TRADE SECRET

Study Title

INDEPENDENT LABORATORY VALIDATION OF DUPONT-38604 "ANALYTICAL METHOD FOR THE DETERMINATION OF RIMSULFURON (DPX-E9636) AND ITS METABOLITES IN SOIL AND WATER USING HPLC/MS/MS"

Test Guidelines

European Commission, Directorate General Health and Consumer Protection. "Guidance Document on Residue Analytical Methods", SANCO/825/00 rev. 8.1, November 16, 2010

U.S. EPA Ecological Effects Test Guidelines: OPPTS 850.7100: Data Reporting for Environmental Chemistry Methods (Draft, April, 1996)

LIST OF ABBREVIATIONS AND SYMBOLS

AVG	Average
°C	Degrees Celsius
Cc	Cubic centimeter
CAS	*Chemical Abstracts* Service
Cm	Centimeter
EEC	European Economic Community
EPA	Environmental Protection Agency
g	Gram(s)
Σ	Total
GLP	Good Laboratory Practice
HPLC	High-performance liquid chromatography
i.d.	Inside diameter
L	Liter
ESI-MS/MS	Electrospray mass spectrometry/mass spectrometry analysis
LOD	Limit of detection
LOQ	Limit of quantitation
M	Molar
MeOH	Methanol
mL	Milliliter(s)
mM	Millimolar
min	Minute(s)
mm	Millimeter(s)
n	Total number of samples analyzed
OECD	Organization for Economic Co-operation and Development
ppb	Parts per billion
rpm	Revolution(s) per minute
RSD	Relative standard deviation
S/N	Signal-to-noise ratio
SD	Standard deviation
SPE	Solid-phase extraction
TIC	Total ion current
t_R	Retention time
µL	Microliter(s)
µm	Micrometer(s)

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-38604 entitled "Analytical Method for the Determination of Rimsulfuron (DPX-E9636) and Its Metabolites in Soil and Water Using HPLC/MS/MS" is applicable for the quantitation of rimsulfuron (DPX-E9636) and it metabolites (IN-70941, IN-70942, and IN-E9260) in water and soil.

Rimsulfuron and its metabolites were extracted from surface water fortified with the analytes at LOQ (0.1 µg/L (ppb)) and 10x LOQ. Rimsulfuron and its metabolites were also extracted from soil fortified with the analytes at LOQ (0.2 µg/kg (ppb)) and 10x LOQ by solid phase extraction. The analytes were eluted from the SPE cartridges using acetonitrile followed by a solution of 9:1 acetonitrile:0.5 M ammonium hydroxide. Aqueous ammonium acetate (5 mM) was added to the eluate and it was evaporated down to a volume of ~1 mL. Next, 0.5 mL of acetonitrile was added and the extracts were then diluted to 5 mL with 5 mM aqueaous ammonium acetate. The purified extract was analyzed by reversed phase LC/MS/MS. Two transitions were monitored for each analyte. Both transition states of rimsulfuron and the metabolites IN-70941, IN-70942, and IN-E9260 were detected by positive ion MS/MS.

The analytical method was designed to achieve a LOQ of 0.1 µg/L (ppb) for water and 0.2 µg/kg (ppb) for soil. The independent validation thus evaluated recoveries of rimsulfuron and its metabolites, IN-70941, IN-70942, and IN-E9260, in samples fortified at 1x and 10x the LOQ level. The method was used as written.

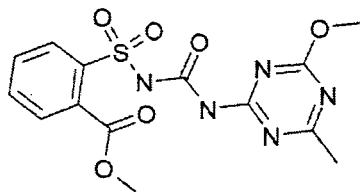
3.0 MATERIALS AND METHODS

3.1 Test Substance

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

DuPont code: DPX-E9636 (Rimsulfuron)

Chemical Structure:



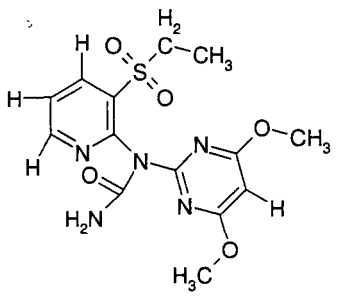
DPX-E9636

CAS Name:	N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-3-(ethylsulfonyl)-2-pyridinesulfonamide
Molecular weight:	431.45 amu
Formula:	$C_{14}H_{17}N_5O_6S$
Source:	DuPont
CAS Number:	122931-48-0
Batch/Lot Number:	E58246-070D
Purity:	99.1%*
Receipt date:	20 March, 2014
Expiration date:	10 March, 2020
Storage:	Ambient

*Note : The purity of 99.1% was based on the study protocol and the COA issued on 09 Feb, 2010. An updated COA was issued on 03 Apr, 2014 with a slightly different purity at 98.6%.

DuPont code: IN-70941

Chemical Structure:

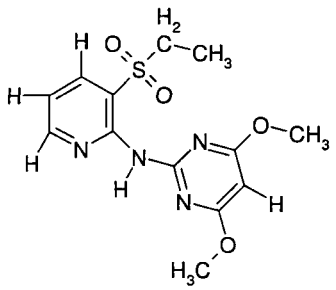


IN-70941

CAS Name:	N-(4,6-Dimethoxy-2-pyrimidinyl)-N-((3-ethylsulfonyl)-2-pyridinyl)urea
Molecular weight:	367.39 amu
Formula:	$C_{14}H_{17}N_5O_5S$
Source:	DuPont
CAS Number:	138724-53-5
Batch/Lot Number:	97331-88C
Purity:	99.4%
Receipt date:	20 March, 2014
Expiration date:	19 June, 2019
Storage:	Ambient

DuPont code: IN-70942

Chemical Structure:

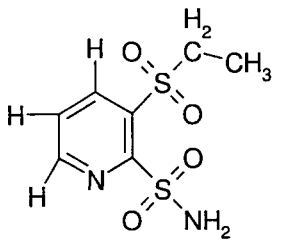


IN-70942

CAS Name:	N-((3-Ethylsulfonyl)-2-pyridinyl)-4,6- dimethoxy-2-pyrimidinamine
Molecular weight:	324.36 amu
Formula:	$C_{13}H_{16}N_4O_4S$
Source:	DuPont
CAS Number:	151331-80-5
Batch/Lot Number:	E76887-5B
Purity:	96.8%
Receipt date:	20 March, 2014
Expiration date:	06 July, 2015
Storage:	Ambient

DuPont code: IN-E9260

Chemical Structure:



IN-E9260

CAS Name:	3-(Ethylsulfonyl)-2-pyridinesulfonamide
Molecular weight:	250.30 amu
Formula:	$C_7H_{10}N_2O_4S_2$
Source:	DuPont
CAS Number:	117671-01-9
Batch/Lot Number:	D102051-178
Purity:	99.8%
Receipt date:	20 March, 2014
Expiration date:	21 August, 2016
Storage:	Ambient

Rimsulfuron and its metabolites (IN-70941, IN-70942, and IN-E9260) 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 and soil. Samples of surface water and soil were sent via UPS from DuPont (Newark, DE) to the testing facility (17 Lee Boulevard, Malvern, PA 19355). The characterization data for the water analyzed is presented in Appendix 2. For the soil sample, its source and characteristics are shown below:

Soil Name	Country	Type	% Clay	% Sand	% Silt	pHw	OM	Notebook
Drummer	U.S.A.	Clay Loam	33	26	41	6.2	5.9	2012-048

Fortifications of water samples were made using 5.0 (±2%) mL of water spiked with 0.01 µg/mL or 0.1 µg/mL standard solutions. Fortifications of soil samples were made using 5.0 (±2%) g of soil spiked with 0.01 µg/mL or 0.1 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-30AD UHPLC was used instead of an Agilent Series 1290 Infinity UHPLC system. An AB SCIEX Triple Quad 5500 was used instead of a SCIEX API5000 triple quad. 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 rimsulfuron and metabolites, as described in the method for DuPont-38604. The following is a summary of the method conducted at Alliance Pharma. The complete description of the method is described in the original method (DuPont-38604).

Rimsulfuron and metabolites were extracted from surface water and soil samples. For surface water, 5.00 mL (± 0.1 mL) of water was measured out into a 15-mL polypropylene centrifuge tube and was fortified with 50 µL of 0.01 µg/mL for LOQ concentration or 0.1 µg/mL for 10x LOQ concentration. To each sample, 500 µL of acetonitrile (ACN) and 25 µL of 1 M ammonium formate were added. All tubes were capped and shaken vigorously to mix. A 2 mL aliquot was taken of each sample and analyzed via reverse-phase HPLC (see section 3.7 for instrumentation).

For soil samples, 5.00 g (± 0.1 g) was measured out into a 50-mL polypropylene centrifuge tube and was fortified with 100 µL of 0.01 µg/mL for LOQ concentration or 0.1 µg/mL for 10x LOQ concentration. Samples were allowed to sit in a fume hood for 10 minutes to allow fortification solvent to evaporate. Next, 25 mL of 9:1 0.1 M aqueous ammonium acetate:methanol (MeOH) was added and the tubes were capped, placed in a mini beadbeater and shaken for 10 minutes to break up the soil and extract the analytes, and then were vortexed. The tubes were centrifuged at 4°C at 3000 rpm for 10 minutes. The supernatant was decanted into a clean 50-mL glass centrifuge tube. Another 25 mL of 9:1 0.1 M aqueous ammonium acetate:MeOH was added and the tubes were again shaken, centrifuged, and the supernatant was decanted into the same 50-mL centrifuge tube as before. The total volume as adjusted to 50 mL with 9:1 0.1 M aqueous ammonium acetate:MeOH and tubes were shaken to mix thoroughly.

The extract from the soil was then purified via solid phase extraction (SPE). A 20 cc, 1-g Oasis HLB cartridge was placed on an SPE manifold and conditioned with 10 mL of

MeOH, followed by 10 mL of 9:1 0.1 M aqueous ammonium acetate:MeOH. Using a measuring pipette, 25 mL of sample extract was passed through the conditioned cartridge at a flow rate of between 2 and 5 mL/min. Before all the solution passed through, the cartridge was rinsed with 10 mL of HPLC-grade water, and all eluates were discarded. Next, 10 mL of 10 mM aqueous ammonium acetate were passed through the cartridge followed by 10 mL of HPLC-grade water. The cartridge was dried under full vacuum for at least 2 minutes and all eluates were discarded. The sample tube was rinsed with acetone and the rinse was discarded. The analytes were eluted into the centrifuge tube under gravity flow with 10 mL of ACN, followed by 5 mL of 9:1 acetonitrile:0.5 M ammonium hydroxide. Full vacuum was then applied for ~15 seconds to remove all liquid. Immediately, 1 mL of 5 mM aqueous ammonium acetate was added to the centrifuge tube and was allowed to evaporate to 1.0 mL (± 0.1 mL) under nitrogen flow in a 30°C water bath. Then 0.5 mL of ACN was added and the tubes were capped and vortexed. The final volume was adjusted to 5.0 mL with 5 mM aqueous ammonium acetate and the tubes were capped, vortexed, sonicated for 5 minutes, and vortexed again. The purified extract was filtered through a 0.2-µm PTFE disc and a 1.5 mL aliquot was taken and analyzed via reverse-phase HPLC.

The purified final extracts were analyzed by reversed-phase HPLC using a Kinetex C18 1.7µm 2.1 x 50 mm column with mobile phases of 0.01 M aqueous formic acid solution and methanol. Rimsulfuron, IN-70941, IN-70942, and IN-E9260 were detected using positive ion mode. Two parent-to-daughter ion transitions of each analyte were monitored as follows: rimsulfuron using 432.1→325.0 and 432.1→182.0 m/z, IN-70941 using 368.1→325.0 and 368.1→231.0 m/z, IN-70942 using 325.1→279.0 and 325.1→231.0 m/z, and IN-E9260 using 251.1→234.0 and 251.1→106.0 m/z. The confirmatory method was based on acceptable calibration curve and fortification recovery data generated from the second/confirmatory ion transition during the validation.

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 LC-30AD UHPLC was used instead of an Agilent Series 1290 Infinity UHPLC system. An AB SCIEX Triple Quad 5500 was used instead of a SCIEX API5000 triple quad.

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

During sample analyses, collision energies (CE) used for rimsulfuron, IN-70941 and IN-70942 were not in optimized conditions in order to obtain sufficient mass spectrometer signal strength and thus preserve the linearity of the instrument response.

3.7 *Instrumentation*

HPLC Conditions

System:	Shimadzu LC-30AD / Sil-30AC Autosampler
Column:	Kinetex C18 1.7µm 2.1 x 50 mm
Column Temperature:	50°C
Injection Volume:	20 µL
Autosampler Temperature:	4°C
Conditions:	A: 0.01 M FA in H_2O
	B: Methanol
	Flow in mL/minute
	Time %A %B Flow 0.0 95 5 0.600 0.3 95 5 0.600 0.4 85 15 0.600 4.9 45 55 0.600 5.0 5 95 1.00 6.0 5 95 1.00 6.1 95 5 0.600 8.0 95 5 0.600
Analyte Retention Times (minutes)	
DPX-E9636	~4.5
IN-70941	~3.3
IN-70942	~4.0
IN-E9260	~1.0

The detection method utilized was LC-MS/MS employing atmospheric pressure electrospray ionization interface in the positive mode on a triple quadrupole instrument. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for rimsulfuron and metabolites are shown below:

SYSTEM:	AB SCIEX TRIPLE QUAD 5500			
ANALYTES	IONS MONITORED	DECLUSTERING POTENTIAL (DP)	COLLISION ENERGY (CE)	EXIT POTENTIAL (CXP)
DPX-E9636	432.1 → 325.0 AMU	90	27	13
	432.1 → 182.0 AMU	90	36	13
IN-70941	368.1 → 325.0 AMU	65	36	13
	368.1 → 231.0 AMU	65	45	13
IN-70942	325.1 → 279.0 AMU	120	45	13
	325.1 → 231.0 AMU	120	58	13
IN-E9260	251.1 → 234.0 AMU	75	15	25
	251.1 → 106.0 AMU	75	23	13
Ion Mode:	Positive			
Turbo Spray Voltage:	4000 V			
Source Temperatures:	500°C			
CUR:	20 psig			
CAD:	10			
GS1:	50 psig			
GS2:	50 psig			

The instrument was operated in the MS/MS (MRM) positive ion mode for quantitative analysis of rimsulfuron, IN-70941, IN-70942 and IN-E9260. The ion chromatograms were integrated and the peak areas were used for quantitation.

For each analytical run, an eight-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. The relative ratio of the fragment ions was evaluated to confirm the presence of an analyte in an unknown sample.

3.8 Calculations

Residue rimsulfuron 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 0.025 ng/mL to 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.

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

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
G :	Sample weight

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

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

Example 1: Table 1, Sample LOQ-1, Rimsulfuron, Surface Water, Fortified @ 0.1 ppb, transition ion: 432.1 → 325.0:

Calibration curve calculated by Analyst software:

y = (2.46e+005) x + (491)

Where:

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

$C_{End} = x = (27071 - 491) / (2.46e+005)$

$= \mathbf{0.1080\ ng/mL}$

$R_{fortified} = 0.1$ ppb

$V_F = 5.525$ mL

5.0 CONCLUSIONS

Alliance Pharma successfully, independently validated the DuPont residue analytical method for rimsulfuron and its metabolites in water and soil, as described in DuPont Study No. DuPont-38604.

An LOQ of 0.1 µg/L (ppb) was demonstrated for all analytes evaluated in water, and an LOQ of 0.2 µg/kg (ppb) was demonstrated for all analytes evaluated in soil. The method was found to be suitable for the determination of rimsulfuron (DPX-E9636) and it metabolites IN-70941, IN-70942, and IN-E9260 in water and soil.