EXPERIMENTAL

Sample Origin, Numbering, Preparation and Storage

Untreated control samples were obtained from the Dow AgroSciences Brazil Regulatory Sciences Laboratory Sample Management Group. All samples were tracked in the Dow AgroSciences Labs Information Management System (RLIMS) database. Unique sample numbers were assigned to the samples to track them during receipt, storage, and analysis. Complete source documentation was included in the study file.

During the course of the study, the samples were stored in temperature-monitored freezers at approximately - 20 °C, except when removed for analysis. Complete documentation may be found in the raw study file.

Determination of Isotopic Crossover

In this method validation, no stable-isotope labeled internal standards were used. In this way, the determination of isotopic crossover assay was not performed and there is no possibility that isotopic contributions could have occured between the transitions used for quantitation of the unlabeled and labeled compounds.

Calculation of Standard Calibration Curve

Calculation of a standard curve begins with the injection of a series of calibration standards described in Appendix I and acquisition of peak areas for the following analytes.

Fluroxypyr-MHE	*m/z* Q1/Q3 367/255 (quantitative)
Fluroxypyr-MHE	*m/z* Q1/Q3 367/209 (confirmatory)
Fluroxypyr acid	*m/z* Q1/Q3 255/181 (quantitative)
Fluroxypyr acid	*m/z* Q1/Q3 255/209 (confirmatory)
Fluroxypyr-DCP	*m/z* Q1/Q3 197/152 (quantitative)
Fluroxypyr-DCP	*m/z* Q1/Q3 197/144 (confirmatory)
Fluroxypyr-MP	*m/z* Q1/Q3 211/196 (quantitative)
Fluroxypyr-MP	*m/z* Q1/Q3 211/113 (confirmatory)

For each analyte, the linearity of detector response was evaluated using matrix-matched standard solutions. In order to generate a standard curve, plot the analyte concentration on the abscissa (x-axis) and the respective peak area on the ordinate (y-axis) in the Analyst software. Using regression analysis, determine the equation for the curve with respect to the abscissa. Refer to

Figures 5 – 12 for example calibration plots. Individual calibration results and analytical set parameters can be found in Table 2 – 9.

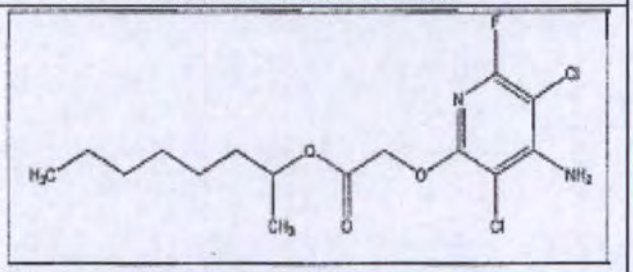
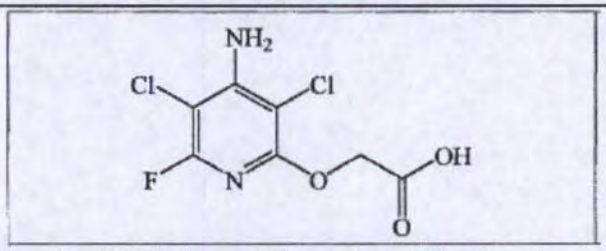
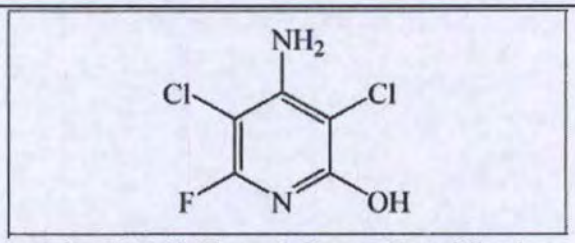
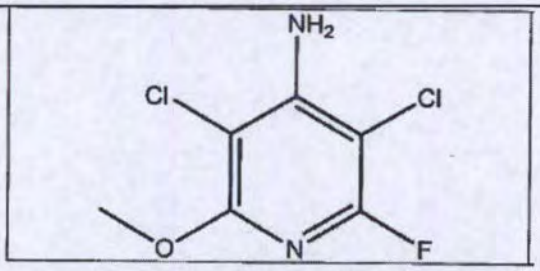
Full-Scan and Product-Ion Mass Spectra

A full scan and two product-ion mass spectra of Fluroxypyr-MHE, Fluroxypyr acid, Fluroxypyr-DCP and Fluroxypyr-MP are illustrated in Figure 17 - 20.

Statistical Treatment of Data

Statistical treatment of data included but was not limited to the calculation of regression equations, correlation coefficients (r) for describing the linearity of calibration curves, and means, standard deviations, and relative standard deviations of the results for the fortified recovery samples.

Table 1. Identities and structures of Fluroxypyr-MHE, Fluroxypyr acid, Fluroxypyr-DCP and Fluroxypyr-MP.

Identifying Information	Structure and CAS Name
Common Name of Compound: Fluroxypyr-MHE Molecular Formula: $C_{15}H_{21}Cl_2FN_2O_3$ Formula Weight: 367.24 Nominal Mass: 367 CAS Number: NA	 ((4-amino-3,5,-dichloro-6-fluoro-2-pyridinyl)oxy)acetic acid l-methylheptyl ester
Common Name of Compound: Fluroxypyr acid Molecular Formula: $C_7H_5Cl_2FN_2O_3$ Formula Weight: 255.03 Nominal Mass: 255 CAS Number: NA	 ((4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy) acetic acid
Common Name of Compound: Fluroxypyr-DCP Molecular Formula: $C_5H_3Cl_2FN_2O$ Formula Weight: 197.0 Nominal Mass: 197 CAS Number: NA	 4-amino-3,5-dichloro-6-fluoro-2-pyridinol
Common Name of Compound: Fluroxypyr-MP Molecular Formula: $C_6H_5Cl_2FN_2O$ Formula Weight: 211.02 Nominal Mass: 211 CAS Number: NA	 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine

APPENDIX I ANALYTICAL METHOD

Analytical Method Validation for the Analysis of Fluroxypyr-MHE, Fluroxypyr acid, Fluroxypyr-DCP and Fluroxypyr-MP in Soil and Sediment

Scope

This method is applicable for the determination of residues of Fluroxypyr-MHE, Fluroxypyr acid, Fluroxypyr-DCP and Fluroxypyr-MP in Soil and Sediment. This method is applicable over a concentration range of 0.0004 – 1 µg/g for Fluroxypyr-MHE and Fluroxypyr acid; 0.01 – 1 µg/g Fluroxypyr-DCP and Fluroxypyr-MP.

Principle

Residues of Fluroxypyr-MHE, Fluroxypyr acid, Fluroxypyr-DCP and Fluroxypyr-MP in soil and sediment were extracted from samples by homogenizing and shaking with acetonitrile/0.5 N HCl (90:10, v/v) and NaCl and $MgSO_4$. Shaked. Centrifuged and transferred an aliquot of extraction solution into a 15 mL tube and added HCl 0.5 N. The aliquot was then purified using an offline Strata-X polymeric sorbent (60 mg, 3 mL) solid phase extraction (SPE) column. After elution from SPE column with acetonitrile, samples were diluted with H_2O/0.2 % acetic acid solution directly into 2 mL HPLC vial. The sample were analyzed for all analytes by liquid chromatography coupled with positive-ion electrospray ionization tandem mass spectrometry (LC-MS/MS).

Safety Precautions

Each analyst must be acquainted with the potential hazards of the equipment, reagents, products, solvents and procedures used in this method before commencing laboratory work. Sources of information include operation manuals, material safety data sheets, literature, and other related data. Safety information should be obtained from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance applicable governmental requirements.

Acetonitrile and methanol are flammable and should be used in well-ventilated areas away from ignition sources. Acetic acid and hydrochloric acid are corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

Laboratory Equipment

Balance, analytical Sartorius

Balance, Mettler – Toledo

Bottle, 250 mL nalgene

Bottle-Top Dispenser, 100 mL, Brand

Strata-X, 33 µm Polymeric Reversed Phase (60mg/3 mL) SPE, part# 8B-S100-UBJ, Phenomenex

Pipette 5-100 µL capacity, Eppendorf

Pipette, 50-1000 µL capacity, Eppendorf

Pipette, 100-5000 µL capacity, Eppendorf

Tomtec Autogizer

Vacuum Manifold, IST VacMaster

Centrifuge, Eppendorf 5810

Chromatographic and Spectrometric Systems

Column, analytical, Eclipse Plus Phenyl Hexyl 3.0 x 50 mm x 1.8 µm (SN: USPHP01280), Agilent Technologies

Liquid chromatography, Model Agilent 1290, Agilent Technologies

Mass spectrometer, QTRAP 6500, Applied Biosystems

Mass spectrometer data system, Analyst 1.6.2, Applied Biosystems

Reagents and solvents

Acetonitrile, HPLC grade, J. T. Baker

Methanol, HPLC grade, J. T. Baker

Acetic acid, Merck

Hydrochloric acid, Merck

Water, Milli Q

Sodium chloride, J.T. Baker

Magnesium sulphate, Dinâmica

Prepared Solutions

Water containing 0.1 % acetic acid (v/v)
Measure 999 mL of HPLC grade water, using a graduated cylinder. Pipette 1.0 mL of acetic acid into the 1000 mL graduated cylinder and mix.

Water containing 0.2 % acetic acid (v/v)
Measure 998 mL of HPLC grade water, using a graduated cylinder. Pipette 2.0 mL of acetic acid into the 1000 mL graduated cylinder and mix.

Methanol containing 0.1 % acetic acid (v/v)
Measure 1000 mL of methanol, using a graduated cylinder. Pipette1.0 mL of acetic acid into the 1000 mL graduated cylinder and mix.

Hydrochloric Acid 0.5 N
Measure 983 mL of HPLC grade water, using a graduated cylinder. Pipette 17 mL of concentrated hydrochloric acid into the 1000 mL graduated cylinder and mix.

Acetonitrile/0.5 N Hydrochloric Acid (90:10, v/v)
Measure 900 mL of acetonitrile, using a graduated cylinder, and transfer into a 1 L bottle. Add 100 mL of 0.5N hydrochloric acid into the 1 L bottle and mix.

Acetonitrile/0.5 N Hydrochloric Acid (10:90, v/v)
Measure 100 mL of acetonitrile, using a graduated cylinder, and transfer into a 1 L bottle. Add 900 mL of 0.5N hydrochloric acid into the 1 L bottle and mix.

Preparation of Fortification Solutions

1. Weigh 0.0127 g of Fluroxypyr-MHE analytical standard and quantitatively transfer into a 25 mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 507.49 µg/mL stock solution of Fluroxypyr-MHE.

2. Weigh 0.0126 g of Fluroxypyr acid analytical standard and quantitatively transfer into a 25 mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 501.98 µg/mL stock solution of Fluroxypyr acid.

3. Weigh 0.0129 g of Fluroxypyr-DCP analytical standard and quantitatively transfer into a 25 mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 510.84 µg/mL stock solution Fluroxypyr-DCP.

4. Weigh 0.0133 g of Fluroxypyr-MP analytical standard and quantitatively transfer into a 25 mL volumetric flask with acetonitrile. Dilute to volume with acetonitrile to obtain a 510.72 µg/mL stock solution Fluroxypyr-MP.

5. Pipette 9.85 mL of Fluroxypyr-MHE and 9.96 mL of Fluroxypyr acid into the same 50 mL volumetric flask. Dilute to volume with acetonitrile to obtain a 100 µg/mL mix of Fluroxypyr-MHE and Fluroxypyr acid spiking solution.

6. Pipette 9.79 mL of Fluroxypyr-DCP and 9.79 mL of Fluroxypyr-MP into the same 50 mL volumetric flask. Dilute to volume with acetonitrile to obtain a 100 µg/mL mix of Fluroxypyr-DCP and Fluroxypyr-MP spiking solution.

7. Pipette 5 mL of the 100 µg/mL mixed spiking solution prepared in step 5 into a 50 mL volumetric flask. Dilute to volume with acetonitrile to obtain a 10 µg/mL mix of Fluroxypyr-MHE and Fluroxypyr acid spiking solution.

8. Pipette 5 mL of the 100 µg/mL mixed spiking solution prepared in step 6 into a 50 mL volumetric flask. Dilute to volume with acetonitrile to obtain a 10 µg/mL mix of Fluroxypyr-DCP and Fluroxypyr-MP spiking solution.

9. Pipette 5 mL of the 10 µg/mL mixed spiking solution prepared in step 7 into a 50 mL volumetric flask. Dilute to volume with acetonitrile to obtain a 1 µg/mL mix of Fluroxypyr-MHE and Fluroxypyr acid spiking solution.

10. Pipette 5 mL of the 10 µg/mL mixed spiking solution prepared in step 8 into a 50 mL volumetric flask. Dilute to volume with acetonitrile to obtain a 1 µg/mL mix of Fluroxypyr-DCP and Fluroxypyr-MP spiking solution.

11. Pipette 5 mL of the 1 µg/mL mixed spiking solution prepared in step 9 into a 50 mL volumetric flask. Dilute to volume with acetonitrile to obtain a 0.1 µg/mL mix of Fluroxypyr-MHE and Fluroxypyr acid spiking solution.

Note: All of the above stock and spiking solutions should be stored in refrigerator.
Stock solutions were corrected by the purity (99.9 % for Fluroxypyr-MHE, 99.6 % for Fluroxypyr acid, 99 % for Fluroxypyr-DCP and 96 % for Fluroxypyr-MP).

Preparation of Calibration Standards

Prepare calibration standards by using the spiking solutions described above as shown in the following table. Dilute the calibrators to volume with acetonitrile. Calibration solutions must be stored in refrigerator.
The final calibration curve were matrix matched. Prior injection, each calibration solution were separetly diluted with extract of control sample (0.05 mL of standard solution + 0.95 mL of control sample extract).

For Fluroxypyr-MHE and Fluroxypyr acid:

Spiking sol (µg/mL)	Aliquot (mL)	Final vol (mL)	Final concentration (ng/mL)
0.1	0.50	25	2
1.0	0.25	25	10
1.0	1.00	25	40
10.0	0.25	25	100
10.0	0.50	25	200
10.0	1.00	25	400
10.0	1.50	25	600

For Fluroxypyr-DCP and Fluroxypyr-MP:

Spiking sol (µg/mL)	Aliquot (mL)	Final vol (mL)	Final concentration (ng/mL)
1	0.500	25	20
10	0.250	25	100
10	0.500	25	200
10	0.750	25	300
10	1.000	25	400
100	0.125	25	500
100	0.150	25	600

Instrumental Conditions

Typical LC-MS/MS Operating Conditions

Instrumentation:	Agilent 1290 Infinity LC System AB SCIEX API 6500 LC/MS/MS System AB SCIEX Analyst 1.6.2 data system
Column:	Eclipse Plus Phenyl Hexyl 3.0 x 50 mm x 1.8 µm (SN:USPHP01280)
Column Temperature:	50 °C
Sample Temperature	15 °C
Injection Volume:	10 µL
Autosampler Wash	30 seconds of acetonitrile/water (80:20, v/v) at the flush port
Mobile Phase:	A – water containing 0.1 % acetic acid B – methanol containing 0.1 % acetic acid
Flow Rate:	500 µL/min

Gradient for Fluroxypyr acid and Fluroxypyr-MHE

Time, min	Solvent A, %	Solvent B, %
0.0	90	10
0.50	90	10
2.00	3	97
4.00	3	97
4.10	90	10
6.00	90	10

Gradient for Fluroxypyr-DCP and Fluroxypyr-MP

Time, min	Solvent A, %	Solvent B, %
0.0	90	10
0.50	90	10
2.00	3	97
3.50	3	97
3.60	90	10
6.00	90	10

Typical Mass Spectrometry Operating Conditions

Ionization Mode:	Electrospray
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Collision Gas (CAD):	Medium
Curtain Gas (CUR):	15
Ion Source Gas 1 (GS1):	40 psi
Ion Source Gas 2 (GS2):	40 psi
Temperature (TEM):	500 °C
Entrance Potential (V):	10
IonSpray Voltage (IS):	5000 volts

MS transitions:

ID	Q1 (Da)	Q3 (Da)	Time (min)	DP (V)	CE (V)	CXP (V)
Fluroxypyr-MHE	367.025	255.000	3.11	30	15	16
		209.000		30	31	14
Fluroxypyr acid	254.910	181.000	2.60	16	31	12
		209.000		16	21	14
Fluroxypyr-MP	210.917	196.000	2.85	40	29	12
		112.900		40	49	14
Fluroxypyr-DCP	196.910	152.000	2.45	50	37	16
		144.000		60	41	8

The instrumental conditions may be modified to obtain optimal chromatographic separation and sensitivity.

Analysis Procedure

For procedural recovery samples:

1. For reagent blank, add 10 mL of extraction solution into 250-mL (8 ounce) nalgene bottle containing no samples.
2. For control samples transfer 10.0 g of each type of sample into a 250-mL (8 ounce) nalgene bottle.
3. For fortified samples, transfer 10.0 g of each type of sample into separate 250-mL (8 ounce) nalgene bottles. Add the appropriate volume of the spiking solution to obtain fortified samples.

Fluroxypyr-MHE and Fluroxypyr acid			
Description	Spiking Volumes (µL)	Spiking Solutions (µg/mL)	Fortification Level (µg/g)
Control	---	---	---
LOD	120	0.01	0.00012
LOQ	40	0.1	0.0004
10× LOQ	40	10	0.04
2500× LOQ	100	100	1

Fluroxypyr-DCP and Fluroxypyr-MP			
Description	Spiking Volumes (µL)	Spiking Solutions (µg/mL)	Fortification Level (µg/g)
Control	---	---	---
LOD	30	1	0.003
LOQ	100	1	0.01
10× LOQ	100	10	0.1
2500× LOQ	100	100	1

For field samples:

4. Weigh 10.0 ± 0.05 g of each soil sample into individual 50 mL polypropylene centrifuge tubes equipped with caps (acceptable stopping point if sample is kept frozen);
5. Add 10 mL of the ACN/0.5 N hydrochloric acid (90:10, v/v) extraction solution;
6. Add 2 g of NaCl and 5 g of $MgSO_4$;
7. Cap the sample vial and shake at approximately 180 excursions/minute for 60 min;
8. Centrifuge the sample for 5 min/2500 rpm;

Clean-up step: Fluroxypyr and Fluroxypyr-MHE

9. For Fluroxypyr acid and Fluroxypyr-MHE transfer 2 mL of the extract (from Step 8) to a 15 mL falcon tube and add 8 mL of 0.5 N hydrochloric acid, pulse vortex mix for about 5 seconds;

10. Clean up samples on the Strata-X polymeric sorbent SPE cartridge using the following procedure:

a. Place a Strata -X polymeric sorbent SPE cartridge (60 mg, 3 mL) on a vacuum manifold.
b. Condition the SPE cartridge with 3 mL of acetonitrile followed by 3 mL of 0.5 N hydrochloric acid, discarding the eluates. Apply full vacuum for about 10 seconds between solvent additions;
c. Transfer the diluted sample (from Step 9) to the SPE cartridge. Pull the sample through the SPE cartridge at approximately 1 mL/min, using vacuum if necessary. Discard the eluate;
d. Rinse with 3 mL of acetonitrile/0.5 N hydrochloric acid (10:90, v/v) solution. Discard the eluate;
e. Elute Fluroxypyr acid and Fluroxypyr-MHE from the SPE cartridge with 2 mL of acetonitrile at a rate of approximately 1 mL/min, using vacuum if necessary. Collect the eluate (acceptable stopping point if sample is kept refrigerated);

Dilution step: Fluroxypyr and Fluroxypyr-MHE

11. Transfer 0.6 mL of extract (Step 10.e) into a 2 mL vial and add 0.4 mL of H2O/0.2 % acetic acid;
12. Analyse by LC-MS/MS;

Clean-up step: Fluroxypyr-DCP and Fluroxypyr-MP

13. For Fluroxypyr-DCP and Fluroxypyr-MP transfer 1 mL of the extract (from Step 8) to a 15 mL falcon tube and add 7 mL of 0.5 N hydrochloric acid, pulse vortex mix for about 5 seconds;

14. Clean up samples on the Strata-X polymeric sorbent SPE cartridge using the following procedure:

a. Place a Strata -X polymeric sorbent SPE cartridge (60 mg, 3 mL) on a vacuum manifold.
b. Condition the SPE cartridge with 3 mL of acetonitrile followed by 3 mL of 0.5 N hydrochloric acid, discarding the eluates. Apply full vacuum for about 10 seconds between solvent additions;
c. Transfer the diluted sample (from Step 13) to the SPE cartridge. Pull the sample through the SPE cartridge at approximately 1 mL/min, using vacuum if necessary. Discard the eluate;
d. Rinse with 3 mL of acetonitrile/0.5 N hydrochloric acid (10:90, v/v) solution. Discard the eluate;

e. Elute Fluroxypyr-DCP and Fluroxypyr-MP from the SPE cartridge with 2 mL of acetonitrile at a rate of approximately 1 mL/min, using vacuum if necessary. Collect the eluate (acceptable stopping point if sample is kept refrigerated);

Dilution step: Fluroxypyr-DCP and Fluroxypyr-MP

15. Transfer 0.6 mL of extract (Step 14.e) into a 2 mL vial and add 0.6 mL of H2O/0.2 % acetic acid;
16. Analyse by LC-MS/MS;

Analyze the calibration standards and samples by LC-MS/MS with positive-ion electrospray tandem mass spectrometry, injecting the calibration standards interspersed with the samples throughout the run. Determine the suitability of the chromatographic system using the following performance criteria:

a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.
b. Peak resolution: Visually determine that sufficient resolution has been achieved for the analyte and internal standard relative to background interferences.
c. Appearance of chromatograms: Visually determine the chromatograms with respect to peak response, baseline noise, and background interference.

For dilution use extract from a control sample.

(**NOTE:** evaporation procedure must be avoided).

Re-analyze any samples with concentrations greater than 80 % of the highest standard with an appropriate amount of dilution solution.
The gross analyte concentration should be at least 30 % above the lowest calibration standard and at least 20 % less than the highest calibration standard.