binari

Signature: 

Date: 8/21/14

Secondary Reviewer: Lisa Muto

Signature: 

Date: 8/21/14

QC/QA Manager: Joan Gaidos

Signature: 

Date: 8/21/14

Analytical method for hexazinone and its transformation products IN-T3937, IN-A3928, IN-T3935, IN-G3453, IN-JS472, and IN-G3170 in soil

- Reports:** ECM (GC-NPD): EPA MRID No.: 45132802 (Appendix 2, pp. 75-92). Brookey, F. and K. Clark (Appendix 2, p. 92). 1996. DETERMINATION OF HEXAZINONE AND ITS METABOLITES IN SOIL. MORSE SOP# Meth-101, Revision #2 (Appendix 2, p. 75). Report prepared by MORSE LABORATORIES, INC., sponsor not specified, submitted by E. I. du Pont de Nemours and Company, Wilmington, Delaware; 18 pages. Final report issued October 24, 1996.
- ECM (LC/MS/MS): EPA MRID No.: 45132802 (Appendix 2, pp. 93-122). Brill, F. and T. Gardner. 1999. Enforcement Analytical Method for the Determination of Hexazinone and Metabolites of Interest in Soil and Water Using Electrospray-LC/MS/MS. Dupont Project and Report No.: DuPont-2292. Report prepared, sponsored, and submitted by E. I. du Pont de Nemours and Company, Wilmington, Delaware; 30 pages. Draft 2 report issued April 23, 1999.
- ILV: EPA MRID No.: 45132802. Zheng, S. 1999. Independent Laboratory Validation of a Proposed Environmental Chemistry Analytical Method for the Determination of Hexazinone and Metabolites in Soil by GC/NPD Analysis and Confirmation by LC/MS/MS. Centre Analytical Laboratories Study No.: 008-036. DuPont Project No.: DuPont-2547. Report prepared by Centre Analytical Laboratories, Inc., State College, Pennsylvania, sponsored and submitted by E. I. DuPont de Nemours and Company, Wilmington, Delaware; 145 pages. Final report issued December 20, 1999.
- Document No.:** MRID 45132802
- Guideline:** 850.6100
- Statements:** ECM (GC-NPD): It was not specified whether or not the study was conducted in compliance with USEPA FIFRA Good Laboratory Practice (GLP) standards (Appendix 2, pp. 75-92). Signed and dated No Data Confidentiality, GLP, Quality Assurance, and Authenticity Certification statements were not provided.
- ECM (LC/MS/MS): It was not specified whether or not the study was conducted in compliance with USEPA FIFRA Good Laboratory Practice (GLP) standards (Appendix 2, pp. 93-122). Signed and dated No Data Confidentiality, GLP, Quality Assurance, and Authenticity Certification statements were not provided.
- ILV: The study was conducted in accordance with USEPA FIFRA GLP standards (p. 3). Signed and dated No Data Confidentiality, GLP, Quality Assurance, and Authenticity Certification statements were provided (pp. 2-5).

Classification: This analytical method is considered unacceptable, for the following reasons.

- No originating ECM performance data were reported for either method. For both methods, complete, finalized ECM reports with implemented ILV major modifications were not provided. Two sets of performance data should be submitted, one for the initial or other internal validation (ECM), and one for the ILV. Furthermore, these reports should be provided separate from each other.
- For ILV analysis of IN-T3935 by GC-NPD, no performance data at 10xLOQ were provided.
- For the LC/MS/MS method, ILV performance data for 2.0 µg/kg (LOQ) fortified IN-JS472 and 20 µg/kg (10x LOQ) fortified IN-G3170 in soil did not meet OCSPP Guideline 850.6100 criteria.
- The determination of LOQ and LOD were not based on scientifically acceptable procedures (for guidance, refer to the references section of this DER).
- Linearity (r^2) of the calibration standards was not always ≥ 0.995 .

Portions of the study appeared to meet guideline criteria and may be upgradable with the submission of additional information. Overall mean method recoveries reported by the ILV met OCSPP Guideline 850.6100 criteria for both precision and accuracy, with the exception of LC/MS/MS analysis of 2.0 µg/kg (LOQ) IN-JS472 (mean 65%) and 20 µg/kg (10x LOQ) IN-G3170 (mean 66%, RSD 30%).

PC Code: 107201

Signature:

Reviewer: José L. Meléndez, USEPA

Date: November 4, 2014

All page citations refer to the page numbers located in the bottom right corner of the MRID.

Executive Summary

Analytical method DuPont-2549 is designed for the quantitative determination of hexazinone and its products IN-T3937, IN-A3928, IN-T3935, IN-G3453, IN-JS472, and IN-G3170 in soil using GC-NPD, and analytical method DuPont-2292 Draft 2 (April 23, 1999) is designed for the confirmatory determination of hexazinone and its products in soil and water using LC/MS/MS. Validation of the LC/MS/MS method with water is addressed in a separate submission (MRID 45132803). The GC-NPD ECM did not establish LOQs for hexazinone and its products in soil. For GC-NPD analysis, the ILV found the method is quantitative at LOQs of 20 µg/kg for hexazinone, IN-T3937, IN-A3928, IN-G3453, IN-JS472 and IN-G3170, and 40 µg/kg for IN-T3935 µg/L. The LC/MS/MS ECM stated LOQs of 2.0 µg/kg for all analytes in soil. For LC/MS/MS analysis, the ILV found the method is quantitative at LOQs of 2.0 µg/kg for hexazinone, IN-T3937, IN-A3928, IN-G3453 and IN-G3170, and 4.0 µg/kg for IN-T3935; recoveries of IN-JS472 at the LOQ (2.0 µg/kg) did not meet OCSPP Guideline 850.6100 criteria.

The lowest toxicological level of concern in soil was not stated in the study. The GC-NPD method specifies the correction of procedural recoveries for residues in the controls; however, no residues were detected in the control samples. The independent laboratory reported that one validation set of soil was extracted and analyzed by each method. For both methods, complete, finalized ECM reports with implemented ILV major modifications were not provided.

Table 1. Analytical Method Summary

Analyte(s) by Pesticide	MRID		EPA Review	Matrix	Method Date (dd/mm/yyyy)	Registrant	Analysis	Limit of Quantitation (LOQ)
	Environmental Chemistry Method	Independent Laboratory Validation						
Hexazinone	45132802	45132802		Soil	GC-NPD: 24/10/1996	E. I. du Pont de Nemours and Company	GC-NPD	20 µg/kg
IN-T3937								20 µg/kg
IN-A3928								20 µg/kg
IN-T3935								40 µg/kg
IN-G3453								20 µg/kg
IN-JS472								20 µg/kg
IN-G3170								20 µg/kg
Hexazinone					LC/MS/MS ¹ : 23/04/1999	LC/MS/MS	2.0 µg/kg	
IN-T3937							2.0 µg/kg	
IN-A3928							2.0 µg/kg	
IN-T3935							4.0 µg/kg	
IN-G3453							2.0 µg/kg	
IN-JS472							2.0 µg/kg	
IN-G3170							2.0 µg/kg	

¹ The LC/MS/MS ECM covers analysis of both water and soil matrices, whereas, this ILV (MRID 45132802) is for analysis of soil only. The ILV for analysis of hexazinone and its products in water is addressed in a separate submission (MRID 45132803).

I. Principle of the Method

GC-NPD Method:

Samples (5.0 g) of soil were extracted twice with 20 mL of acetone:0.1 M monopotassium phosphate/0.5 M sodium chloride solution (1:1, v:v) by shaking (wrist-action shaker, maximum speed) for 10 minutes (Appendix 3, pp. 78, 80). Soil and extract were separated by centrifugation (*ca.* 2,500 rpm, 10 minutes). Extracts were filtered, combined, and the solvent removed under nitrogen (Turbo-Vap, 50°C; Appendix 2, pp. 76, 80-81). The aqueous sample was brought to volume (50 mL) with HPLC grade water, then loaded onto a solid-phase extraction (SPE) cartridge (Supelclean Envi-Carb SPE Tube, Custom, 12 mL polypropylene tube with Teflon frit packed with 1.5 g of Envi-Carb), preconditioned with acetone:3 mM acetic acid (9:1, v:v) and water (Appendix 2, pp. 76, 81). The loaded sample was rinsed with water, followed by hexane, then analytes were eluted with acetone:3 mM acetic acid (9:1, v:v, 10 mL) into a silanized glass tube. The eluate was taken to dryness under nitrogen (N-Evap, 40-50°C) in the presence of acetone (Appendix 2, p. 82). Resulting residues were reconstituted in acetone (200 µL), diluted with water, and all trace of solvent removed under nitrogen (final volume 5 mL). The aqueous sample was loaded onto a reverse-phase SPE cartridge (Varian, C18 Mega Bond Elut, 6 cc/1g) preconditioned with methanol:3mM acetic acid (9:1, v:v) and water. The

loaded sample was rinsed with water, followed by hexane, then analytes were eluted with methanol:3mM acetic acid (9:1, v:v, 10 mL) into a silanized glass tube (Appendix 2, p. 83). The eluate was taken to dryness under nitrogen (N-evap, *ca.* 50°C) in the presence of methanol, with resulting residues reconstituted in acetone, ethyl acetate and toluene (final solvent ratio 20:50:30, v:v:v) for GC-NPD analysis.

Samples were analyzed for hexazinone (DPX-A3674) and its products IN-T3937 (Metabolite A), IN-A3928 (Metabolite B), IN-T3935 (Metabolite C), IN-G3453 (Metabolite A1), IN-JS472 (Metabolite 1), and IN-G3170 (Metabolite G3170) by GC (RTX-35, 0.53 mm x 15 m, 0.5 µm df column) with nitrogen-phosphorous detection (NPD): carrier gas helium, column gas flow rate 5 mL/minute, injector temperature 290°C, detector temperature 285°C, initial column temperature 150°C, ramp rate (1) 25°C/minute to 275°C and hold for 2.2 minutes, or ramp rate (2) 10°C/minute, final temperature 285°C and hold for 1.0 minute (Appendix 2, pp. 75, 84). Injection volume was 2 µL.

LC/MS/MS Method:

This method provides for the quantification of hexazinone and its products in both water and soil matrices; however, only methods regarding the soil matrix are presented in this DER. Methods regarding quantification of analytes in water are addressed in a separate submission (MRID 45132803).

Samples (5.0 g) of soil were extracted twice with 20 mL of acetone:0.1 M monopotassium phosphate/0.5M sodium chloride solution (9:1, v:v), with the resulting extract purified and concentrated using Envi-Carb and C18 Mega Bond Elut SPE as described above (Appendix 2, pp. 96-99, 102-104). The resulting C18 SPE eluate was taken to dryness under nitrogen (N-evap, temperature not specified) in the presence of methanol, with resulting residues reconstituted in HPLC or Milli-Q water and filtered (0.2 µm) for LC/MS/MS analysis.

Samples were analyzed for hexazinone and its products by HPLC (Zorbax RX-C8, 4.6 mm x 25 cm, 5 µm column, with guard column attached, column temperature 30°C) using a mobile phase of (A) aqueous 0.01 M acetic acid and (B) acetonitrile [percent A:B at 0.0 min. 100:0, 3.0 min. 90:10, 10.0 min. 50:50, 15.0 min. 25:75, 15.1-20.0 min. 5:95, 20.1-30.0 min. 0:100] with MS/MS-ESI (electrospray ionization, positive) detection and multiple reaction monitoring (MRM; Appendix 2, pp. 97, 104-106). Injection volume was 50 µL. Analytes were identified using one ion transition. Ion transitions monitored were as follows: m/z 253.2→171.0 for hexazinone, m/z 269.3→171.0 for IN-T3937, m/z 239.2→157.0 for IN-A3928, m/z 255.3→157.0 for IN-T3935, m/z 269.3→171.0 for IN-G3453, m/z 267.2→171.0 for IN-JS472, and m/z 171.1→71.0 for IN-G3170 (Appendix 2, pp. 105-106).

ILV:

Samples (5 g) of soil were extracted (acetone:aqueous buffer ratio used not specified) and the resulting extract purified and concentrated using Envi-Carb and C18 SPE as described above (p. 14). The resulting C18 SPE eluate was evaporated to *ca.* 1 mL, brought to 2.0 mL with methanol, and the sample divided into two 1.0-mL aliquots. One sample was evaporated and the resulting residues reconstituted in acetone, ethyl acetate and toluene (final volume 1.0 mL) as described above for GC-NPD analysis (p. 15). GC-NPD analysis was conducted as described above with

the following modification: initial column temperature 140°C and hold for 1 minute, ramp rate 20°C/minute, final temperature 290°C and hold for 1 minute (pp. 15-16). The other sample was evaporated and the resulting residues reconstituted in water and filtered as described above for LC/MS/MS analysis (pp. 14-15). LC/MS/MS analysis was conducted as described above with the following modifications: LC column temperature 35°C and changing the re-equilibration solvent from 100% acetonitrile (mobile phase B) to 100% aqueous 0.01M acetic acid (mobile phase A) [percent A:B at 0.0 min. 100:0, 3.0 min. 90:10, 10.0 min. 50:50, 15.0 min. 25:75, 15.1-20.0 min. 5:95, 20.1-30.0 min. 100:0], plus minor changes to optimize MS/MS instrument conditions (pp. 15-16).

In the ILV, LOQs for GC-NPD analysis were reported at 20 µg/kg (ppb) for hexazinone, IN-T3937, IN-A3928, IN-G3453, IN-JS472 and IN-G3170, and 40 µg/kg for IN-T3935 (p. 20); LOQs for the analytes were not reported in the ECM (Appendix 2, pp. 75-92). For GC-NPD analysis, LODs for the analytes were not specified in either the ECM or ILV. For LC/MS/MS analysis, LOQs for all analytes were reported at 2.0 µg/kg in the ECM, with LODs estimated at 0.008-0.02 µg/kg (Appendix 2, p. 110). In the ILV, LOQs for LC/MS/MS analysis were reported at 2.0 µg/kg for hexazinone, IN-T3937, IN-A3928, IN-G3453, IN-JS472 and IN-G3170, and 4.0 µg/kg for IN-T3935, with LODs set at 0.67 µg/kg (p. 20; Appendix 5, pp. 139-145).

II. Recovery Findings

ECMs (Appendix 2, pp. 75-126 of MRID 45132802): Originating ECM performance data were not reported for either the GC-NPD (quantitation) method or LC/MS/MS (confirmation) method.

ILV (MRID 45132802): For GC-NPD (quantitation) analysis, mean recoveries and relative standard deviations (RSDs) were within guideline requirements (mean 70-120%; RSD ≤20%) for analysis of hexazinone and its products IN-T3937, IN-A3928, IN-G3452, IN-JS472 and IN-G3170 at fortification levels of 20 µg/kg (LOQ) and 200 µg/kg (10x LOQ, DER Attachment 2). For product IN-T3935, mean recoveries and RSDs were within guideline criteria at fortification levels of 40 µg/kg (LOQ) and 200 µg/kg (5x LOQ); performance data at 10x LOQ were not reported.

For LC/MS/MS (confirmation) analysis, mean recoveries and RSDs were within guideline criteria at fortification levels of 2.0 µg/kg (LOQ) and 20 µg/kg (10x LOQ) for hexazinone, IN-T3937, IN-A3928, IN-G3452, IN-J472 and IN-G3170, with the exception of 2.0 µg/kg IN-JS472 (mean 65%) and 20 µg/kg IN-G3170 (mean 66%, RSD 30%). For product IN-T3935, mean recoveries and RSDs were within guideline criteria at fortification levels of 4.0 µg/kg (LOQ) and 40 µg/kg (10x LOQ).

Calculation of recovery values allowed for the correction of residues in the matrix control samples; however, no residues were detected in the control samples (Appendix 5, pp. 132-145). The independent laboratory reported that one validation set of soil was extracted and analyzed by both GC-NPD and LC/MS/MS (p. 9). The soil was fully characterized, except the soil moisture content was not reported (Appendix 4, p. 130).

Table 2. Initial Validation Method Recoveries for Analytes in Soil

Analyte	Fortification Level (µg/L)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%) ¹	Relative Standard Deviation (%)
Hexazinone	LOQ					
	10x LOQ					
IN-T3937	LOQ					
	10x LOQ					
IN-A3928	LOQ					
	10x LOQ					
IN-T3935	LOQ		No originating ECM performance data were reported for either method (GC-NPD or LC/MS/MS).			
	10x LOQ					
IN-G3453	LOQ					
	10x LOQ					
IN-JS472	LOQ					
	10x LOQ					
IN-G3170	LOQ					
	10x LOQ					

Table 3. Independent Validation Method Recoveries for Analytes in Loamy Sand Soil

Analyte	Fortification Level (µg/kg)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
Hexazinone (DPX-A367)	GC-NPD (quantitation)					
	20 (LOQ)	5	84-114	99	11	11
	200	5	64-99	86	13	16
	LC/MS/MS (confirmation)					
	2.0 (LOQ)	5	92-105	97	5	5
	20	5	92-101	96	3	4
IN-T3937	GC-NPD (quantitation)					
	20 (LOQ)	5	94-120	106	11	11
	200	5	63-90	79	10	13
	LC/MS/MS (confirmation)					
	2.0 (LOQ)	5	84-99	93	6	7
	20	5	94-101	97	3	3
IN-A3928	GC-NPD (quantitation)					
	20 (LOQ)	5	88-118	98	12	13
	200	5	69-99	84	11	13
	LC/MS/MS (confirmation)					
	2.0 (LOQ)	5	91-104	98	6	6
	20	5	91-100	96	4	4
IN-T3935	GC-NPD (quantitation)					
	40 (LOQ)	5	100-126	112	11	10
	200	5	77-114	96	13	14
	LC/MS/MS (confirmation)					
	4.0 (LOQ)	5	72-87	79	6	7
	40	5	82-93	85	4	5
IN-G3453	GC-NPD (quantitation)					
	20 (LOQ)	5	99-123	108	10	9
	200	5	68-97	83	11	14
	LC/MS/MS (confirmation)					
	2.0 (LOQ)	5	81-95	87	6	7
	20	5	88-92	90	2	2
IN-JS472	GC-NPD (quantitation)					
	20 (LOQ)	5	83-108	95	11	11
	200	5	64-88	76	9	12
	LC/MS/MS (confirmation)					
	2.0 (LOQ)	5	57-70	65	5	8
	20	5	79-88	84	4	4
IN-G3170	GC-NPD (quantitation)					
	20 (LOQ)	5	80-119	105	15	14
	200	5	73-91	80	9	11
	LC/MS/MS (confirmation)					
	2.0 (LOQ)	5	81-87	84	3	3
	20	5	37-88	66	20	30

Data were obtained from Tables 1-2, pp. 22-23 and DER Attachment 2 (means, SDs and RSDs per fortification level were not provided by the study author).

III. Method Characteristics

GC-NPD method: In the ILV, LOQs were reported at 20 µg/kg (ppb) for hexazinone, IN-T3937, IN-A3928, IN-G3453, IN-JS472, and IN-3170, and 40 µg/kg for IN-T3935 (p. 20); LOQs for the analytes were not reported in the ECM (Appendix 2, pp. 75-92). No justification for the selected LOQs were provided. LODs for the analytes were not specified in either the ECM or ILV; however, the ILV may have set LODs at the lowest calibration standard (0.03 µg/mL for hexazinone, IN-A3928, IN-G3453, IN-T3937, IN-JS472, and IN-3170, and 0.06 µg/mL for IN-T3935; Appendix 4, pp. 132-138).

LC/MS/MS method: In the ILV, LOQs were reported at 2.0 µg/kg for hexazinone, IN-T3937, IN-A3928, IN-G3453, IN-JS472 and IN-G3170, and 4.0 µg/kg for IN-T3935, with LODs set at 0.67 µg/kg (p. 20; Appendix 5, pp. 139-145). In the ECM, LOQs for all analytes were reported at 2.0 µg/kg, with LODs estimated at 0.008-0.02 µg/kg (Appendix 2, p. 110). No justification for the selected LOQs was provided in the ILV or ECM. The ECM defined the LOD as the level where the signal to noise ratio is in the range of 3:1 to 5:1 at the expected retention time of the analyte (Appendix 2, p. 110).

Table 4. Method Characteristics

	Hexazinone	IN-T3937	IN-A3928	IN-T3935	IN-G3453	IN-JS472	IN-G3170
Loamy Sand Soil							
GC-NPD (quantitation)							
Limit of Quantitation (LOQ)	20 µg/kg	20 µg/kg	20 µg/kg	40 µg/kg	20 µg/kg	20 µg/kg	20 µg/kg
Limit of Detection (LOD)	Not reported.						
Linearity (calibration curve r^2 and concentration range) ¹	$r^2 = 0.9508$ (0.03-0.20 µg/mL)	$r^2 = 0.9817$ (0.03-0.375 µg/mL)	$r^2 = 0.9902$ (0.03-0.375 µg/mL)	$r^2 = 0.9773$ (0.06-0.75 µg/mL)	$r^2 = 0.9853$ (0.03-0.375 µg/mL)	$r^2 = 0.9856$ (0.03-0.375 µg/mL)	$r^2 = 0.9788$ (0.03-0.25 µg/mL)
Repeatable	Yes	Yes	Yes	Yes ³	Yes	Yes	Yes
Reproducible	Yes; however, performance data establishing the LOQs for the analytes were not provided with the ECM.						
Specific	Yes						
LC/MS/MS (confirmation)							
Limit of Quantitation (LOQ)	2.0 µg/kg	2.0 µg/kg	2.0 µg/kg	4.0 µg/kg	2.0 µg/kg	2.0 µg/kg	2.0 µg/kg
Limit of Detection (LOD)	0.67 µg/kg						
Linearity (calibration curve r^2 and concentration range) ²	$r^2 = 0.9957$ (3.33-50 ng/mL)	$r^2 = 0.9968$ (3.33-50 ng/mL)	$r^2 = 0.9975$ (3.33-50 ng/mL)	$r^2 = 0.9931$ (3.33-50 ng/mL)	$r^2 = 0.998$ (3.33-50 ng/mL)	$r^2 = 0.9981$ (3.33-50 ng/mL)	$r^2 = 0.9873$ (3.33-50 ng/mL)
Repeatable	Yes	Yes	Yes	Yes	Yes	No at LOQ (mean 65%) Yes at 10x LOQ	Yes at LOQ No at 10x LOQ (mean 66%, RSD 30%)
Reproducible ^{4,5}	Yes	Yes	Yes	Yes	Yes	No	Yes
Specific	Yes						

Data were obtained from p. 20; Appendix 5, pp. 132-145.

1 Reviewer-calculated calibration curves verified reported linearity for the ILV ($r^2 = 0.9508-0.9902$; see DER Attachment 2). Linearity is satisfactory when $r^2 \geq 0.995$.

2 Calibration curve r^2 values determined by reviewer (DER Attachment 2). Calibrations standards were analyzed, but calibration curves were not provided (Appendix 4, pp. 139-145). Linearity is satisfactory when $r^2 \geq 0.995$.

3 Performance data at 10x LOQ were not reported (Table 2, p. 23)

4 The ILV validated the method at the LOQ, with the exception of 2.0 µg/kg (LOQ) IN-JS472 (mean 65%).

5 Performance data establishing the LOQs for the analytes were not provided with the ECM.

IV. Method Deficiencies and Reviewer's Comments

1. For both the GC-NPD (quantitation) and LC/MS/MS (confirmation) methods, no originating ECM performance data were reported (Appendix 2, pp. 110-159).
2. For GC-NPD analysis of product IN-T3935, no performance data at 10x LOQ were provided. A validation sample set should consist of, at a minimum, a reagent blank, two unspiked matrix control samples, five matrix control samples spike at the LOQ, and five matrix control samples spiked at 10x LOQ for each analyte and matrix.
3. For the ILV, the following fortifications did not meet OCSPP Guideline 850.6100 criteria for precision and accuracy (mean recoveries for replicates at each spiking level between 70% and 120%, and relative standard deviations (RSD) $\leq 20\%$): LC/MS/MS analysis of 2.0 µg/kg (LOQ) fortified IN-JS472 (mean 65%) and 20 µg/kg (10x LOQ) fortified IN-G3170 (mean 66%, RSD 30%; DER Attachment 2).
4. The determination of the LOQ and LOD were not based on scientifically acceptable procedures. For either method (GC-NPD, LC/MS/MS), no justification for the selected LOQs for the analytes (20 or 40 µg/kg for GC-NPD, 2.0 or 4.0 µg/kg for LC/MS/MS) were provided. LODs were not reported for GC-NPD analysis. For LC/MS/MS analysis, LODs for all analytes were set at 0.67 µg/kg in the ILV (Appendix 4, pp. 139-145) and estimated at 0.008-0.020 µg/kg in the ECM (Appendix 2, p. 110), with the ECM defining the LOD as the level where the signal to noise ratio is in the range of 3:1 to 5:1 at the expected retention time of the analyte. Detection limits should not be based on the arbitrarily selected lowest concentration in the spiked samples. Additionally, the lowest toxicological level of concern in soil was not reported. An LOQ above toxicological levels of concern results in an unacceptable method classification.
5. For the ILV, calculation of recovery values allowed for the correction of residues in the matrix control samples; however, no residues were detected in the control samples (Appendix 5, pp. 132-145).

The GC-NPD ECM residue calculations specified for the correction of sample recoveries for any residues found in the matrix control samples (Appendix 2, pp. 85-86); however, the LC/MS/MS ECM residue calculations did not specify for correction of sample recoveries (Appendix 2, p. 107).

6. For both the GC-NPD and LC/MS/MS methods, complete, finalized ECM reports with implemented ILV major modifications were not provided.
 - a) For the GC-NPD analysis method, DuPont-2549, "Analytical Method for the Determination of Hexazinone and Its Metabolites in Soil by GC-NPD Analysis"

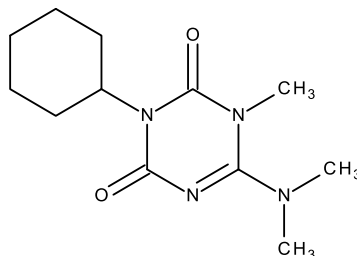
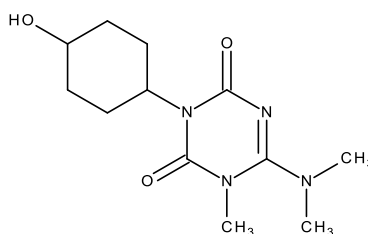
(p. 13), the ECM report was either not adequately identified and/or a complete ECM report was not provided (Appendix 2, pp. 75-92). The ECM is appended to the ILV, and, it appears that in preparation of the ILV report, pages 1-11 of the ECM were omitted. The GC-NPD ECM appended to the ILV report is titled MORSE LABORATORIES SOP# Meth-101, Revision #2, Determination of Hexazinone and Its Metabolites in Soil, but does appear to be analytical method DuPont-2549 based on the general description of the method provided by the independent laboratory (pp. 11, 14). The modification to method DuPont-2549 to include a sample splitting procedure at step 4.4.5 of the GC-NPD method to allow for LC/MS/MS confirmation (Appendix 2, p. 125), plus the required LC/MS/MS conditions should be included as part of the finalized GC-NPD ECM. It should also be noted that the modification to method DuPont-2549 (Appendix 2, pp. 124-125) appears to be appended to the LC/MS/MS method (Appendix 2, pp. 93-122), rather than the GC-NPD method to which it pertains (Appendix 2, pp. 75-92).

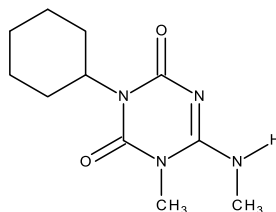
- b) The LC/MS/MS method appended to the ILV report is labeled as a Draft (Appendix 2, pp. 93-122). The major ILV modification to the method, changing the re-equilibration solvent from 100% acetonitrile (mobile phase B) to 100% aqueous 0.1M acetic acid (mobile phase A), was not implemented in, or even appended to, the ECM report (pp. 15-16; Appendix 2, pp. 104-105). In addition, the ECM covers analysis of both water and soil matrices, whereas, this ILV (MRID 45132802) is for analysis of soil only. The ILV for analysis of hexazinone and its products in water is addressed in a separate submission (MRID 45132803).
7. The soil used in the ILV was fully characterized, except the soil moisture content was not reported (Appendix 4, p. 130).
8. Linearity (r^2) of the calibration standards was not always ≥ 0.995 (see Table 4 above). Calibration curves were not provided for LC/MS/MS analyses. Calibration standards were analyzed, but calibration curves were not generated as part of the ILV report (Appendix 4, pp. 139-145). Fortification recoveries were determined based on average response factor (RF) of bracketed standards (p. 20). The RSD value of the average RF was $\leq 20\%$ for all samples (Appendix 4, pp. 139-145).
9. It was reported for the ILV that a single analyst completed a sample set consisting of eighteen samples (one reagent blank, two matrix control samples, and fifteen fortified samples) in *ca.* 5 days; 3 days, or 24 person hours, to complete extraction, plus *ca.* 5 hours for GC-NPD analysis and *ca.* 11.5 hours for LC/MS/MS analysis (p. 19).
10. A typographical error was noted in section 3.7 Instrumentation of the ILV report (p. 15); "DuPont-2550" should read DuPont-2549.

V. References

U.S. Environmental Protection Agency. 2012. Ecological Effects Test Guidelines, OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation. Office of Chemical Safety and Pollution Prevention, Washington, DC. EPA 712-C-001.

40 CFR Part 136. Appendix B. Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11, pp. 317-319.

Attachment 1: Chemical Names and Structures**Hexazinone (DPX-A367)****IUPAC Name:** 3-Cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione**CAS Name:** 3-Cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione**CAS Number:** 51235-04-2**SMILES String:** O=C(N=C(N(C1=O)C)N(C)C)N1C(CCCC2)C2**IN-T3937 (Metabolite A)****IUPAC Name:** 6-(Dimethylamino)-3-(4-hydroxycyclohexyl)-1-methyl-1,3,5-triazine-2,4-dione**CAS Name:** 3-(4-Hydroxycyclohexyl)-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)dione**CAS Number:** Not reported**SMILES String:** Cn1c(nc(=O)n(c1=O)C2CCC(CC2)O)N(C)C**IN-A3928 (Metabolite B)****IUPAC Name:** 3-Cyclohexyl-1-methyl-6-(methylamino)-1,3,5-triazine-2,4-dione**CAS Name:** 3-Cyclohexyl-6-(methylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)dione**CAS Number:** 56611-54-2**SMILES String:** [H]N(C)c1nc(=O)n(c(=O)n1C)C2CCCCC2

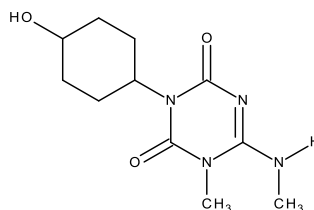
**IN-T3935 (Metabolite C)**

IUPAC Name: 3-(4-Hydroxycyclohexyl)-1-methyl-6-(methylamino)-1,3,5-triazine-2,4-dione

CAS Name: 3-(4-Hydroxycyclohexyl)-6-(methylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)dione

CAS Number: 72585-88-7

SMILES String: [H]N(C)c1nc(=O)n(c(=O)n1C)C2CCC(O)C2

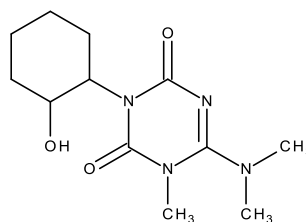
**IN-G3453 (Metabolite A1)**

IUPAC Name: 6-(Dimethylamino)-3-(2-hydroxycyclohexyl)-1-methyl-1,3,5-triazine-2,4-dione

CAS Name: 3-(2-Hydroxycyclohexyl)-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)dione

CAS Number: Not reported

SMILES String: Cn1c(nc(=O)n(c1=O)C2CCCC(O)C2)N(C)C

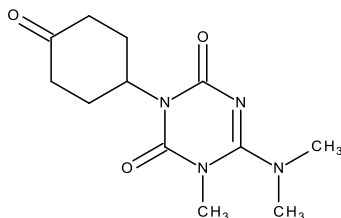
**IN-JS472 (Metabolite 1)**

IUPAC Name: 6-(Dimethylamino)-1-methyl-3-(4-oxocyclohexyl)-1,3,5-triazine-2,4-dione

CAS Name: 3-(4-Ketocyclohexyl)-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)dione

CAS Number: Not reported

SMILES String: Cn1c(nc(=O)n(c1=O)C2CCC(=O)CC2)N(C)C



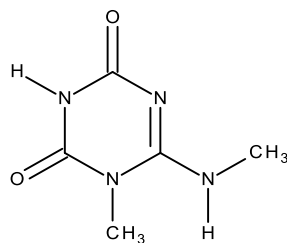
IN-G3170 (Metabolite G3170)

IUPAC Name: 1-Methyl-6-(methylamino)-1,3,5-triazine-2,4-dione

CAS Name: 6-(Dimethylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)dione

CAS Number: Not reported

SMILES String: [H]n1c(=O)nc(n(c1=O)C)N([H])C



Attachment 2: Calculations

Chemical: Hexazinone

PC: 107201

MRIDs: 45132802

Guideline: 850.6100

ILV for Determination of Hexazinone and its Products in Loamy Sand Soil

Fortified (µg a.i./kg)	Hexazinone (DPX-A3674)							IN-T3937							IN-A3928						
	Recovery (%)	Mean (%)	SD ¹ (%)	RSD ² (%)	Max	Min	n =	Recovery (%)	Mean (%)	SD ¹ (%)	RSD ² (%)	Max	Min	n =	Recovery (%)	Mean (%)	SD ¹ (%)	RSD ² (%)	Max	Min	n =
	GC-NPD (quantitation)							GC-NPD (quantitation)							GC-NPD (quantitation)						
20.0 (LOQ)	98 84 101 99 114	99	11	11	114	84	5	94 94 109 111 120	106	11	11	120	94	5	88 88 102 96 118	98	12	13	118	88	5
200	87 87 94 99 64	86	13	16	99	64	5	90 77 77 86 63	79	10	13	90	63	5	87 81 84 99 69	84	11	13	99	69	5
	LC/MS/MS (confirmation)							LC/MS/MS (confirmation)							LC/MS/MS (confirmation)						
2.0 (LOQ)	92 93 98 98 105	97	5	5	105	92	5	84 91 94 99 99	93	6	7	99	84	5	91 93 101 100 104	98	6	6	104	91	5
20	101 97 94 95 92	96	3	4	101	92	5	101 97 94 98 94	97	3	3	101	94	5	100 98 93 97 91	96	4	4	100	91	5

Results from Tables 1-2, pp. 22-23 of MRID 45132802.

Means and standard deviations calculated using Microsoft program functions =AVERAGE(A1:A2) and =STDEV(A1:A2).

Any discrepancies between reviewer calculated values and reported results most likely due to rounding.

1 SD = Standard Deviation; determined using the "unbiased" or "n-1" method.

2 RSD = Relative Standard Deviation; calculated as (SD/mean) x 100.

Chemical: Hexazinone
 PC: 107201
 MRIDs: 45132802
 Guideline: 850.6100

ILV for Determination of Hexazinone and its Products in Loamy Sand Soil

Fortified (µg a.i./kg)	IN-G3453							IN-JS472							IN-G3170							
	Recovery (%)	Mean (%)	SD ¹ (%)	RSD ² (%)	Max	Min	n =	Recovery (%)	Mean (%)	SD ¹ (%)	RSD ² (%)	Max	Min	n =	Recovery (%)	Mean (%)	SD ¹ (%)	RSD ² (%)	Max	Min	n =	
	GC-NPD (quantitation)							GC-NPD (quantitation)							GC-NPD (quantitation)							
20.0 (LOQ)	99							85							80							
	99							83							103							
	109							96							108							
	112							102							119							
	123	108	10	9	123	99	5	108	95	11	11	108	83	5	113	105	15	14	119	80	5	
200	91							82							89							
	78							73							73							
	82							75							74							
	97							88							91							
	68	83	11	14	97	68	5	64	76	9	12	88	64	5	75	80	9	11	91	73	5	
	LC/MS/MS (confirmation)							LC/MS/MS (confirmation)							LC/MS/MS (confirmation)							
2.0 (LOQ)	83							57							81							
	81							65							81							
	84							68							83							
	95							65							86							
	92	87	6	7	95	81	5	70	65	5	8	70	57	5	87	84	3	3	87	81	5	
20	91							88							76							
	92							87							73							
	88							82							58							
	91							86							88							
	88	90	2	2	92	88	5	79	84	4	4	88	79	5	37	66	20	30	88	37	5	

Results from Tables 1-2, pp. 22-23 of MRID 45132802.

Means and standard deviations calculated using Microsoft program functions =AVERAGE(A1:A2) and =STDEV(A1:A2).

Any discrepancies between reviewer calculated values and reported results most likely due to rounding.

1 SD = Standard Deviation; determined using the "unbiased" or "n-1" method.

2 RSD = Relative Standard Deviation; calculated as (SD/mean) x 100.

Chemical: Hexazinone

PC: 107201

MRIDs: 45132802

Guideline: 850.6100

ILV for Determination of Hexazinone and its Products in Loamy Sand Soil

IN-T3935							
Fortified (µg a.i./kg)	Recovery (%)	Mean (%)	SD ¹ (%)	RSD ² (%)	Max	Min	n =
GC-NPD (quantitation)							
40.0 (LOQ)	100 102 115 117 126	112	11	10	126	100	5
200	114 95 96 100 77	96	13	14	114	77	5
LC/MS/MS (confirmation)							
4.0 (LOQ)	72 75 81 80 87	79	6	7	87	72	5
40	93 85 83 82 83	85	4	5	93	82	5

Results from Tables 1-2, pp. 22-23 of MRID 45132802.

Means and standard deviations calculated using Microsoft program functions =AVERAGE(A1:A2) and =STDEV(A1:A2).

Any discrepancies between reviewer calculated values and reported results most likely due to rounding.

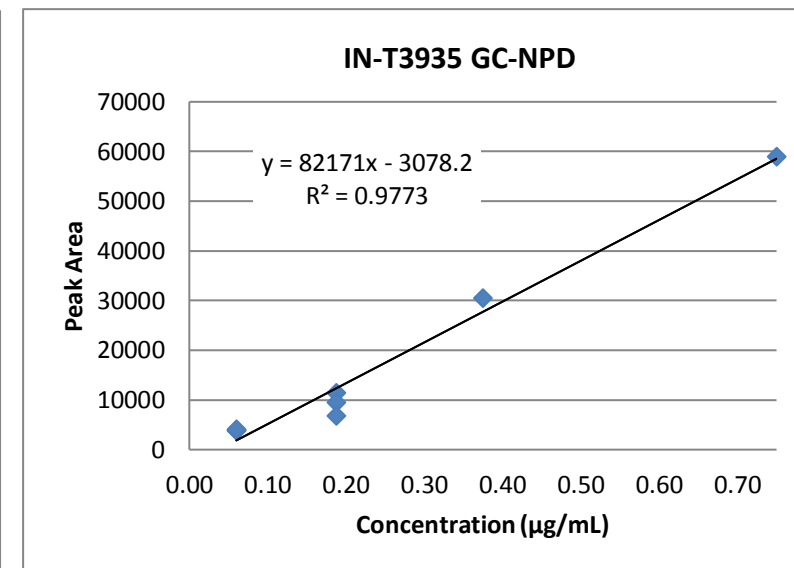
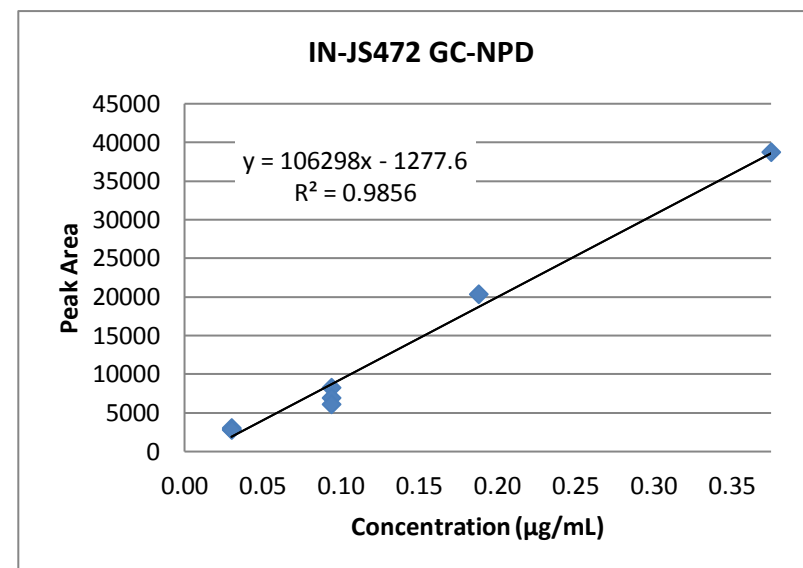
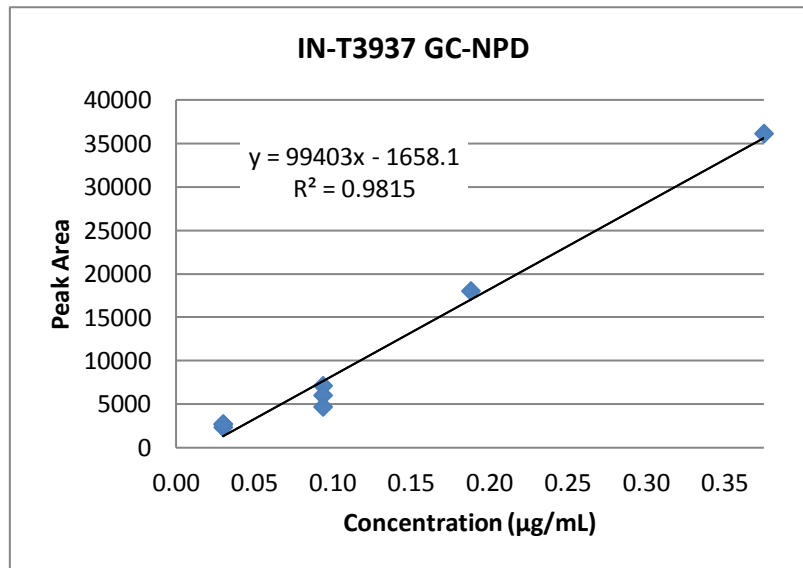
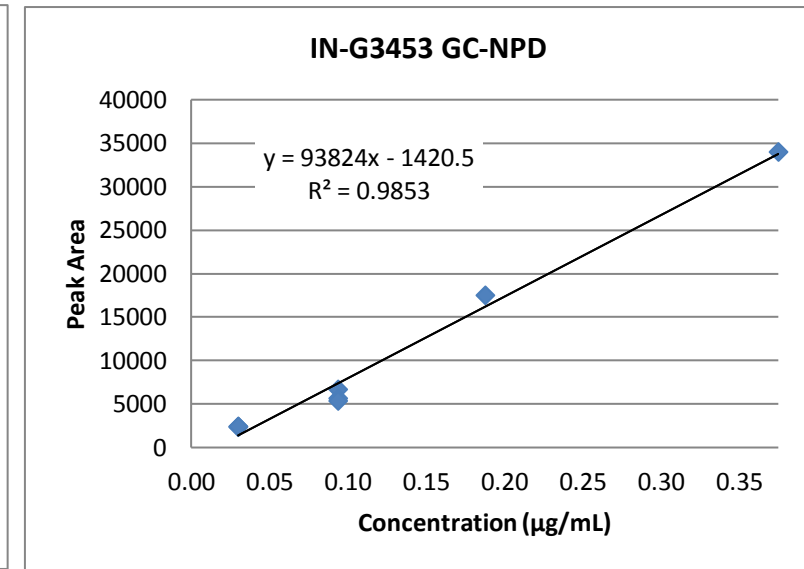
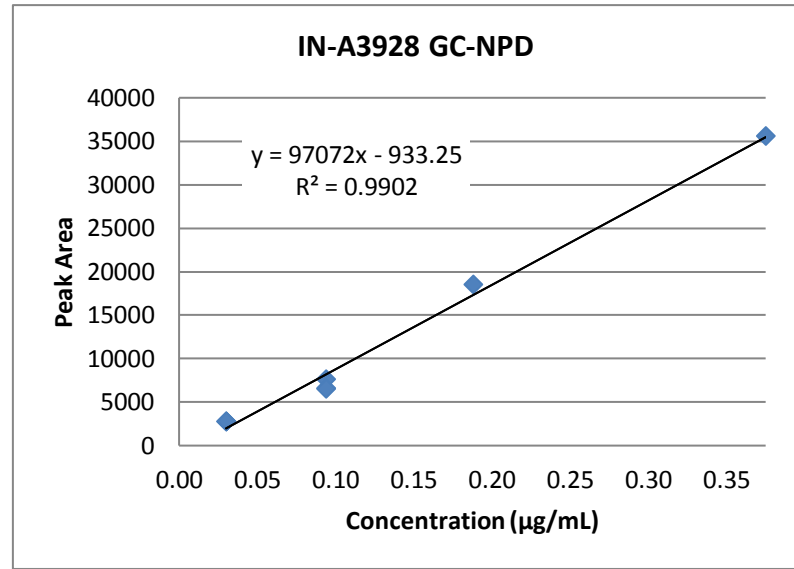
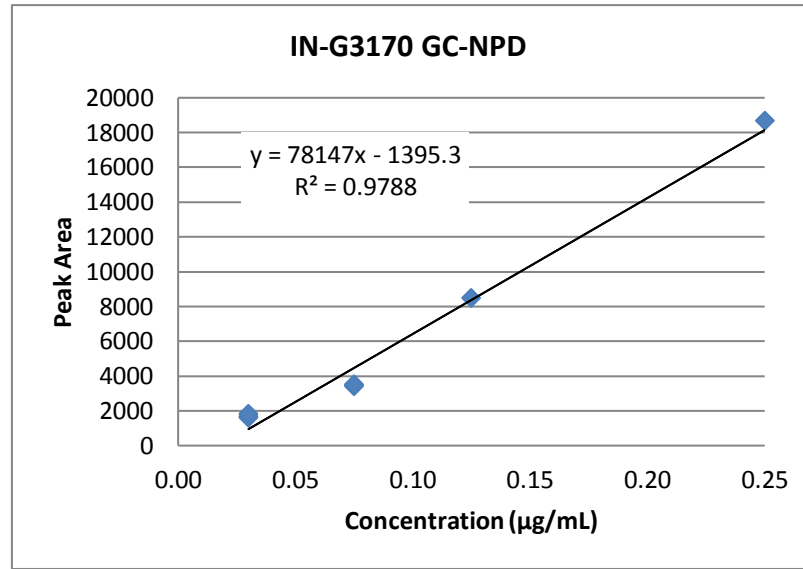
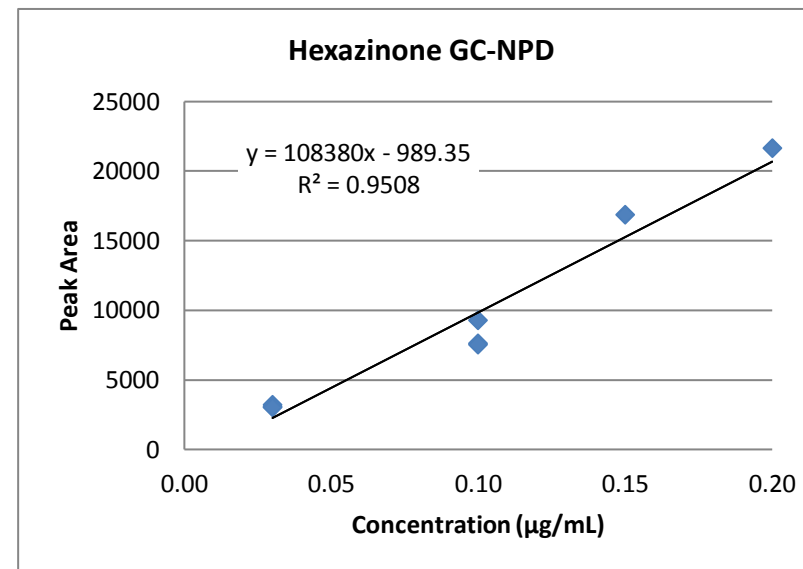
1 SD = Standard Deviation; determined using the "unbiased" or "n-1" method.

2 RSD = Relative Standard Deviation; calculated as (SD/mean) x 100.

Chemical: Hexazinone
 PC: 107201
 MRIDs: 45132802
 Guideline: 850.6100
 ILV Calibration Curves: GC-NPD Method (quantitation)

Calibration Curve Data	Hexazinone (DPX-A3674)		IN-G3170		IN-A3928		IN-G3453		IN-T3937		IN-JS472		IN-T3935	
	Conc. (µg/mL)	Peak Area counts	Conc. (µg/mL)	Peak Area counts	Conc. (µg/mL)	Peak Area counts	Conc. (µg/mL)	Peak Area counts	Conc. (µg/mL)	Peak Area counts	Conc. (µg/mL)	Peak Area counts	Conc. (µg/mL)	Peak Area counts
	0.030	3220.859	0.030	1685.510	0.030	2777.342	0.030	2396.116	0.030	2682.469	0.030	3016.086	0.060	3780.745
	0.030	3078.197	0.030	1607.823	0.030	2776.415	0.030	2343.816	0.030	2315.980	0.030	2872.859	0.060	3861.379
	0.030	3007.855	0.030	1848.706	0.030	2811.735	0.030	2425.796	0.030	2691.092	0.030	2825.563	0.060	4029.162
	0.100	9299.115	0.075	3536.648	0.094	7660.336	0.094	6716.244	0.094	7149.832	0.094	8293.451	0.188	11376.614
	0.100	7630.561	0.075	3411.360	0.094	6577.093	0.094	5633.914	0.094	5981.884	0.094	6970.067	0.188	9513.360
	0.100	7528.331	0.075	3479.047	0.094	6561.873	0.094	5321.220	0.094	4722.735	0.094	6110.617	0.188	6854.974
	0.150	16872.859	0.125	8492.876	0.188	18508.570	0.188	17515.537	0.188	17989.633	0.188	20374.148	0.375	30519.539
	0.200	21648.553	0.250	18697.352	0.375	35623.160	0.375	34009.078	0.375	36143.805	0.375	38705.172	0.750	59016.730

Results (Peak Area) from Appendix 5, pp. 132-138.



Chemical: Hexazinone
 PC: 107201
 MRIDs: 45132802
 Guideline: 850.6100
 ILV Calibration Curves: LC/MS/MS Method (confirmation)

Calibration Curve Data	Hexazinone (DPX-A3674)		IN-G3170		IN-A3928		IN-G3453		IN-T3937		IN-JS472		IN-T3935	
	Conc. (ng/mL)	Peak Area counts	Conc. (ng/mL)	Peak Area counts	Conc. (ng/mL)	Peak Area counts	Conc. (ng/mL)	Peak Area counts	Conc. (ng/mL)	Peak Area counts	Conc. (ng/mL)	Peak Area counts	Conc. (ng/mL)	Peak Area counts
	3.33	17378	3.33	3571	3.33	12320	3.33	8272	3.33	7340	3.33	7774	3.33	4992
	3.33	14174	3.33	2760	3.33	10697	3.33	7587	3.33	6431	3.33	6756	3.33	4081
	10.0	46731	10.0	8746	10.0	34042	10.0	24098	10.0	20499	10.0	22087	10.0	13110
	10.0	40732	10.0	8189	10.0	32013	10.0	22598	10.0	18969	10.0	20403	10.0	11922
	25.0	104716	25.0	21873	25.0	76836	25.0	58035	25.0	47512	25.0	53484	25.0	30777
	25.0	97691	25.0	16905	25.0	74953	25.0	54185	25.0	44875	25.0	49154	25.0	26979
	50.0	189348	50.0	39359	50.0	142195	50.0	107408	50.0	85763	50.0	99170	50.0	53964
	50.0	181197	50.0	36173	50.0	137779	50.0	104533	50.0	83368	50.0	96965	50.0	51029

Results (Peak Area) from Appendix 4, pp. 139-145.

