Independent Laboratory Validation of an Analytical Method for the Determination of Fluroxypyr and its Metabolites in Soil using LC-MS/MS

## INTRODUCTION

### Scope

This method is applicable for the quantitative determination of residues of fluroxypyr and its metabolites in soil. The method is detailed in the report for Study ID 160535 'Analytical Method Validation for the Analysis of Fluroxypyr-MHE, Fluroxypyr acid, Fluroxypyr-DCP and Fluroxypyr-MP in Soil and Sediment' (*1*) and was included in the appendix to amendment 1 of the study plan. The method was independently validated over the concentration range of 0.0004-1.0 mg/kg (µg/g), with a validated limit of quantitation of 0.0004 mg/kg (µg/g) for fluroxypyr 1-MHE and fluroxypyr acid. The method was also validated over the concentration range of 0.01-1.0 mg/kg (µg/g), with a validated limit of quantitation of 0.01 mg/kg (µg/g) for fluroxypyr-DCP (X061784) and fluroxypyr-MP (X61420). Common names, chemical names, and structural formulas for the analytes are given in Table 1.

This study was conducted to fulfil data requirements outlined in the EPA Ecological Effects Test Guidelines, OCSPP 850.6100 (*2*). The validation also complies with the requirements of SANCO/825/00 rev.8.1 (*3*). A maximum of three sample set attempts were allowed in order to validate the method for the independent laboratory validation (ILV).

### Method Principle

Residues of fluroxypyr-MHE, fluroxypyr acid, fluroxypyr-DCP (X061784) and fluroxypyr-MP (X61420) are extracted from samples by homogenising and shaking with acetonitrile/0.5 N hydrochloric acid (90:10, v/v), adding sodium chloride and magnesium sulfate. An aliquot of extraction solution is acidified with 0.5 N hydrochloric acid and purified using an offline Strata-X polymeric sorbent solid phase extraction (SPE) column. Eluted samples in acetonitrile are diluted with water containing 0.2% acetic acid (v/v) solution to a final solution of acetonitrile/water containing 0.2% acetic acid (60:40, v/v). The final sample is analysed for fluroxypyr-MHE, fluroxypyr acid, fluroxypyr-DCP (X061784) and fluroxypyr-MP (X61420) by liquid chromatography coupled with positive-ion electrospray tandem mass spectrometry (LC-MS/MS).

Reference Compounds/Analytical Standards

| Test Substance | TSN | Percent Purity | Recertification Date | Lot No. |
|---|---|---|---|---|
| Fluroxypyr 1-MHE | TSN308719 | 99.5 | 08 Oct 2018 | YC2-142695-96 |
| Fluroxypyr acid | TSN301238 | 98.6 | 31 Oct 2017 | LP-SCF-II-052 |
| Fluroxypyr-2-pyridinol (Fluroxypyr-DCP (X061784)) | TSN306123 | 99 | 22 Oct 2017 | SYN-FS10329-53 |
| DMP Fluroxypyr metabolite (Fluroxypyr-MP (X61420)) | TSN027993-0001 | 96 | 31 Oct 2020 | SYN-6477-51-01 |

The Certificates of Analysis for the reference substances can be found in Figures 1-4. The above standards may be obtained free of charge from Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

# EXPERIMENTAL

Sample Origin, Numbering, Preparation, Storage, and Characterisation

Prepared soil sample (CSR-7909-001) was supplied by the Sponsor. The soil sample was characterised by Agvise Laboratories, 604 Highway 15 West, P.O. Box 510, Northwood, ND 58267. The Characterisation Report for the soil sample can be found in Figure 5.

During the course of the study, the sample was stored in a temperature-monitored coldroom set to maintain a sample temperature of 2 - 8 °C, except when removed for analysis.

Calculation of Standard Calibration Curve

Calculation of a standard curve began with the injection of eight calibration standards, which were within the concentration range of 0.1 – 30 ng/mL for fluroxypyr 1-MHE and fluroxypyr acid and 1.0 – 30 ng/mL for fluroxypyr-MP (X61420) and fluroxypyr-DCP (X061784) respectively. This was performed according to the method taken from the report for Study ID 160535 'Analytical Method Validation for the Analysis of Fluroxypyr-MHE, Fluroxypyr acid, Fluroxypyr-DCP and Fluroxypyr-MP (X61420) in Soil and Sediment' (*1*). Instrument conditions are described in Appendix 2 and acquisition of peak areas were made for the following analytes.

| | |
|---|---|
| Fluroxypyr 1-MHE | *m/z* Q1/Q3 367.1/255.1 (quantitative)<br>*m/z* Q1/Q3 367.1/209.0 (confirmatory) |
| Fluroxypyr acid | *m/z* Q1/Q3 255.1/181.0 (quantitative)<br>*m/z* Q1/Q3 255.1/209.0 (confirmatory) |
| Fluroxypyr-MP (X61420) | *m/z* Q1/Q3 210.9/196.0 (quantitative)<br>*m/z* Q1/Q3 210.9/112.9 (confirmatory) |
| Fluroxypyr-DCP (X061784) | *m/z* Q1/Q3 196.9/152.0 (quantitative)<br>*m/z* Q1/Q3 196.9/144.0 (confirmatory) |

For each analyte, the linearity of detector response was evaluated using matrix-matched standards. In order to generate a standard curve, the analyte concentration was plotted on the abscissa (x-axis) and the respective peak area on the ordinate (y-axis) in Excel. Using regression analysis the equation for the curve with respect to the abscissa was determined. From this standard curve the correlation coefficient (r) and the coefficient of determination ($r^2$) were determined. Linear regression analysis with 1/x weighting was then used to generate a standard calibration equation for determination of analyte concentrations.

Refer to Figures 6-21 for example calibration curves and response factor plots.

Individual calibration results can be found in Tables 2-13.

Determination of Fluroxypyr 1-MHE in a Fortified Sample

An example calculation for the quantitative recovery of fluroxypyr 1-MHE in a 0.0004 mg/kg fortified sample is shown below:

Sample reference ASR 0276/17/04

Fluroxypyr 1-MHE peak area = 21389

From the 1/x weighted calibration curve, where standard concentration in ng/mL is plotted against the peak area:
Slope = 69641.5264
Intercept = 4607.9292

Residue of fluroxypyr 1-MHE in final volume of sample
= (Peak area – Intercept) ÷ Slope = Residue in Final Volume (ng/mL)
= (21389 – 4607.9292) ÷ 69641.5264 = 0.240964 ng/mL

Sample concentration = 0.6 g/mL

Fluroxypyr 1-MHE residue in sample = 0.240964 ng/mL ÷ 0.6 g/mL = 0.40 µg/kg
0.40 µg/kg ÷ 1000 = 0.00040 mg/kg

Recovery of fluroxypyr 1-MHE in 0.0004 mg/kg fortified sample
= Residue in Sample ÷ Recovery Level × 100%
= 0.00040 mg/kg ÷ 0.0004 mg/kg × 100%
= 100%

Determination of Fluroxypyr acid in a Fortified Sample

An example calculation for the quantitative recovery of fluroxypyr acid in a 0.0004 mg/kg fortified sample is shown below:

Sample reference ASR 0276/17/05

Fluroxypyr acid peak area = 2498

From the 1/x weighted calibration curve, where standard concentration in ng/mL is plotted against the peak area:
Slope = 12407.1025
Intercept = -501.1218

Residue of fluroxypyr acid in final volume of sample
= (Peak area – Intercept) ÷ Slope = Residue in Final Volume (ng/mL)
= (2498 + 501.1218) ÷ 12407.1025 = 0.241726 ng/mL

Sample concentration = 0.6 g/mL

Fluroxypyr acid residue in sample = 0.241726 ng/mL ÷ 0.6 g/mL = 0.40 µg/kg
0.40 µg/kg ÷ 1000 = 0.00040 mg/kg

Recovery of fluroxypyr acid in 0.0004 mg/kg fortified sample
= Residue in Sample ÷ Recovery Level × 100%
= 0.00040 mg/kg ÷ 0.0004 mg/kg × 100%
= 100%

Determination of Fluroxypyr-DCP (X061784) in a Fortified Sample

An example calculation for the quantitative recovery of fluroxypyr-DCP (X061784) in a 0.01 mg/kg fortified sample is shown below:

Sample reference ASR 0053/17/04

Fluroxypyr-DCP (X061784) peak area = 131167

From the 1/x weighted calibration curve, where standard concentration in ng/mL is plotted against the peak area:
Slope = 54765.831
Intercept = 9914.041

Residue of fluroxypyr-DCP (X061784) in final volume of sample
= (Peak area – Intercept) ÷ Slope = Residue in Final Volume (ng/mL)
= (131167 – 9914.041) ÷ 54765.831 = 2.214026 ng/mL

Sample concentration = 0.25 g/mL

Fluroxypyr-DCP (X061784) residue in sample = 2.214026 ng/mL ÷ 0.25 g/mL = 8.86 µg/kg
8.86 µg/kg ÷ 1000 = 0.00886 mg/kg

Recovery of fluroxypyr-DCP (X061784) in 0.01 mg/kg fortified sample
= Residue in Sample ÷ Recovery Level × 100%
= 0.00886 mg/kg ÷ 0.01 mg/kg × 100%
= 89%

Determination of Fluroxypyr-MP (X61420) in a Fortified Sample

An example calculation for the quantitative recovery of fluroxypyr-MP (X61420) in a 0.01 mg/kg fortified sample is shown below:

Sample reference ASR 0053/17/05

Fluroxypyr-MP (X61420) peak area = 21790

From the 1/x weighted calibration curve, where standard concentration in ng/mL is plotted against the peak area:
Slope = 9840.513
Intercept = -586.582

Residue of fluroxypyr-MP (X61420) in final volume of sample
= (Peak area – Intercept) ÷ Slope = Residue in Final Volume (ng/mL)
= (21790 + 586.582) ÷ 9840.513 = 2.273924 ng/mL

Sample concentration = 0.25 g/mL

Fluroxypyr-MP (X61420) residue in sample = 2.273924 ng/mL ÷ 0.25 g/mL = 9.10 µg/kg
9.10 µg/kg ÷ 1000 = 0.00910 mg/kg

Recovery of fluroxypyr-MP (X61420) in 0.01 mg/kg fortified sample
= Residue in Sample ÷ Recovery Level × 100%
= 0.00910 mg/kg ÷ 0.01 mg/kg × 100%
= 91%

Confirmation of Residue Identity

The method is specific for the determination of fluroxypyr 1-MHE, fluroxypyr acid, fluroxypyr-DCP (X061784) and fluroxypyr-MP (X61420) by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, an additional MS/MS transition was monitored for each analyte.

| | |
|---|---|
| Fluroxypyr 1-MHE | *m/z* Q1/Q3 367.1/255.1 (quantitative) |
| | *m/z* Q1/Q3 367.1/209.0 (confirmatory) |
| Fluroxypyr acid | *m/z* Q1/Q3 255.1/181.0 (quantitative) |
| | *m/z* Q1/Q3 255.1/209.0 (confirmatory) |
| Fluroxypyr-MP (X61420) | *m/z* Q1/Q3 210.9/196.0 (quantitative) |
| | *m/z* Q1/Q3 210.9/112.9 (confirmatory) |
| Fluroxypyr-DCP (X061784) | *m/z* Q1/Q3 196.9/152.0 (quantitative) |
| | *m/z* Q1/Q3 196.9/144.0 (confirmatory) |

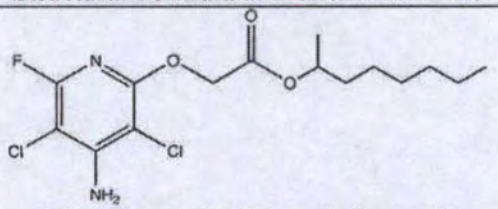
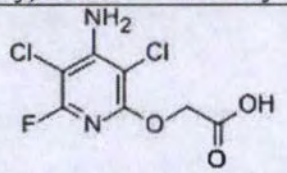
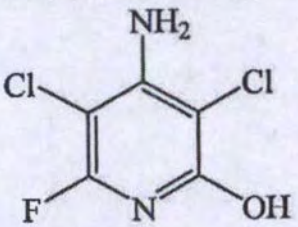
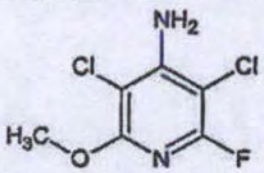
Statistical Treatment of Data

Statistical treatment of the data included but was not limited to the calculation of regression equations, correlation coefficient (r) and coefficient of determination ($r^2$) for describing the linearity of calibration curves, means, standard deviations and relative standard deviations of the results for the fortified recovery samples.

Acceptance/Rejection of Calibration Linearity Curves

The acceptance criteria for the linearity of response was the coefficient of determination ($r^2$). This had to be equal to or greater than 0.995 for a minimum of a five point calibration curve. If this criteria was not met, then calibration data points were removed, until the coefficient of determination ($r^2$) was $\geq$0.995 with at least five calibrations points remaining.

Table 1. Identities and Structures

| Common Name | Structural Formula and Chemical Name |
|---|---|
| Fluroxypyr 1-MHE<br><br>Molecular Formula: $C_{15}H_{21}Cl_2FN_2O_3$<br>Molecular weight: 367.24 |  <br>((4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy) acetic acid 1-methylheptyl ester |
| Fluroxypyr acid<br><br>Molecular Formula: $C_7H_5Cl_2FN_2O_3$<br>Molecular weight: 255.03 |  <br>((4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy) acetic acid |
| Fluroxypyr-DCP (X061784)<br><br>Molecular Formula: $C_5H_3Cl_2FN_2O$<br>Molecular weight: 197.0 |  <br>4-amino-3,5-dichloro-6-fluoro-2-pyridinol |
| Fluroxypyr-MP (X61420)<br><br>Molecular Formula: $C_6H_5Cl_2FN_2O$<br>Molecular weight: 211.02 |  <br>4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine |

**Appendix 1 – Analytical Method**

Number of pages: 4

1. 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);

2. For recovery samples, add appropriate aliquots of the spiking solution to obtain concentration ranging from 0.0004 - 1.0 µg/g (for Fluroxypyr acid and Fluroxypyr-MHE) and 0.01 – 1.0 µg/g (for Fluroxypyr-DCP and Fluroxypyr-MP) according to table below:

| Analyte | Concentration (µg/mL) | Aliquot (mL) | Fortification level (µg/g) |
|---|---|---|---|
| Fluroxypyr acid | 0.1 | 0.04 | 0.0004 |
| | 10 | | 0.04 |
| | 100 | 0.1 | 1[a] |
| Fluroxypyr-MHE | 0.1 | 0.04 | 0.0004 |
| | 10 | | 0.04 |
| | 100 | 0.1 | 1[a] |
| Fluroxypyr-DCP | 1 | 0.1 | 0.01 |
| | 10 | | 0.1 |
| | 100 | | 1[b] |
| Fluroxypyr-MP | 1 | 0.1 | 0.01 |
| | 10 | | 0.1 |
| | 100 | | 1[b] |

[a] must be diluted (0.03 mL of sample from Step 8.e + 1.47 mL of control extract).
[b] must be diluted (0.05 mL of sample from Step 12.e+ 0.95 mL of control extract).

3. Add 10 mL of the ACN/0.5 N hydrochloric acid (90:10, v/v) extraction solution;

4. Add 2 g of NaCl and 5 g of $MgSO_4$;

5. Cap the sample vial and shake at approximately 180 excursions/minute for 60 min;

6. Centrifuge the sample for 5 min/2500 rpm;

Clean-up step: Fluroxypyr and Fluroxypyr-MHE

7. For Fluroxypyr acid and Fluroxypyr-MHE transfer 2 mL of the extract (from Step 6) to a 15 mL falcon tube and add 8 mL of 0.5 N hydrochloric acid, pulse vortex mix for about 5 seconds;

8. 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 7) 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

9. Transfer 0.6 mL of extract (Step 8.e) into a 2 mL vial and add 0.4 mL of $H_2O$/0.2 % acetic acid;
10. Analyse by LC-MS/MS;

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

11. For Fluroxypyr-DCP and Fluroxypyr-MP transfer 1 mL of the extract (from Step 6) to a 15 mL falcon tube and add 7 mL of 0.5 N hydrochloric acid, pulse vortex mix for about 5 seconds;

12. 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 11) 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

13. Transfer 0.6 mL of extract (Step 12.e) into a 2 mL vial and add 0.6 mL of $H_2O$/0.2 % acetic acid;
14. Analyse by LC-MS/MS;

Calibration curve

Calibration curve must be diluted with control sample extract (matrix match) for each soil and sediment sample.

Calibration curve for Fluroxypyr acid and Fluroxypyr-MHE.

| Start concentration (ng/mL) | Aliquot (mL) | Matrix volume (mL) | Final volume (mL) | Final concentration (ng/mL) |
|---|---|---|---|---|
| 2 | 0.05 | 0.95 | 1.0 | 0.1 |
| 10 | | | | 0.5 |
| 40 | | | | 2.0 |
| 100 | | | | 5.0 |
| 200 | | | | 10 |
| 400 | | | | 20 |
| 600 | | | | 30 |

Calibration curve for Fluroxypyr-DCP and Fluroxypyr-MP.

| Start concentration (ng/mL) | Aliquot (mL) | Matrix volume (mL) | Final volume (mL) | Final concentration (ng/mL) |
|---|---|---|---|---|
| 20 | 0.05 | 0.95 | 1.0 | 1 |
| 100 | | | | 5 |
| 200 | | | | 10 |
| 300 | | | | 15 |
| 400 | | | | 20 |
| 500 | | | | 25 |
| 600 | | | | 30 |

Chromatographic condition

Column: Eclipse Plus Phenyl Hexyl 3.0 x 50 mm x 1.8 µm (SN: USPHP01290)
Mobile phase A: $H_2O$ + 0.1 % acetic acid
Mobile phase B: MeOH + 0.1 % acetic acid
Injection volume: 10 µL
Column oven temperature: 50 °C

Gradient:

For Fluroxypyr acid and Fluroxypyr-MHE

| Time (min) | Flow (µL/min) | A (%) | B (%) |
|---|---|---|---|
| 0.00 | 500 | 90 | 10 |
| 0.50 | 500 | 90 | 10 |
| 2.00 | 500 | 3 | 97 |
| 4.00 | 500 | 3 | 97 |
| 4.10 | 500 | 90 | 10 |
| 6.00 | 500 | 90 | 10 |

For Fluroxypyr-DCP and Fluroxypyr-MP

| Time (min) | Flow (µL/min) | A (%) | B (%) |
|---|---|---|---|
| 0.00 | 500 | 90 | 10 |
| 0.50 | 500 | 90 | 10 |
| 2.00 | 500 | 3 | 97 |
| 3.50 | 500 | 3 | 97 |
| 3.60 | 500 | 90 | 10 |
| 6.00 | 500 | 90 | 10 |

MS condition

Curtain gas (CUR): 15 psi
Collision gas (CAD): Medium
Ion spray voltage (IS): 5000 V
Temperature (TEM): 500 °C
GS1: 40 psi
GS2: 40 psi
Entrance potential: 10 V

| ID | Q1 (Da) | Q3 (Da) | Time (min) | DP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|
| Fluroxypyr acid | 367.025 | 255.000 | 3.11 | 30 | 15 | 16 |
| | | 209.000 | | 30 | 31 | 14 |
| Fluroxypyr-MHE | 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 |

**Appendix 1 – Analytical Method**

Number of pages: 11

**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

Torntec 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<br>AB SCIEX API 6500 LC/MS/MS System<br>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<br>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.

# APPENDIX 2 TYPICAL LC-MS/MS INSTRUMENT CONDITIONS

(4 Pages)

**LC-MS/MS Instrument Description - for Fluroxypyr 1-MHE and Fluroxypyr acid**

| | | |
|---|---|---|
| Instrument | : | Agilent 1290 HPLC system |
| | : | AB Sciex Triple Quad 5500 Q-Trap MS/MS system |
| | : | Analyst 1.6.2 data system |

**Typical Chromatography Conditions**

| | | |
|---|---|---|
| Column | : | Agilent, Poroshell 120, Phenyl Hexyl, 50 × 2.1 mm, 2.7 µm particle size * |
| Column Temperature | : | 50 °C |
| Injection Volume | : | 5 µL |
| Run Time | : | 7.0 minutes |
| Mobile Phase | : | A – Water + 0.1% acetic acid<br>B – Methanol + 0.1% acetic acid |
| Flow Rate | : | 700 µL/min |

Mobile Phase Composition

| Time (min) | Solvent A (%) | Solvent B (%) |
|---|---|---|
| 0.50 | 90 | 10 |
| 3.00 | 3 | 97 |
| 4.00 | 3 | 97 |
| 4.10 | 90 | 10 |
| 7.00 | 90 | 10 |

Under these conditions the retention times of Fluroxypyr 1-MHE and Fluroxypyr acid are approximately 3.48 minutes and 2.51 minutes, respectively.

Note: For the analysis of Fluroxypyr 1-MHE and Fluroxypyr acid, interfering peaks and poor chromatography were observed. A few minor method modifications were therefore applied for these analytes. The flow rate was increased from 500 to 700 µL/min, the injection volume was decreased from 10 µL to 5 µL and the mobile phase gradient times were adjusted. The modified conditions are shown above.

* The column specified in the method (Eclipse Plus Phenyl Hexyl, 50 × 3.0 mm, 1.8 µm) has the same stationary phase but slightly different dimensions and particle size. The column used for this ILV is detailed above.

**Typical Mass Spectrometer Conditions - for Fluroxypyr 1-MHE and Fluroxypyr acid**

| | | |
|---|---|---|
| Ionisation Mode | : | Turbo Spray |
| Polarity | : | Positive |
| Scan Type | : | MRM |
| Resolution | : | Q1 – unit, Q3 – unit |
| Curtain Gas (CUR) | : | 35 |
| Collision Gas (CAD) | : | Medium |
| IonSpray Voltage (IS) | : | 5500 V |
| Temperature (TEM) | : | 500°C |
| Ion Source Gas 1 (GS1) | : | 50 |
| Ion Source Gas 2 (GS2) | : | 50 |
| Entrance Potential (EP) | : | 10 V |

| Compound: | Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | Declustering Potential (DP) | Collision Energy (CE) | Collision exit potential (CXP) |
|---|---|---|---|---|---|---|
| Fluroxypyr 1-MHE Quantitation | 367.1 | 255.1 | 100 | 40 | 16 | 10 |
| Fluroxypyr 1-MHE Confirmation | 367.1 | 209.0 | 100 | 40 | 31 | 10 |
| Fluroxypyr acid Quantitation | 255.1 | 181.0 | 100 | 40 | 29 | 10 |
| Fluroxypyr acid Confirmation | 255.1 | 209.0 | 100 | 40 | 19 | 10 |

**LC-MS/MS Instrument Description – for Fluroxypyr-DCP (X061784) and Fluroxypyr-MP (X61420)**

| | | |
|---|---|---|
| Instrument | : | Agilent 1260/1290 HPLC system |
| | : | AB Sciex Triple Quad 5500 Q-Trap MS/MS system |
| | : | Analyst 1.6.2 data system |

**Typical Chromatography Conditions**

| | | |
|---|---|---|
| Column | : | Agilent, Poroshell 120, Phenyl Hexyl, 50 × 2.1 mm, 2.7 µm particle size * |
| Column Temperature | : | 50°C |
| Injection Volume | : | 10 µL |
| Run Time | : | 7.0 minutes |
| Mobile Phase | : | A – Water + 0.1% acetic acid<br>B – Methanol + 0.1% acetic acid |
| Flow Rate | : | 500 µL/min |

Mobile Phase Composition

| Time (min) | Solvent A (%) | Solvent B (%) |
|---|---|---|
| 0.00 | 90 | 10 |
| 0.50 | 90 | 10 |
| 2.00 | 3 | 97 |
| 3.50 | 3 | 97 |
| 3.60 | 90 | 10 |
| 7.00 | 90 | 10 |

Under these conditions the retention times of Fluroxypyr-DCP (X061784) and Fluroxypyr-MP (X61420) are approximately 3.31 minutes and 3.97 minutes, respectively.

* The column specified in the method (Eclipse Plus Phenyl Hexyl, 50 × 3.0 mm, 1.8 µm) has the same stationary phase but slightly different dimensions and particle size. The column used for this ILV is detailed above.

**Typical Mass Spectrometer Conditions – for Fluroxypyr-DCP (X061784) and Fluroxypyr-MP (X61420)**

| | | |
|---|---|---|
| Ionisation Mode | : | Turbo Spray |
| Polarity | : | Positive |
| Scan Type | : | MRM |
| Resolution | : | Q1 – unit, Q3 – unit |
| Curtain Gas (CUR) | : | 20 |
| Collision Gas (CAD) | : | Medium |
| IonSpray Voltage (IS) | : | 5000 V |
| Temperature (TEM) | : | 500 °C |
| Ion Source Gas 1 (GS1) | : | 40 |
| Ion Source Gas 2 (GS2) | : | 40 |
| Entrance Potential (EP) | : | 10 V |

| Compound: | Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | Declustering Potential (DP) | Collision Energy (CE) | Collision exit potential (CXP) |
|---|---|---|---|---|---|---|
| Fluroxypyr-DCP (X061784) Quantitation | 196.9 | 152.0 | 100 | 60 | 37 | 16 |
| Fluroxypyr-DCP (X061784) Confirmation | 196.9 | 144.0 | 100 | 60 | 41 | 8 |
| Fluroxypyr-MP (X61420) Quantitation | 210.9 | 196.0 | 100 | 40 | 29 | 12 |
| Fluroxypyr-MP (X61420) Confirmation | 210.9 | 112.9 | 100 | 40 | 49 | 14 |