INDEPENDENT LABORATORY VALIDATION OF DUPONT-35446 "ANALYTICAL METHOD FOR THE DETERMINATION OF TRIBENURON METHYL AND METABOLITES IN SOIL USING LC/ MS/MS"

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1.0 SUMMARY

The objective of this study was to conduct an independent laboratory validation of analytical method DuPont-35446, entitled "Analytical Method for the Determination of Tribenuron Methyl and Metabolites in Soil Using LC/MS/MS", as written. This study was designed to fulfill the requirements of U.S. EPA guidelines found in OPPTS 850.6100 and EEC Directive 91/414/EEC, Annex IIA 4.2.1 as amended by EC Directive 96/46/EC; SANCO/825/00 rev.8.1 (16/11/2010) - Guidance Document on Residue Analytical Methods.

The method under evaluation has a stated Limit of Quantitation (LOQ) of 0.001 mg/kg (ppm). In this study, the method was validated at the LOQ and 10×LOQ in soil for both of the analytes.

The first validation trial for the determination of the corresponding analytes in the test system using the method as written was successful for both analytes in soil. No communication between the study monitor and the study director was required during the laboratory trial.

All interferences were negligible at <30% of the LOQ. All individual percent recoveries were within the U.S. EPA guideline acceptance range of 70-120%. The average recovery at each fortification level for both primary and confirmation MS/MS transition was within the European Commission guideline acceptance range for an average recovery per fortification level of 70-120%, with RSDs ≤20% in each case. Average ratios for LOQ samples of the two corresponding transitions for each analyte all fell within the required range (≤ ±30%). Thus, the residue analytical method as described in DuPont-35446 was demonstrated to be applicable for the determination of tribenuron methyl (DPX-L5300) and IN-L5296 in soil.

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 (8 hours), with LC-MS/MS analysis performed unattended the next day.

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-35446 entitled "Analytical Method for the Determination of Tribenuron Methyl and Metabolites in Soil Using LC/MS/MS" is applicable for the quantitation of tribenuron methyl and its metabolites in soil.

Tribenuron methyl (DPX-L5300) and IN-L5296 were extracted from soil by homogenization in 4:1 acetone:0.1 M aqueous ammonium carbonate. The samples were then centrifuged, extract aliquots were evaporated, diluted with 4:1 acetone:0.1 M aqueous ammonium carbonate, and filtered through carbon solid phase extraction cartridges. The analytes were eluted with 1:1 acetone:0.1 M aqueous ammonium carbonate and the eluates were evaporated and reconstituted with HPLC grade water. The purified extracts were analyzed by reverse phase LC/MS/MS. Two transitions were monitored per analyte, and both were detected by positive ion MS/MS.

The analytical method was designed to achieve a LOQ of 0.001 mg/kg (ppm) for all analytes, and the Limit of Detection (LOD) was estimated to be 0.0003 mg/kg (ppm). The independent validation thus evaluated tribenuron methyl (DPX-L5300) and IN-L5296 recoveries of samples fortified at 1x and 10x the LOQ level. The method was used as written.

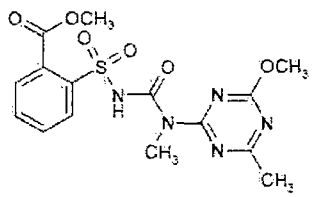
3.0 MATERIALS AND METHODS

3.1 Test Substance

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

DuPont code: DPX-L5300 (Tribenuron methyl)

Chemical Structure:

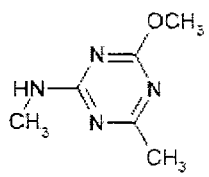


DPX-L5300

CAS Name:	methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) methylamino]carbonyl]amino]sulfonyl]benzoate
Molecular weight:	Average, 395.40 amu
Formula:	$C_{15}H_{17}N_5O_6S$
Source:	E. I. du Pont de Nemours and Company
CAS Number:	101200-48-0
Batch/Lot Number:	NOV00MA207
Purity:	98.7%
Receipt date:	29 March, 2013
Expiration date:	29 March, 2017
Storage:	Freezer (≤ -10°C)

DuPont code: IN-L5296 (Triazine Amine)

Chemical Structure:



IN-L5296

CAS Name:	4-methoxy-N,6-dimethyl-1,3,5-triazin-2-amine
Molecular weight:	Average, 154.17 amu
Formula:	$C_6H_{10}N_4O$
Source:	E. I. du Pont de Nemours and Company
CAS Number:	5248-39-5
Batch/Lot Number:	E79048-55
Purity:	99.6%
Receipt date:	29 March, 2013
Expiration date:	25 January, 2021
Storage:	Ambient temperature

Tribenuron methyl (DPX-L5300) and IN-L5296 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. Soil was obtained at the location of testing facility (17 Lee Boulevard, Malvern, PA 19355).

Fortifications of the samples were made using 5.0 g of soil spiked with 0.10 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. An ACE 3 C18 2.1x 50 mm column was used instead of a Mac Mod ACE 3 C18 150 x 3.0 mm 3-µm column. A Shimadzu LC-20AD HPLC was used instead of HP1200 HPLC systems. An AB SCIEX Triple Quad 5500 was used instead of an API 5000 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 tribenuron methyl (DPX-L5300) and IN-L5296, as described in the method for DuPont-35446. 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-35446).

Tribenuron methyl (DPX-L5300) and IN- L5296 were extracted from 5 grams of soil sample. Those requiring fortification were fortified with the appropriate standard solution and placed in a hood for 15 minutes. Fifteen milliliters of 4:1 acetone:0.1 M aqueous ammonium carbonate were added to each sample. The samples were then placed on a GenoGrinder shaker set at 1100 strokes per minute for three minutes. Once shaken, the samples were centrifuged for 10 minutes at approximately 3000 rpm. The steps of adding fifteen milliliters of 4:1 acetone:0.1 M aqueous ammonium carbonate to centrifugation were repeated two more times, with the extracts being combined before each repeat.

The supernatant was decanted before each repeat into a polypropylene tube, the final volume of supernatant was diluted to 50 mL of 4:1 acetone:0.1 M aqueous ammonium carbonate. The extracts were vortexed for approximately 30 seconds. A pipette volume of 20 mL was transferred from each sample into a 50 mL centrifuge tube and evaporated down to 5-7 mL using a flow of nitrogen at 25-30°C. The diluted sample extracts were loaded onto Envi-Carb cartridges, which were initially conditioned with methanol and 1:1 acetone:0.1M aqueous ammonium carbonate. After the cartridges were washed with 1:1 acetone:0.1M aqueous ammonium carbonate, the analytes were eluted with 1:1 acetone:0.1M aqueous ammonium carbonate. The eluates were then evaporated to dryness using a nitrogen evaporator at 25-30 °C, reconstituted in 6 mL of HPLC grade water and vortexed. An aliquot of the sample extract was then transferred into a HPLC vial. The purified final extracts were analyzed by reversed-phase HPLC using an ACE 3 C18 2.1x 50 mm column and 0.01 M formic acid in water and methanol as the mobile phases. The analytes were detected by mass spectrometry/mass spectrometry (MS/MS) in the positive ion mode. Two parent-to-daughter ion transitions of tribenuron methyl (DPX-L5300) (396.1→155.1 and 396.1→181.0, m/z) and IN-L5296 (155.0→57.1 and 155.0→71.1, m/z) were monitored. The confirmatory method was based on the relative ratios of the two MS/MS ion transitions during the validation.

Method validation was accomplished by analyzing both of 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:

1. An ACE 3 C18 2.1 x 50 mm column was used instead of a Mac Mod ACE 3 C18 150 x 3.0 mm 3-µm column. A short HPLC gradient run time (Section 3.7) was used because only two compounds were monitored in this independent lab validation.

2. A Shimadzu HPLC was used instead of Waters or Agilent HPLC. An AB SCIEX Triple Quad 5500 was used instead of an API 5000 triple quad.

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

3.7 *Instrumentation*

HPLC Conditions

System:	Shimadzu LC-20AD / Sil-20AC Autosampler
Column:	ACE 3 C18 2.1x50 mm
Column Temperature:	4°C
Injection Volume:	10 µL
Autosampler Temperature:	4°C
Conditions:	A: 0.01 M Formic Acid in Water B: Methanol Flow in mL/minute Time %A %B Flow 0.5 95 5 1.0 5.0 10 90 1.0 7.0 10 90 1.0 7.1 95 5 1.0 10 5 STOP
Analyte Retention Times (minutes)	
DPX-L5300	~4.45
IN-L5296	~2.15

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 tribenuron methyl (DPX-L5300) and IN-L5296 are shown below:

SYSTEM:	AB SCIEX TRIPLE QUAD 5500			
ANALYTES	IONS MONITORED	DECLUSTERING POTENTIAL (DP)	COLLISION ENERGY (CE)	EXIT POTENTIAL (CXP)
DPX-L5300	396.1 → 155.1 AMU	80	10	10
	396.1 → 181.0 AMU	80	28	10
IN-L5296	155.0 → 57.1 AMU	80	30	10
	155.0 → 71.1 AMU	80	26	10
Ion Mode: Turbo Spray Voltage: Source Temperatures:	Positive 4500 V 600 ^{0}C			

CUR:	30 psig	
CAD:	10	
GS1:	40 psig	
GS2:	50 psig	
Dwell:	75 ms	

The instrument was operated in the MS/MS (MRM) positive ion mode for quantitative analysis. 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. The relative ratio of the fragment ions was evaluated to confirm the presence of an analyte in an unknown sample.

3.8 Calculations

Residues tribenuron methyl (DPX-L5300) and IN- L5296 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 0.2 ng/mL to 5.0 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.

$R = [(C_{End} * V_F) / (AF*DF)] / (W / 1000)$

Where:

R: Analyte residue in mg/kg (ppm)

$R_{fortified}$: Amount of analyte residue fortified in mg/kg (ppm)

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

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

DF: Dilution factor = Aliquot volume (V_{aliq}) / Total volume (V_{Total})

V_F: Final volume

W: Soil matrix sample weight: 5 g

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

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

Example: Table 1, Sample LOQ-1, DPX-L5300, Soil, Fortified @ 0.001 ppm, transition ions 396.1-155.1.

Calibration curve calculated by Analyst software:

y = 2.25e+005 x + - 993

Where:

y: Peak area

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

C_{End} = x = (y + 993) / 2.25e+005

= (56045 + 993) / 2.25e+005 = 0.254 ng/mL

$R_{fortified}$ = 0.001 ppm

V_F = 6 mL

AF = $V_{aliq\ Ex}$ / $V_{Total\ Ex}$ = 20 mL / 50 mL = 0.400

DF = V_{aliq} / V_{Total} = 20 mL / 20 mL = 1

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

= 0.254 (ng/mL) * (6 mL) / (0.400*1) / 5 g / 1000= 0.00076 ppm

Rec. = $(R / R_{fortified})$ x 100 % = (0.00076 / 0.001) x 100% = 76%