## 2.0 INTRODUCTION

To satisfy US regulatory ILV requirements, a residue analytical method must be validated at an independent laboratory prior to its submission to the appropriate regulatory authority. This study was conducted to fulfill those requirements.

The analytical method DuPont-39487 entitled "Analytical Method for the Determination of Chlorsulfuron and Metabolites in Soil Using LC/MS/MS" is applicable for the quantitation of chlorsulfuron (DPX- W4189) and its metabolites (IN-JJ998, IN-A4097, IN-UND13, IN-M6957, IN-D5293and IN-A4098) in soil.

The structure, CAS name, CAS registry number, and various properties of chlorsulfuron and metabolites IN-D5293, IN-A4097, IN-JJ998, IN-M6957, IN-UND13 and IN-A4098 can be found in section 3.0. The method was validated on soil, which was collected from Pennsylvania, U. S. A. (17 Lee Blvd. Malvern PA 19355).

Chlorsulfuron and metabolites IN-D5293, IN-A4097, IN-JJ998, IN-M6957, IN-UND13 and IN-A4098 were extracted from soil samples using a solution of ammonium carbonate and acetonitrile. An aliquot of the extracts were purified using a dispersive SPE step and by filtration through a graphitized carbon SPE cartridge. The volume of the purified extracts were evaporated to less than 4-mL and diluted to 4-mL using aqueous ammonia carbonate. The purified extracts were analyzed using reversed phase liquid chromatography (LC) and electrospray mass spectrometry/mass spectrometry (MS/MS). The Limit of Quantitation (LOQ) was 1.0 µg/kg (ppb). The Limit of Detection (LOD) was estimated to be 0.25 µg/kg (ppb) for the least responsive analyte, IN-A4097.

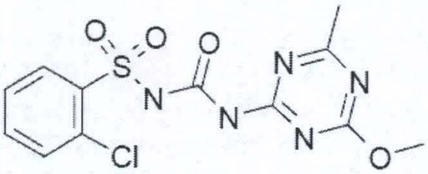
## 3.0 MATERIALS AND METHODS

### *3.1 Test Substance*

The reference analytical standards (test substances) used for this study were:

**DuPont code: DPX-W4189 (Chlorsulfuron)**

Chemical Structure:

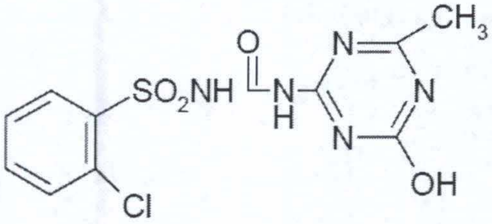


DPX-W4189

| | |
|---|---|
| CAS Name: | 2-Chloro-N-[4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl]benzenesulfonamide |
| Molecular weight: | 357.78 amu |
| Formula: | $C_{12}H_{12}N_5O_4SCl$ |
| Source: | Du Pont |
| CAS Number: | 64902-72-3 |
| Batch/Lot Number: | AG0486-112 |
| Purity: | 99.4% |
| Receipt date: | 12 June, 2014 |
| Expiration date: | 03 November, 2019 |
| Storage: | Ambient |

**DuPont code: IN-M6957**

Chemical Structure:

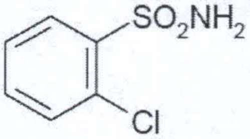


IN-M6957

| | |
|---|---|
| Molecular weight: | 343.75 amu |
| Formula: | $C_{11}H_{10}N_5O_4SCl$ |
| Source: | Du Pont |
| Batch/Lot Number: | E79181-4D |
| Purity: | 82.9% |
| Receipt date: | 12 June, 2014 |
| Expiration date: | 31 May, 2016 |
| Storage: | Ambient temperature under nitrogen |

**DuPont code: IN-A4097**

Chemical Structure:

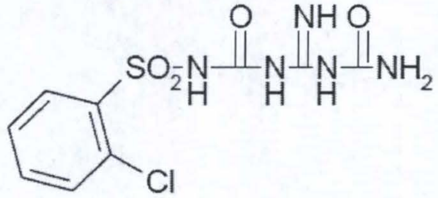


IN-A4097

| | |
|---|---|
| Molecular weight: | 191.64 amu |
| Formula: | $C_6H_6NO_2SCl$ |
| Source: | Du Pont |
| Batch/Lot Number: | AG0416-107 |
| Purity: | 99.7% |
| Receipt date: | 12 June, 2014 |
| Expiration date: | 22 January, 2021 |
| Storage: | Ambient |

**DuPont code: IN-JJ998**

Chemical Structure:



IN-JJ998

Molecular weight: 319.73 amu

Formula: $C_9H_{10}N_5O_4SCl$

Source: Du Pont

Batch/Lot Number: E100118-61

Purity: 90.0%

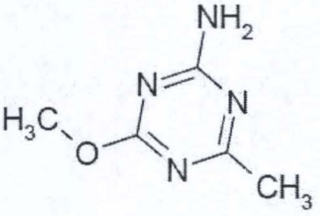
Receipt date: 12 June, 2014

Expiration date: 06 November, 2017

Storage: Ambient

**DuPont code: IN-A4098**

Chemical Structure:



IN-A4098

Molecular weight: 140.15 amu

Formula: $C_5H_8N_4O$

Source: Du Pont

Batch/Lot Number: 050942-015

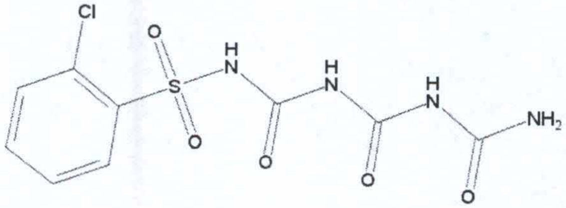
Purity: 98.7%

Receipt date: 12 June, 2014

| | |
|---|---|
| Expiration date: | 02 September, 2019 |
| Storage: | Ambient |

**DuPont code: IN-UND13**

Chemical Structure:

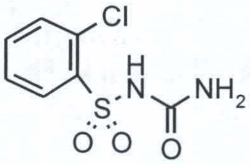


IN-UND13

| | |
|---|---|
| Molecular weight: | 320.71 amu |
| Formula: | $C_9H_9ClN_4O_5S$ |
| Source: | Du Pont |
| Batch/Lot Number: | E119561-8 |
| Purity: | 98.2% |
| Receipt date: | 04 December, 2014 |
| Expiration date: | 16 September, 2017 |
| Storage: | Ambient |

**DuPont code: IN-D5293**

Chemical Structure:



IN-D5293

| | |
|---|---|
| Molecular weight: | 234.66 amu |
| Formula: | $C_7H_7ClN_2O_3S$ |
| Source: | Du Pont |
| Batch/Lot Number: | KJR-02-66A |

| | |
|---|---|
| Purity: | 99.6% |
| Receipt date: | 04 December, 2014 |
| Expiration date: | 25 January, 2021 |
| Storage: | Ambient |

Chlorsulfuron, IN-JJ998, IN-A4097, IN-M6957, IN-UND13, IN-D5293and IN-A4098 were supplied by E. I. du Pont de Nemours and Company, DuPont Crop Protection, Newark, DE. Information pertaining to the characterization and stability of the test substances is archived by DuPont Crop Protection, E. I. du Pont de Nemours and Company, Newark, Delaware.

### *3.2 Test System*

In this study, the analytical method was validated in soil. Samples of soils were collected at the test facility (17 Lee Blvd. Malvern PA 19355). Fortifications of the samples were made using 7.5 g (±1%) of soil spiked with 0.075mL of 0.10 µg/mL standard solution for LOQ and 0.750 mL of 0.10 µg/mL standard solution for 10 x LOQ. The samples were assigned unique identification by the laboratory, an alpha-numeric sample ID along with additional designations such as "control" and "LOQ", as appropriate.

### *3.3 Equipment*

Equipment used was either the same as that specified in the analytical method or the equivalent. A Shimadzu LC-20AD HPLC was used instead of an Agilent 1290 HPLC system. An AB SCIEX 5500 was used instead of an AB SCIEX 5000. The changes were demonstrated as equivalent to that specified in the method.

### *3.4 Reagents*

Reagents used were either the same as those specified in the analytical method or equivalent grade of quality.

### *3.5 Principles of the Analytical Method*

The analyses in this study followed the analytical method for chlorsulfuron and metabolites, as described in the method DuPont-39487. The following is a summary of the method conducted at Alliance Pharma.

Chlorsulfuron and metabolites were extracted from 7.5 g (± 1%) soil samples. After fortification, 15-mL of 50:50 0.1 M aqueous ammonium carbonate: acetonitrile solution was added to each sample. The samples were placed on a genogrinder and homogenized for 3 minutes at a rate of approximately 1000 strokes per minute. The samples were centrifuged at a rate of approximately 3000 RPM for 10 minutes to drive the particulates to the bottom of the tube. The supernatants were transferred into a clean 50-mL centrifuge tubes. These steps were repeated for an additional two times combining all extracts into same 50-mL centrifuge tubes. The extracts were diluted to 45-mL using 50:50 0.1 M aqueous ammonium carbonate: acetonitrile. The extract were mixed using a vortex mixer for approximately 30 seconds. A 10.0-mL aliquot of each extract was transferred into a clean 14-mL centrifuge tube. Bulk bondesil SAX material (0.25 grams) was added to each extract. The extracts were mixed using a vortex mixer for approximately 30 seconds. The extracts were centrifuged at a rate of approximately 3000 RPM for 5 minutes to drive the particulates to the bottom of the tube. The resulting extracts were further purified by filtering them through 6-mL, 0.25-g Supelclean Envi Carb cartridges. The purified extracts were collected into a clean 14-mL glass tube. The purified extracts were evaporated to less than 4 mL under a stream of nitrogen in evaporator at approximate 35°C. The extracts were diluted to 4-mL using 0.10 M aqueous ammonium carbonate. The purified final extracts were filtered by syringe filter (0.45 um, PTFE) and transfer to HPLC sampler vial for LC/MS/MS analysis.

The analytes were separated from co-extracts using a Phenomenex Luna 3µ C18(2) 2.0x150 mm column and were detected by electrospray ionization mass spectrometer.

Two parent-to-daughter ion transitions of each analyte were monitored as follows: chlorsulfuron: 358.0→141.1 (Q) and 358.0→167.1 (C) m/z, IN-A4098: 141.1→57.0 (Q) and 141.1→85.1 (C) m/z. These MRM transitions were measured and analyzed by a positive mode ESI mass spectrometer. Whereas, the associated metabolites: IN-A4097 (189.9→77.9 (Q) and 189.9→125.8 (C) m/z), IN-M6957 (342.0→125.0 (Q) and 342.0→182.0 (C) m/z), IN-JJ998 (318.0 →274.9 (Q) and 318.0→ 189.9 (C) m/z), IN-D5293 (233.1→78.0 (Q) and 233.1→125.9 (C) m/z), IN-UND13 (319.0→189.8 (Q) and 319.0→232.7 (C) ), were analyzed and quantified by a negative mode electrospray ionization mass spectrometer. The confirmatory method was based on the recovery of secondary MS/MS ion transitions.

Method validation was accomplished by analyzing the analytes in validation sets consisting of 2 blank control specimens, 5 replicate specimens fortified at the LOQ, and 5 replicate specimens fortified at 10xLOQ.

### *3.6 Modifications, Interpretations, and Critical Steps*

The analytical method was run exactly as written except for the following:

A Shimadzu HPLC was used instead of HP1290 with temperature controlled autosampler (Agilent Technologies). An AB SCIEX 5500 was used instead of an AB SCIEX 5000.

The substitutions were demonstrated to be equivalent to the equipment specified in the method and did not impact the analytical results.

The stock solutions of IN-M6957 and IN-A4098 were made in DMSO, then were largely diluted in acetonitrile because of compound solubility issues.

A confirmatory transition of IN-A4098, 141.1→58.0 m/z was changed to 141.1→85.1 m/z.

The Certificate of Analysis for analytical standard IN-M6957 states the compound should be stored at ambient temperature under nitrogen. The vial was stored at ambient temperature with desiccant, but not under nitrogen. Method Deviation 01 was issued and kept in study file.

## 3.7 *Instrumentation*

***HPLC Conditions for Positive Mode ESI***

| | |
|---|---|
| System: | Shimadzu LC-20AD / Sil-20AC Autosampler |
| Column: | Phenomenex Luna 3u C18(2) 2.0x150 mm |
| Column Temperature: | 40°C |
| Injection Volume: | 10 µL |
| Autosampler Temperature: | 6°C |
| Conditions: | A: 0.02 M Formic Acid in $H_2O$ |
| | B: Methanol |
| | Flow in 0.4 mL/minute |
| | Step / Time (min) / Event / Parameter<br>1 / 0.01 / B. Conc. / 10<br>2 / 1 / B. Conc. / 10<br>3 / 14 / B. Conc. / 99<br>4 / 16 / B. Conc. / 99<br>5 / 16.1 / B. Conc. / 10<br>6 / 20 / B. Conc. / 10<br>7 / 20 / STOP / |
| Analyte Retention Times (minutes) | |
| CHLORSULFURON | ~10.4 |
| IN-A4098 | ~3.1 |

The detection method utilized was LC-MS/MS employing atmospheric pressure electrospray ionization interface in the positive mode. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for chlorsulfuron and metabolite, IN-A4098 are shown below, (bold as quantification transition, while, the other for confirmation):

| **SYSTEM:** | | **AB SCIEX 5500** | | | | |
|---|---|---|---|---|---|---|
| **Compounds** | **Parent Ion (m/z)** | **Product Ion (m/z)** | **Dwell Time (ms)** | **DP** | **CE** | **CXP** |
| Chlorsulfuron | 358.1 | **141.1** | 150 | 66 | 20 | 15 |
| | | 167.0 | 150 | 70 | 20 | 15 |
| IN-A4098 | 141.0 | **57.0** | 150 | 86 | 25 | 12 |
| | | 85.1 | 150 | 86 | 21 | 15 |
| Ion Mode: | | Positive | | | | |
| Turbo Spray Voltage: | | 4500 V | | | | |
| Source Temperatures: | | 600 °C | | | | |
| CUR: | | 30 psig | | | | |
| CAD: | | 8 | | | | |
| GS1: | | 40 psig | | | | |
| GS2: | | 50 psig | | | | |
| Dwell: | | 150 ms | | | | |
| EP: | | 10 | | | | |

The instrument was operated in the MS/MS (MRM) positive ion mode for quantitative analysis of chlorsulfuron and its metabolite, IN-A4098. The ion chromatograms were integrated and the peak areas were used for quantitation.

For each analytical run, a seven-point standard (0.1~7.5ng/mL) curve was prepared by injecting constant volumes of mixed standard solutions composed of each analyte of interest. Constant volume injections were used for sample extracts, as well.

### *HPLC Conditions for Negative Mode ESI*

| | |
|---|---|
| System: | Shimadzu LC-20AD / Sil-20AC Autosampler |
| Column: | Phenomenex Luna 3u C18(2) 2.0x150 mm |
| Column Temperature: | 40°C |
| Injection Volume: | 10 µL |
| Autosampler Temperature: | 6°C |
| Conditions: | A: 0.02 M Formic Acid in $H_2O$ |
| | B: Methanol |
| | Flow in 0.4 mL/minute |
| | Step / Time (min) / Event / Parameter<br>1 / 0.01 / B. Conc. / 10<br>2 / 1 / B. Conc. / 10<br>3 / 2 / B. Conc. / 30<br>4 / 14 / B. Conc. / 99<br>5 / 16 / B. Conc. / 99<br>6 / 16.1 / B. Conc. / 10<br>7 / 20 / B. Conc. / 10<br>8 / 20 / STOP |
| Analyte Retention Times (minutes) | |
| IN-JJ998 | ~4.9 |
| IN-A4097 | ~4.9 |
| IN-M6957 | ~5.6 |
| IN-D5293 | ~4.9 |
| IN-UND13 | ~7.3 |

The detection method utilized was LC-MS/MS employing atmospheric pressure electrospray ionization interface in the negative mode. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for metabolites, IN-JJ998, IN-A4097, IN-M6957, IN-D5293 and IN-UND13 are shown below, (bold as quantification transition, while, the other for confirmation):

| **SYSTEM:** | | **AB SCIEX 5500** | | | | |
|---|---|---|---|---|---|---|
| **Compounds** | ***Parent Ion (m/z)*** | **Product Ion (m/z)** | **Dwell Time (ms)** | **DP** | **CE** | **CXP** |
| IN-JJ998 | 318.0 | **274.9** | 150 | -45 | -16 | -15 |
| | | 189.9 | 150 | -45 | -30 | -21 |
| IN-A4097 | 189.9 | **77.9** | 150 | -65 | -29 | -10 |
| | | 125.8 | 150 | -65 | -20 | -10 |
| IN-M6957 | 342.0 | **125.0** | 150 | -55 | -17 | -15 |
| | | 82.0 | 150 | -55 | -47 | -10 |
| IN-D5293 | 233.0 | **78.0** | 150 | -65 | -38 | -13 |
| | | 125.9 | 150 | -65 | -28 | -11 |
| IN-UND13 | 319.0 | **189.8** | 150 | -35 | -22 | -19 |
| | | 232.7 | 150 | -35 | -20 | -23 |
| Ion Mode: | | Negative | | | | |
| Turbo Spray Voltage: | | -4500 V | | | | |
| Source Temperatures: | | 600 $^{0}$C | | | | |
| CUR: | | 30 psig | | | | |
| CAD: | | 8 | | | | |
| GS1: | | 40 psig | | | | |
| GS2: | | 50 psig | | | | |
| Dwell: | | 150 ms | | | | |
| EP: | | -10 | | | | |

The instrument was operated in the MS/MS (MRM) negative ion mode for quantitative analysis of metabolites, IN-JJ998, IN-A4097, IN-M6957, IN-D5293 and IN-UND13. The ion chromatograms were integrated and the peak areas were used for quantitation.

For each analytical run, a seven-point standard (0.1~7.5 ng/mL) curve was prepared by injecting constant volumes of mixed standard solutions composed of each analyte of interest. Constant volume injections were used for sample extracts, as well.

### *3.8 Calculations*

Residue chlorsulfuron and metabolites were quantitated by external standards. A calibration curve for each analyte was generated by plotting the detector's response in peak area versus the concentration (ng/mL) of standard injected. The data system derived an equation for the fit of the standard curve with a weighted [$(1/x^2)$ where $x =$ concentration] linear regression, and this equation was used to calculate intercept and slope of the linear regression curve.

The calibration curve was obtained by direct injection of 10 µL of standard (ranging from 0.1 ng/mL to 7.5 ng/mL) into the LC-MS/MS for each analyte. In a given injection run, the same injection volume was used for all samples and standards.

Peak integration and quantitation were performed using Applied Biosystems' Analyst software version 1.6. Calculations of recovery results were computed for each set of samples in a Microsoft Excel® spreadsheet. The equations used for quantitation are shown below.

$R = (C_{End} * V_F * AF) / W$

Where:

$R$: Analyte residue in µg/kg (ppb)

$R_{fortified}$: Amount of analyte residue fortified in µg/kg (ppb)

$C_{End}$: Final concentration of analyte derived from calibration curve in ng/mL

$AF$: Aliquot factor = Total extraction volume ($V_{Total\ Ex}$) / Aliquot extraction volume ($V_{aliq\ Ex}$)

$V_F$: Final volume

$W$: Soil sample weight: 7.5 g

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$Rec. = (R / R_{fortified}) \times 100\ \%$

Example: Table 1, Sample LOQ-1, Chlorsulfuron, soil, Fortified @ 1.0 ppb, transition ions 358.0→141.1:

Calibration curve calculated by Analyst software:

$y = 3.01e{+}005\ x + -657$

Where:

$y$: Peak area

$x$: $C_{End}$, final concentration of analyte derived from calibration curve

$C_{End} = x = (118343 + 657) / 3.01e{+}005$

$= \mathbf{0.3953 ng/mL}$

$R_{fortified} = 1.0$ ppb

$V_F = 4.0$ mL

$AF = V_{Total\ Ex} / V_{aliq\ Ex} = 45\ mL / 10\ mL = 4.5$

$R = (C_{End} * V_F * AF) / W$

= 0.395 (ng/mL) * (4.0 mL) * (4.5) / 7.5 g = 0.948 ng/g = **0.948 µg/kg (ppb)**

Rec. = $(R / R_{\text{fortified}})$ x 100 % = (0.948 / 1.0) x 100% = **95%**

NOTE: Slight rounding differences may be noted when using a hand calculator. Full computer/calculator precision was used in any intermediate calculations. Only the final value was rounded.

## 5.0 CONCLUSIONS

Alliance Pharma successfully, independently validated the DuPont residue analytical method for chlorsulfuron and its metabolites in soil, as described in DuPont Study No. DuPont-39487.

An LOQ of 1.0 µg/kg (ppb) was demonstrated for chlorsulfuron (DPX- W4189) and its metabolites IN-JJ998, IN-A4098, IN-M6957, IN-D5293, IN-UND13 in soil, however, the sensitivity is not sufficient to quantify compound IN-A4097 confirmatory transition (189.9→125.8 m/z) in soil using current method. Overall, the method was found to be suitable for the determination of chlorsulfuron (DPX-W4189) and its metabolites IN-JJ998, IN-M6957, IN-D5293, IN-UND13 and IN-A4098 in soil.