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-8579 Revision 1 entitled "Analytical Method for the Determination of Chlorsulfuron and Metabolites IN-JJ998, IN-A4097, IN-M6957, and IN-A4098 in Water Using LC/MS/MS" is applicable for the quantitation of chlorsulfuron (DPX- W4189) and its metabolites (IN-JJ998, IN-A4097, IN-M6957, and IN-A4098) in water.

Chlorsulfuron and its metabolites were extracted from water fortified with the analytes at LOQ (0.050 µg/L (ppb)) and 10x LOQ by solid phase extraction. The analytes were eluted from the SPE cartridges using a basic acetonitrile (ACN) solution. The volume collected was diluted to 20 mL and a 10-mL aliquot was removed. Two hundred and fifty microliters of water was added and the extract was evaporated under a flow of nitrogen until the volume was approximately 250 µL. The extracts were diluted to 2.5 mL using an aqueous ammonium acetate buffer. The purified extract was analyzed by reversed phase LC/MS/MS. Two transitions were monitored for each analyte. Both transitions were detected by positive ion MS/MS.

The analytical method was designed to achieve an LOQ of 0.050 µg/L and the Limit of Detection (LOD) was estimated to be 0.02 µg/L. The independent validation thus evaluated recoveries of chlorsulfuron and its metabolites, IN-JJ998, IN-A4097, IN-M6957, and IN-A4098, in samples fortified at 1x and 10x the LOQ level. The method was used as written.

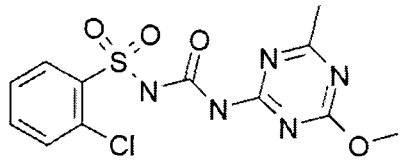
3.0 MATERIALS AND METHODS

3.1 Test Substance

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

DuPont code: DPX-W4189 (Chlorsulfuron)

Chemical Structure:

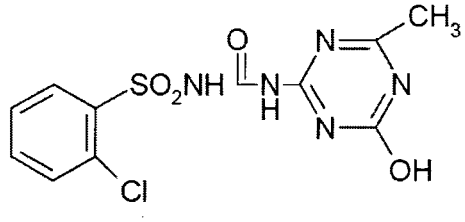


DPX-W4189

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:



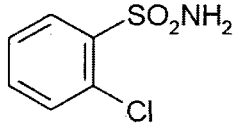
IN-M6957

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:

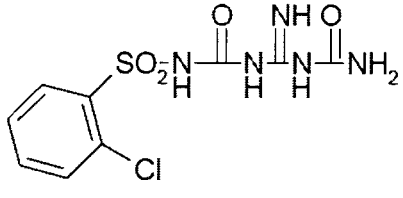


IN-A4097

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:



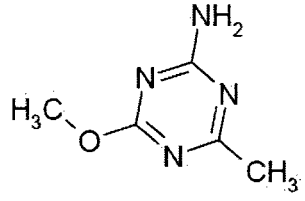
IN-JJ998

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

Chlorsulfuron, IN-JJ998, IN-A4097, IN-M6957, and 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 water. Samples of drinking water were collected at the test facility. Surface water was collected from French Creek, Phoenixville, PA. Ground water was collected at Charlestown Township, PA. The characterization data for the surface water analyzed is presented in Appendix 2.

Fortifications of the samples were made using 100 mL (±1%) of water spiked with 0.050 µg/mL or 0.5 µg/mL standard solutions. 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 Hewlett-Packard HP1100 HPLC system. An AB SCIEX 4000 was used instead of a Micromass Quattro II. 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-8579 Revision 1. The following is a summary of the method conducted at Alliance Pharma. The complete description of the method is described in the original method.

Chlorsulfuron and metabolites were extracted from 100 mL (± 1%) of water sample. An aliquot of 50 µL concentrated formic acid was added to each sample and those requiring fortification were fortified with the appropriate standard solution and mixed thoroughly. On an SPE vacuum manifold, 1.0g/20-mL Waters Oasis HLB cartridges were conditioned with 20 mL of methanol followed by 20 mL of HPLC grade water. The samples were filtered via gravity through the cartridges. The cartridges were dried using vacuum for approximately 1 minutes, and the eluate was disposed of. Wash the cartridge with 10 mL of 70:30 hexane: ethyl acetate. The cartridges were dried using vacuum for approximately 3 minutes, and the eluate was disposed of. A volume of 20 mL of basic acetonitrile (ACN) was measured into each sample tube, and then was loaded into the SPE cartridges and placed under slight vacuum. The eluate was collected in 50 mL centrifuge tubes.

The eluate was diluted to 20 mL with basic ACN and a 10 mL aliquot was transferred to a 15 mL centrifuge tube. The extract was then evaporated under nitrogen flow in a water bath set to approximately 30°C. When the extract volume was approximately 3 mL, 0.25 mL of water was added and the extract was allowed to continue evaporating until the volume was approximately 0.25 mL. The extracts were removed from the water bath. Tubes were vortexed and all samples were transferred to HPLC injection vials via a syringe filter.

The purified final extracts were analyzed by reversed-phase HPLC using a Phonomenex Luna Phenyl Hexyl 150 x 4.6 mm column with mobile phases of 0.02 M aqueous formic acid and methanol. The analytes chlorsulfuron and metabolites were detected by mass spectrometry/mass spectrometry (MS/MS) in the positive ion mode. Two parent-to-daughter ion transitions of each analyte were monitored as follows: chlorsulfuron using 358.0→141.1 and 358.0→167.1 m/z, IN-A4098 using 141.1→85 and 141.1→57.1 m/z, IN-A4097 using 192.0→111.0 and 194.2→113.1 m/z, IN-M6957 using 343.8→127.2 and 343.8→153.0 m/z, and IN-JJ998 using 320.1→86.0 and 320.1→277.2 m/z. 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 Hewlett-Packard HPLC. A SCIEX 4000 was used instead of a Micromass Quattro II.

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

The stock solution of IN-JJ998 was made in methanol instead of acetonitrile because of compound solubility issues.

3.7 *Instrumentation*

HPLC Conditions

System:	Shimadzu LC-20AD / Sil-20AC Autosampler
Column:	Phenomenex Luna Phenyl Hexyl 4.6x150 mm
Column Temperature:	Ambient
Injection Volume:	50 µL
Autosampler Temperature:	4°C
Conditions:	A: 0.02 M Formic Acid in H_2O
	B: Methanol
	Flow in mL/minute
	Time %A %B Flow 0.01 95 5 0.800 1 95 5 0.800 3 45 55 0.800 11 15 85 0.800 14 15 85 0.800 14.2 5 95 1.000 17 5 95 1.000 17.5 95 5 1.000 20 STOP
Analyte Retention Times (minutes)	
IN-JJ998	~6.4
IN-A4097	~6.6
IN-M6957	~8.4
CHLORSULFURON	~11.6
IN-A4098	~5.1

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 metabolites are shown below:

SYSTEM:	AB SCIEX 4000			
ANALYTES	IONS MONITORED	DECLUSTERING POTENTIAL (DP)	COLLISION ENERGY (CE)	EXIT POTENTIAL (CXP)
CHLORSULFURON (DPX- W4189)	358.0 → 141.1 AMU	66	29	13
	358.0 → 167.1 AMU	66	25	13
IN-JJ998	320.1 → 86.0 AMU	71	44	13
	320.1 → 277.2 AMU	71	20	13
IN-A4097	192.2 → 111.0 AMU	47	31	13
	194.2 → 113.1 AMU	54	31	13
IN-A4098	141.1 → 85.0 AMU	65	25	13
	141.1 → 57.1 AMU	65	33	13
IN-M6957	343.8 → 127.2 AMU	66	25	13
	343.8 → 153.0 AMU	66	23	13
Ion Mode:	Positive			
Turbo Spray Voltage:	5500 V			
Source Temperatures:	300 ^{0}C			
CUR:	20 psig			
CAD:	8			
GS1:	50 psig			
GS2:	50 psig			
Dwell:	100 ms			

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

For each analytical run, a six-point standard 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 50 µL of standard (ranging from 0.6 ng/mL to 15 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 1.4.1. 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:	Water sample weight: 100 g

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

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

Example: Table 1, Sample LOQ-1, Chlorsulfuron, Drinking Water, Fortified @ 0. 05 ppb, transition ions 358.0→141.1:

Calibration curve calculated by Analyst software:

$y = 6.47e{+}03\ x + 11.4$

Where:

y:	Peak area
x:	C_{End}, final concentration of analyte derived from calibration curve

$C_{End} = x = (6337 - 11.4) / 6.47e{+}03$

$= \mathbf{0.9777\ ng/mL}$

$R_{fortified} = 0.05$ ppb

$V_F = 2.5$ mL

$AF = V_{Total\ Ex} / V_{aliq\ Ex} = 20\ mL / 10\ mL = 2$

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

$= 0.978\ (ng/mL) * (2.5\ mL) * (2) / 100\ g = 0.0489\ ng/g =$ **0.0489 µg/kg (ppb)**

Rec. = (R / $R_{fortified}$) x 100 % = (0.0489 / 0.5) x 100% = **98%**

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.

4.2 Communication

The independent laboratory method validation of method DuPont-8579 Revision 1 was successfully completed without technical communication with the study monitor aside from approval of each validation set as stated in the study protocol. In a test for ground water, it was found water softeners were added to the blank matrix by a water treatment system, which may have affected the mass spec response. Thus, data is not reported. Ground water was later collected from the line before the water treatment system. The first validation trial for the determination of the corresponding analytes in the test system using the method as written was successful for all analytes in drinking water, surface water and ground water.

4.3 Time Requirements

A single analyst completed two sample sets (one set consisting of 1 control and 5 LOQs, one set consisting of 1 control and 5 10xLOQs) in the course of an eight-hour workday, with LC-MS/MS analysis performed unattended the same day. One validation set containing two control blank samples and ten validation samples can thus be completed within one working day's turnaround time.

5.0 CONCLUSIONS

Alliance Pharma successfully, independently validated the DuPont residue analytical method for chlorsulfuron and its metabolites in water, as described in DuPont Study No. DuPont-8579 Revision 1.

An LOQ of 0.050 µg/L (ppb) was demonstrated for all analytes evaluated in water. The method was found to be suitable for the determination of chlorsulfuron (DPX-W4189) and its metabolites IN-JJ998, IN-A4097, IN-M6957, and IN-A4098 in water.

6.0 RETENTION OF RECORDS

Originals or exact copies of all raw data and pertinent information, including the original protocol, any amendments and the final report will be retained at:

E. I. du Pont de Nemours and Company
DuPont Agricultural Products
Global Technology Division
Stine-Haskell Research Center
Newark, DE 19714-0030

Laboratory-specific or site-specific raw data such as personnel files, instrument, equipment, refrigerator, and/or freezer raw data will be retained at:

Alliance Pharma
17 Lee Boulevard
Malvern, PA 19355