Validation Report for the Determination of Residues of Fluroxypyr 1-Methylheptyl Ester, Fluroxypyr and its Major Metabolites in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry

## INTRODUCTION

### Scope

This method is applicable for the quantitative determination of residues of fluroxypyr 1-methylheptyl ester (fluroxypyr 1-MHE) and fluroxypyr, along with its major metabolites, fluroxypyr dichloropyridinol (fluroxypyr-DCP) and fluroxypyr methoxypyridine (fluroxypyr-MP) in surface water, ground water and drinking water. The method was validated over the concentration range of 0.05-5.00 µg/L with a validated limit of quantification of 0.05 µg/L. Common and chemical names, molecular formulas, and the nominal masses for the analyte and related compounds are given in Table 1.

This study was conducted to fulfill data requirements outlined in the U. S. EPA Residue Chemistry Test Guidelines, OPPTS 850.7100 (1), the European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev.7 (2) and SANCO/3029/99 rev.4 (3), and PMRA Residue Chemistry Guidelines as Regulatory Directive Dir98-02 (4).

### Method Principle

Residues of fluroxypyr 1-MHE and fluroxypyr are extracted from the water sample matrices by acidifying the sample using concentrated formic acid, saturating the water with sodium chloride, then partitioning it twice against ethyl acetate. The two ethyl acetate layers are drawn off and combined in the same graduated Nalgene tube. A 1.0 mL aliquot of a methanol/water (50:50) solution containing 0.1% acetic acid solution is added. The combined ethyl acetate extracts are concentrated under a stream of nitrogen until approximately 0.7-0.9 mL remains. The final sample is adjusted to 2.0 mL using a methanol/water (50:50) solution containing 0.1% acetic acid. The sample is analyzed by liquid chromatography with negative-ion electrospray ionization (ESI) tandem mass spectrometry (LC/MS/MS).

For the determination of residues of fluroxypyr-DCP and fluroxypyr-MP, the water samples are buffered to pH 7 using a potassium dihydrogen phosphate buffer solution. The water sample is loaded onto a Strata -X polymeric sorbent SPE column and eluted with acetonitrile into a graduated Nalgene tube containing a 1.0-mL aliquot of a methanol/water (50:50) solution with 0.1% acetic acid solution is added. The acetonitrile eluate is concentrated under a stream of nitrogen until approximately 0.7-0.9 mL remains. The final sample is adjusted to 1.5 mL using a methanol/water (50:50) solution containing 0.1% acetic acid. The sample is analyzed by liquid

chromatography with positive-ion atmosphereic pressure chemical ionization (APCI) 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, ethyl acetate and methanol are flammable and should be used in well-ventilated areas away from ignition sources. Acetic acid and formic acid are corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

Test Substance/Analytical Standard and Internal Standard

| Test Substance/ Analytical Standard | AGR/TSN Number | Percent Purity | Certification Date | Reference |
|---|---|---|---|---|
| fluroxypyr 1-MHE[a] | AGR228289 | 99.5 | 21-Oct-2008 | FA&PC 08 193889 |
| fluroxypyr (acid)[b] | AGR222210 | 99.6 | 17-Jul-2007 | 07-072-L |
| fluroxypyr-DCP[c] | TSN101651 | 99 | 21-Aug-2007 | FA&PC 073378 |
| fluroxypyr-MP[d] | AGR250194 | 99.9 | 08-Jul-2009 | FA&PC 09 225596 |

[a] [(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid 1-methylheptyl ester
[b] [(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid
[c] 4-amino-3,5-dichloro-6-fluoro-2-pyridinol
[d] 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine

The above standards may be obtained from the Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

Equipment, Glassware, and Materials

Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, class A volumetric glassware is used to prepare analytical standards, fortification solutions, and calibration standards.

Laboratory Equipment

Balance, analytical, Model AE100, Mettler-Toledo, Inc.

Balance, pan, Model BB2440, Mettler-Toledo, Inc.

Centrifuge, with rotor to accommodate 8-oz wide-mouth bottles, Model CU-5000, Thermo International Equipment Company.

Centrifuge, with rotor to accommodate 45-mL vials, Model Centra-GP8, Thermo International Equipment Company.

Evaporator, TurboVap LV, Caliper Life Sciences.

Pipet, positive-displacement, 20-50 µL capacity, catalog number M50, Gilson Inc.

Pipet, positive-displacement, 10-100 µL capacity, catalog number M100, Gilson Inc.

Pipet, positive-displacement, 100-1000 µL capacity, catalog number M1000, Gilson Inc.

Shaker, variable speed reciprocating with box carrier, Model 6000, Eberbach Corporation.

Vacuum manifold, Model spe-12G, Mallinckrodt Baker, Inc.

Vortex mixer, Model G-560, Scientific Industries, Inc.

Chromatographic System

Column, analytical, Zorbax SB-C8, 4.6 x 75 mm, 3.5-µm particle size, catalog number 7995208-344, Agilent Technologies.

Liquid chromatograph, Model 1100, Agilent Technologies.

Mass spectrometer, Model API 4000, MDS/Sciex.

Mass spectrometer data system, Model Analyst 1.4.2, MDS/Sciex.

Glassware and Materials

Bottle, 8-oz (237-mL), glass, round, wide-mouth, with PTFE-lined screw cap, catalog number 7984, Qorpak, Bridgeville, PA 15017.

Bottle, 500-mL, media bottle, catalog number 06-423-3C, Fisher Scientific.

Bottle, 1.0-L, media bottle, catalog number 06-423-3D, Fisher Scientific.

Caps, PTFE-lined, 24-400, (fits 45-mL screw-cap vials), catalog number B7815-24, National Scientific Company.

Collection plate, 96-deep well, 2-mL, catalog number 121-5203, International Sorbent Technology Ltd.

Collection plate sealing cap, catalog number 121-5205, Biotage.

Column, Strata-X 33 µm polymeric sorbent SPE, 60-mg, 3-mL, catalog number 8B-S100-UBJ, Phenomenex.

Pipet, disposable serological, 25-mL, catalog number 56800-25210, Kimble/Kontes.

Pipet, disposable serological, 50-mL, catalog number 56800-50510, Kimble/Kontes.

Pipet, 3.2-mL disposable transfer, catalog number 13-711-7, Fisher Scientific.

Pipet tip, positive-displacement, 50-µL capacity, catalog number CP50, Gilson Inc.

Pipet tip, positive-displacement, 100-µL capacity, catalog number CP100, Gilson Inc.

Pipet tip, positive-displacement, 1000-µL capacity, catalog number CP1000, Gilson Inc.

SPE columns, Strata-X 33 µm polymeric sorbent, 60-mg, 3-mL, catalog number 8B-S100-UBJ, Phenomenex.

Tube, round-bottle, polypropylene, graduated, 14-mL (17 x 100 mm), catalog number 352059, Becton Dickinson Labware, Franklin Lakes, NJ 07417

Vial, 45-mL, without PTFE-lined screw cap, catalog number 60958A 11, Kimble/Kontes.

Reagents

Acetic acid, glacial, purified by double distillation, catalog number 380121-500ML, Sigma-Aldrich.

Acetonitrile, ChromaSolv for HPLC gradient grade, ≥99.9%, catalog number 34851-4L, Sigma-Aldrich.

Buffer, potassium dihydrogen phosphate, pH 7.00, 0.05 M, ACS reagent grade, certified concentration, catalog number SB108-500, Fisher Scientific Fisher Scientific.

Ethyl acetate, OmniSolv grade, catalog number EX-0241-1, EMD Chemicals.

Formic acid, 96%, ACS grade, catalog number 251364-500G, Sigma-Aldrich.

Methanol, ChromaSolv for HPLC gradient grade, ≥99.9%, catalog number 34885-4L-R, Sigma-Aldrich.

Nitrogen, refrigerated liquid, catalog number LQNI, BOC Gases.

Sodium chloride, granular, catalog number S642-500, Fisher Scientific.

Water, ChromaSolv for HPLC gradient grade, ≥99.9%, catalog number 270733-4L, Sigma-Aldrich.

Prepared Solutions

acetonitrile/methanol (50:50) (v/v) + 0.1% formic acid (autosampler valve wash solution)

Using a 500-mL graduated cylinder, measure 500 mL of acetonitrile and transfer to a 1000-mL media bottle. Using a 500-mL graduated cylinder, measure 500 mL of methanol and transfer to the same 1000-mL media bottle. Pipet 1.0 mL of formic acid into the 1000-mL bottle. Cap the bottle and invert multiple times to mix well. Allow the solution to equilibrate to room temperature before use.

methanol containing 0.1% acetic acid (mobile phase A)

Pipet 2.0 mL of glacial acetic acid into a 2000-mL graduated mixing cylinder containing approximately 1900 mL of methanol. Dilute to volume with methanol. Cap the cylinder and invert it multiple times to mix well prior to use.

methanol/water (50:50 v/v) containing 0.1% acetic acid (calibration standard and final sample diluent)

Using a 500-mL graduated cylinder, measure 100 mL of methanol containing 0.1% acetic acid (prepared above as mobile phase A) into a 250-mL media bottle. Using a 500-mL graduated cylinder, measure 100 mL of water containing 0.1% acetic acid (prepared below as mobile phase B) into the same 250-mL media bottle. Cap the bottle and invert several times to mix well. Allow solution to equilibrate to room temperature before use.

water containing 0.1% acetic acid (mobile phase B)

Pipet 2.0 mL of glacial acetic acid into a 2000-mL graduated mixing cylinder containing approximately 1900 mL of HPLC grade water. Dilute to volume with HPLC grade water. Cap the cylinder and invert it multiple times to mix well prior to use.

## EXPERIMENTAL

Instrumental Conditions

Typical LC/MS/MS Operating Conditions for Fluroxypyr 1-MHE and Fluroxypyr

| | |
|---|---|
| Instrumentation: | Agilent Model 1100 autosampler<br>Agilent Model 1100 binary pump<br>Agilent Model 1100 degasser<br>MDS/Sciex API 4000 LC/MS/MS System<br>MDS/Sciex Analyst 1.4.2 data System |
| Column: | ZORBAX SB-C8<br>4.6 x 75 mm, 3.5-µm |
| Column Temperature: | Ambient |
| Injection Volume: | 30 µL |
| Run Time: | 16.0 minutes |
| Mobile Phase: | A – methanol/acetic acid (99.9:0.1)<br>B – water/acetic acid (99.9:0.1) |
| Flow Rate: | 900 µL/min (approx 200 µL/min split to source) |

Gradient:

| Time, min | Solvent A, % | Solvent B, % |
|---|---|---|
| 0.0 | 60 | 40 |
| 2.0 | 60 | 40 |
| 10.0 | 100 | 0 |
| 12.0 | 100 | 0 |
| 14.0 | 60 | 40 |
| 16.0 | 60 | 40 |

| | |
|---|---|
| Flow Diverter Program: | 1) 0.0 to 1.0 min: flow to waste<br>2) 1.0 to 10.0 min: flow to source<br>3) 10.0 min: flow to waste |
| Equilibration Time: | 2.0 minutes |

Typical Mass Spectrometry Operating Conditions for Fluroxypyr 1-MHE and Fluroxypyr

| | |
|---|---|
| Interface: | ESI |
| Polarity: | Negative |
| Scan Type: | MRM |
| Resolution: | Q1 – unit, Q3 – unit |
| Curtain Gas (CUR): | 20 |
| Collision Gas (CAD): | 4 |
| Temperature (TEM): | 500°C |
| Ion Source Gas 1 (GS1): | 30 |
| Ion Source Gas 2 (GS2): | 60 |

| | |
|---|---|
| Period 1 | |
| Pre-acquisition Delay: | 0.0 min |
| Acquisition Time | 10.0 min |
| IonSpray Voltage (IS): | -4500 volts |
| Entrance Potential (EP): | -10 volts |

| Analytes: | Precursor Ion Q1 | Product Ion Q3 | Dwell Time (ms) | Collision Energy (CE) | Declustering Potential (DP) | Cell Exit Potential (CXP) |
|---|---|---|---|---|---|---|
| Fluroxypyr 1-MHE (quantification) | 365.1 | 194.1 | 150 ms | -36 V | -95 V | -15 V |
| Fluroxypyr 1-MHE (confirmation) | 367.0 | 196.0 | 150 ms | -34 V | -90 V | -13 V |
| Fluroxypyr (acid) (quantification) | 252.9 | 232.9 | 150 ms | -10 V | -55 V | -11 V |
| Fluroxypyr (acid) (confirmation) | 255.0 | 196.8 | 150 ms | -16 V | -50 V | -11 V |

Typical LC/MS/MS Operating Conditions for Fluroxypyr-DCP and Fluroxypyr-MP

| | |
|---|---|
| Instrumentation: | Agilent Model 1100 autosampler<br>Agilent Model 1100 binary pump<br>Agilent Model 1100 degasser<br>MDS/Sciex API 4000 LC/MS/MS System<br>MDS/Sciex Analyst 1.4.2 data System |
| Column: | ZORBAX SB-C8<br>4.6 x 75 mm, 3.5-µm |
| Column Temperature: | Ambient |

Injection Volume: 20 µL

Run Time: 14.0 minutes

Mobile Phase: A – methanol/acetic acid (99.9:0.1)
B – water/acetic acid (99.9:0.1)

Flow Rate: 900 µL/min (approx 200 µL/min split to source)

Gradient:

| Time, min | Solvent A, % | Solvent B, % |
|---|---|---|
| 0.0 | 40 | 60 |
| 2.0 | 40 | 60 |
| 10.0 | 100 | 0 |
| 12.0 | 40 | 60 |
| 14.0 | 40 | 60 |

Flow Diverter Program: 1) 0.0 to 3.0 min: flow to waste
2) 3.0 to 9.0 min: flow to source
3) 9.0 min: flow to waste

Equilibration Time: 2.0 minutes

Typical Mass Spectrometry Operating Conditions for Fluroxypyr-DCP and Fluroxypyr-MP

Interface: APCI
Polarity: Positive
Scan Type: MRM
Resolution: Q1 – unit, Q3 – unit
Curtain Gas (CUR): 20
Collision Gas (CAD): 4
Temperature (TEM): 550°C
Ion Source Gas 1 (GS1): 30
Ion Source Gas 2 (GS2): 60

Period 1
Pre-acquisition Delay: 0.0 min
Acquisition Time 9.0 min
IonSpray Voltage (IS): 5.00 volts
Entrance Potential (EP): 10 volts

| Analytes: | Precursor Ion Q1 | Product Ion Q3 | Dwell Time (ms) | Collision Energy (CE) | Declustering Potential (DP) | Cell Exit Potential (CXP) |
|---|---|---|---|---|---|---|
| Fluroxypyr-DCP (quantification) | 199.0 | 181.0 | 150 ms | 31 V | 61 V | 4V |
| Fluroxypyr-DCP (confirmation) | 199.0 | 154.1 | 150 ms | 37V | 61 V | 14V |
| Fluroxypyr-MP (quantification) | 210.9 | 113.2 | 150 ms | 49 V | 66 V | 10 V |
| Fluroxypyr-MP (confirmation) | 210.9 | 196.1 | 150 ms | 29 V | 66 V | 16 V |

Full-scan and product-ion mass spectra of fluroxypyr 1-MHE and fluroxypyr, along with its major metabolites, fluroxypyr-DCP and fluroxypyr-MP are shown in Figures 1 through 4, respectively.

Typical calibration curves for the quantitative determination of fluroxypyr 1-MHE, fluroxypyr, fluroxypyr-DCP, and fluroxypyr-MP in surface water, ground water and drinking water are shown in Figures 5-8, respectively.

Typical chromatograms of standards, control samples, a 0.05-µg/L (LOQ) recovery samples, and 5.0-µg/L (high) recovery samples for the quantitative determination of fluroxypyr 1-MHE, fluroxypyr, fluroxypyr-DCP, and fluroxypyr-MP in surface water, ground water and drinking water are illustrated in Figures 9-20.

Typical chromatograms of standards, control samples, a 0.05-µg/L (LOQ) recovery samples, and 5.0-µg/L (high) recovery samples for the confirmation of residues of fluroxypyr 1-MHE, fluroxypyr, fluroxypyr-DCP, and fluroxypyr-MP in surface water, ground water and drinking water are illustrated in Figures 21-32.

Preparation of Standard Solutions

Preparation of Fluroxypyr Stock Solutions and Spiking Solutions

1. Weigh 0.0100 g of fluroxypyr 1-methylheptyl ester and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a stock solution containing 100 µg/mL of fluroxypyr 1-methylheptyl ester.

2. Weigh 0.0100 g of fluroxypyr (acid) and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a stock solution containing 100 µg/mL of fluroxypyr.

3. Weigh 0.0100 g of fluroxypyr-DCP and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a stock solution containing 100 µg/mL of fluroxypyr-DCP.

4. Weigh 0.0100 g of fluroxypyr-MP and quantitatively transfer to a 100-mL volumetric flask with methanol. Dilute to volume with methanol to obtain a stock solution containing 100 µg/mL of fluroxypyr-MP.

5. Accurately pipet 25.0 mL of each of the 100-µg/mL stock standard solutions prepared in Steps 1 through 4 above and quantitatively transfer each into the same 125-mL clear glass bottle equipped with a PTFE-lined screw cap. This will yield a mixed fluroxypyr spiking solution containing 25.0 µg/mL of each analyte.

6. Pipet 10.0 mL of the 25.0-µg/mL fluroxypyr mixed spiking solution prepared in Step 5 above into a 100-mL volumetric flask. Dilute to volume with methanol to obtain a 2.50-µg/mL mixed fluroxypyr spiking solution.

7. Pipet 10.0 mL of the 2.50-µg/mL mixed standard solution prepared in Step 6 above into a 100-mL volumetric flask. Dilute to volume with methanol to obtain a 0.250-µg/mL fluroxypyr mixed spiking solution.

8. Pipet 1.0 mL of the 2.50-µg/mL standard solution prepared in Step 6 above into a 100-mL volumetric flask. Dilute to volume with methanol to obtain a 0.025-µg/mL fluroxypyr mixed spiking solution.

Preparation of Fluroxypyr Calibration Standards for Quantification

1. Pipet 5.0 mL of each of the 100-µg/mL fluroxypyr stock solutions prepared in Steps 1 through 4 in the section above into the same 100-mL volumetric flask. Dilute to volume using a methanol/water/ (50:50) solution containing 0.1% acetic acid to obtain a 5.0-µg/mL mixed fluroxypyr calibration stock solution.

2. Pipet 2.0 mL of the 5.0-µg/mL mixed fluroxypyr standard solution prepared in Step 1 above into a 100-mL volumetric flask. Dilute to volume using a methanol/water/ (50:50) solution containing 0.1% acetic acid to obtain a 0.10-µg/mL mixed fluroxypyr calibration stock solution.

3. Prepare calibration standards for the mixed fluroxypyr analytes by following the table below. Use a volumetric pipet to dispense the appropriate amount of fluroxypyr stock solution into a volumetric flask and dilute to volume with a methanol/water/ (50:50) solution containing 0.1% acetic acid.

| Concentration of Stock Solution | Aliquot Of Stock Solution | Final Solution Volume | Calibration Solution Final Concentration | Equivalent Sample Concentration[a] | Equivalent Sample Concentration[b] |
|---|---|---|---|---|---|
| µg/mL | mL | mL | ng/mL | µg/L | µg/L |
| 0.10 | 0.25 | 100 | 0.250 | 0.025 | 0.00625 |
| 0.10 | 0.5 | 100 | 0.500 | 0.050 | 0.0125 |
| 0.10 | 1.0 | 100 | 1.00 | 0.100 | 0.0250 |
| 0.10 | 2.5 | 100 | 2.50 | 0.250 | 0.0625 |
| 0.10 | 5.0 | 100 | 5.00 | 0.500 | 0.125 |
| 0.10 | 10.0 | 100 | 10.0 | 1.00 | 0.250 |
| 5.00 | 0.5 | 100 | 25.0 | 2.50 | 0.625 |
| 5.00 | 1.0 | 100 | 50.0 | 5.00 | 1.25 |

[a] This is the equivalent concentration of fluroxypyr 1-MHE and/or fluroxypyr in an actual water sample based on taking a 20 mL initial aliquot to a final volume of 2.0 mL.

[b] This is the equivalent concentration of fluroxypyr-DCP and/or fluroxypyr-MP in an actual water sample based on taking a 60 mL initial aliquot to a final volume of 1.5 mL.

Sample Origin, Numbering, Preparation and Storage

Untreated control samples of the water were obtained from the Dow AgroSciences LLC Sample Management Group. All samples were tracked in the Dow AgroSciences LLC Regulatory 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 is included in the study file.

No sample preparation was required prior to sample analysis. Samples were stored refrigerated, except when removed for aliquotting and analysis.

Analysis Procedure for Fluroxypyr-1-MHE and Fluroxypyr

1. Using a pipet, measure 20-mL portions of water into individual 11-dram (45-mL) glass vials equipped with PTFE-lined caps.

2. For preparing fortified samples, add aliquots of the appropriate stock solutions to encompass the necessary concentration range as described in the table below:

| Description | Spiking Volume (µL) | Stock Solution (µg/mL) | Fortification Level (µg/L) |
|---|---|---|---|
| Control | --- | --- | --- |
| LOD | 12 | 0.025 | 0.015 |
| LOQ | 40 | 0.025 | 0.050 |
| Mid | 40 | 0.250 | 0.500 |
| High | 40 | 2.50 | 5.00 |

1. Add 200 µL of concentrated formic acid solution to the water sample.

2. Add approximately 12 grams of sodium chloride (or enough to totally saturate the sample) to the water sample.

3. Add 5 mL of ethyl acetate to the sample.

4. Cap the samples and shake for approximately 10 minutes on a flat-bed shaker at about 180 excursions/minute.

5. Centrifuge the samples for about 5 minutes at approximately 2000 rpm.

6. Carefully draw off the ethyl acetate layer (avoid picking any water) and transfer this to a graduated Nalgene tube. (Note: This first partition can be evaporated to about 1-2 mL to reduce the volume in the tube before the addition of the second partition portion to the tube.) (Critical Step: Do not allow the sample to evaporate to dryness.)

7. Repeat the extraction process by adding a new 5 mL aliquot of ethyl acetate to the water sample.

8. Repeat steps 6 and 7, transferring the second ethyl acetate partition layer to the same Nalgene tube containing the first extract in order to combine the extracts.

9. Add 1.0 mL of a methanol/water (50:50) solution containing 0.1% acetic acid solution. Pulse vortex for about 10 seconds to mix well.

10. Concentrate the sample using a TurboVap evaporator set at 35-40 °C and a nitrogen pressure of 10-15 psi until approximately 0.9 mL of the sample remains. (Critical Step: Do not allow the sample to evaporate to dryness.)

11. Adjust the final sample volume to 2.0 mL using a methanol/water (50:50) solution containing 0.1% acetic acid. Pulse vortex for about 10 seconds to mix well. (Note: If any recovery sample concentration is expected to exceed 80% of the highest standard on the calibration curve, dilute the sample to bring it within the linear range of the calibration curve before it is injected for analysis. Combine an aliquot of the prepared recovery sample with a known proportional volume of methanol/water (50:50) solution containing 0.1% acetic acid and place in an 8-mL vial. Cap the vial, vortex mix.)

12. Transfer a portion of the sample to a 96-deep well plate for analysis.

13. Add approximately 1 mL of each of the calibration standards to the same plate and seal the plate. (Acceptable stopping point if sample is kept refrigerated).

14. Analyze the calibration standards and samples by negative-ion ESI (electrospray ionization) LC/MS/MS for determination of fluroxpyr 1-MHE and fluroxpyr, injecting the calibration standards interspersed with the samples throughout the run.

    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 each analyte relative to background interferences.

    c. Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 9-20 with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for each analyte in the 0.5-ng/mL calibration standard.

15. If any analytical sample concentrations exceed 80% of the highest standard on the calibration curve, reanalyze the sample by performing a dilution to bring the sample within the linear range of the calibration curve. Combine an aliquot of the prepared sample from Step 11 with a known proportional volume of methanol/water (50:50) solution containing 0.1% acetic acid and place in an 8-mL vial. Cap the vial, vortex mix and transfer the diluted sample to a 96-deep well plate for reinjection.

Analysis Procedure for Fluroxypyr-DCP and Fluroxypyr-MP

1. Using a pipet, measure 60.0-mL of the water sample into a 4-ounce (125-mL) glass jar equipped with a PTFE-lined cap.

2. For preparing fortified samples, add aliquots of the appropriate stock solutions to encompass the necessary concentration range as described in the table below:

| Description | Spiking Volume (µL) | Stock Solution (µg/mL) | Fortification Level (µg/L) |
|---|---|---|---|
| Control | --- | --- | --- |
| LOD | 36 | 0.025 | 0.015 |
| LOQ | 120 | 0.025 | 0.050 |
| Mid | 120 | 0.250 | 0.500 |
| High | 120 | 2.50 | 5.00 |

3. Add 6.0 mL of a pH 7.00 buffer solution to the sample bottle.

4. Cap the sample and shake by hand to mix well. Proceed to Step 5.

5. Concentrate and purify the sample using the following SPE procedure:

   a. Place a Phenomenex Strata-X (200-mg, 6-mL) SPE column on the vacuum manifold box.

   b. Condition the SPE column slowly (using no vacuum) with 6 mL of methanol followed by 6 mL of pH 7.00 buffer, discarding the eluates. Dry the column under full vacuum (approx. -380 mm Hg) for 10 seconds between solvent additions.

   c. Attach an empty 75-mL reservoir to the top of the column using an adaptor.

   d. Transfer the entire sample from Step 4 to the 75-mL reservoir attached to the top of the SPE column. Draw the entire sample through the column at a flow rate of approximately 3-5 mL/min (fast drip), using vacuum if necessary. Apply full vacuum to the column for about 10 seconds after the sample has eluted. Discard the eluate.

   e. Rinse the original containers with 2 mL of pH 7.00 buffer and apply rinse to SPE column. Pull the rinse solution through the SPE column at approximately 3-5 mL/min (fast drip), using vacuum if necessary. Apply full vacuum to the column for about 10 seconds after elution. Discard the eluate.

   f. Remove the 75-mL reservoir and column adaptor from the top of the column.

   g. Dry the column under full vacuum for about 20 minutes.

h. Add 1.0 mL of a methanol/water (50:50) solution containing 0.1% acetic acid to graduated Nalgene tubes into which each sample will be eluted.

i. Slowly elute the analytes (using no vacuum) from the SPE column with two 2.0-mL aliquots of acetonitrile collecting the two elutates in the same graduated Nalgene tube (containing 1.0 mL of a methanol/water (50:50) solution containing 0.1% acetic acid). Apply full vacuum for about 10 seconds between solvent additions.

6. Concentrate the sample using a TurboVap evaporator set at 35-40 °C and a nitrogen pressure of 10-15 psi until approximately 0.9 mL of the sample remains. (Critical Step: Do not evaporate the sample to complete dryness.

7. Adjust the final sample fractions to 1.5 mL using a methanol/water (50:50) solution containing 0.1% acetic acid. Pulse vortex for about 10 seconds to mix well. (Note: If any recovery sample concentration is expected to exceed 80% of the highest standard on the calibration curve, dilute the sample to bring it within the linear range of the calibration curve before it is injected for analysis. Combine an aliquot of the prepared recovery sample with a known proportional volume of methanol/water (50:50) solution containing 0.1% acetic acid and place in an 8-mL vial. Cap the vial, vortex mix.)

8. Transfer a portion of each sample to a 96-deep well plate for analysis.

9. Add approximately 1 mL of each of the calibration standards to the same plate and seal the plate. (Acceptable stopping point if sample is kept refrigerated.)

10. Analyze the calibration standards and samples by positive-ion APCI (atmospheric pressure chemical ionization) LC/MS/MS for determination of fluroxpyr-DCP and fluroxpyr-MP, injecting the calibration standards interspersed with the samples throughout the run.

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 each analyte relative to background interferences.

c. Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 9-20 with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for each analyte in the 2.5-ng/mL calibration standard.

11. If any analytical sample concentrations exceed 80% of the highest standard on the calibration curve, reanalyze the sample by performing a dilution to bring the sample within the linear range of the calibration curve. Combine an aliquot of the prepared sample from Step 7 with a known proportional volume of methanol/water (50:50) solution containing 0.1% acetic acid and place in an 8-mL vial. Cap the vial, vortex mix and transfer the diluted sample to a 96-deep well plate for reinjection.

Calculations

Inject the series of calibration standards described in the standard preparation section using the conditions listed in the instrument section and determine the peak areas for each analyte.

Prepare a standard curve using linear regression analysis (5) with 1/x weighting (6) by plotting the equivalent analyte concentration on the abscissa (x-axis) and the respective peak area on the ordinate (y-axis) as shown in Figures 5-8. Power regression or quadratic curve fit may also be used where appropriate.

Determine the concentration (ng/mL) of the sample and/or the recovery (%) from the recovery sample as described in the example calculation outlined in Figures 33-36.

Confirmation of Residue Identity

The method is specific for the determination of fluroxypyr 1-MHE) and fluroxypyr, along with its major metabolites, fluroxypyr-DCP and fluroxypyr-MP in surface water, ground water and drinking water by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, additional fluroxypyr 1-MHE and fluroxypyr, fluroxypyr-DCP and fluroxypyr-MP ion transitions can be monitored as follows:

| | |
|---|---|
| Fluroxypyr 1-MHE | Q1/Q3 *m/z* 365.1/194.1 (quantification) |
| Fluroxypyr 1-MHE | Q1/Q3 *m/z* 367.0/196.0 (confirmation) |
| Fluroxypyr | Q1/Q3 *m/z* 252.9/232.9 (quantification) |
| Fluroxypyr | Q1/Q3 *m/z* 255.0/196.8 (confirmation) |
| Fluroxypyr-DCP | Q1/Q3 *m/z* 199.0/181.0 (quantification) |
| Fluroxypyr-DCP | Q1/Q3 *m/z* 199.0/154.1 (confirmation) |
| Fluroxypyr-MP | Q1/Q3 *m/z* 210.9/113.2 (quantification) |
| Fluroxypyr-MP | Q1/Q3 *m/z* 210.9/196.1 (confirmation) |

1. Prepare samples for analysis by following the steps the Analysis Procedure sections.

2. Transfer a portion of the sample to a 96-deep well plate and firmly seal the plate.

3. Analyze the calibration standards and samples by liquid chromatography as described in the Instrumental Conditions section.

4. For each standard, calculate a confirmation ratio. Use the average confirmation ratio to confirm the presence of the analyte in the water samples.

$$\text{Confirmation Ratio} = \frac{\text{peak area of confirmation ion transition}}{\text{peak area of quantitation ion transition}}$$

For example, using the data for the confirmation of fluroxypyr from the 0.50-ng/mL standards in Figures 12 and 24, sample index number 6:

$$\text{Confirmation Ratio} = \frac{\text{fluroxypyr peak area at } m/z \text{ 255.0/196.8}}{\text{fluroxypyr peak area at } m/z \text{ 252.9/232.9}}$$

$$\text{Confirmation Ratio} = \frac{7306}{2504}$$

$$\text{Confirmation Ratio} = 2.9177$$

Confirmation of the presence of the analyte is indicated when the retention time of the samples matches that of the standards and the confirmation ratio is in the range of ±20% of the average found for the standards.

Statistical Treatment of Data

Statistical treatment of data included the calculation of regression equations, coefficients of determination ($r^2$) for describing the linearity of calibration curves, and means, standard deviations, and relative standard deviations of the results for the fortified recovery samples.

Standardization of SPE Elution Profile for Fluroxypyr-DCP and Fluroxypyr-MP

There is a possibility that lot-to-lot variation in the Phenomenex Strata-X SPE columns could influence the elution profile of fluroxypyr-DCP and fluroxypyr-MP. In order to obtain an elution profile for each lot of SPE columns used and to ensure optimum recovery and clean-up efficiency, the following procedure can be used:

1. Using a pipet, measure 60.0-mL of the water sample into a 4-ounce (125-mL) glass jar equipped with a PTFE-lined cap.

2. Fortifiy the water with 20 µL of the 2.5 µg/mL fortification solution (equivalent to a total of 50 ng of each analyte).

3. Add 6.0 mL of a pH 7.00 buffer solution to the sample jar.

4. Cap the sample and shake by hand to mix well. Proceed to Step 5.

5. Concentrate and purify the sample using the following SPE procedure:

    a. Place a Phenomenex Strata-X (200-mg, 6-mL) SPE column on the vacuum manifold box.

    b. Condition the SPE column slowly (using no vacuum) with 6 mL of methanol followed by 6 mL of pH 7.00 buffer, discarding the eluates. Dry the column under full vacuum (approx. -380 mm Hg) for 10 seconds between solvent additions.

    c. Attach an empty 75-mL reservoir to the top of the column using an adaptor.

    d. Transfer the entire sample from Step 4 to the 75-mL reservoir attached to the top of the SPE column. Draw the entire sample through the column at a flow rate of approximately 3-5 mL/min (fast drip), using vacuum if necessary. Apply full vacuum to the column for about 10 seconds after the sample has eluted. Discard the eluate.

    e. Rinse the original sample jar with 2 mL of pH 7.00 buffer and apply rinse to SPE column. Pull the rinse solution through the SPE column at approximately 3-5 mL/min (fast drip), using vacuum if necessary. Apply full vacuum to the column for about 10 seconds after elution. Discard the eluate.

    f. Remove the 75-mL reservoir and column adaptor from the top of the column.

    g. Dry the column under full vacuum for about 20 minutes.

h. Slowly elute the analytes (using no vacuum) from the SPE column with five 2.0-mL aliquots of acetonitrile collecting each 2.0-mL fraction eluted in a separate graduated Nalgene tube. Apply full vacuum for about 10 seconds between solvent additions.

6. Adjust the final volume of each fraction to 4.0 mL using a methanol/water (50:50) solution containing 0.1% acetic acid. Cap the tubes and pulse vortex for about 10 seconds to mix well.

7. Transfer a portion of each elution fraction to a 96-deep well plate for analysis.

8. Add approximately 1 mL of each of the calibration standards to the same plate and seal the 96-deep well plate. (Acceptable stopping point if sample is kept refrigerated.)

9. Analyze the calibration standards and samples by positive-ion APCI (atmospheric pressure chemical ionization) LC/MS/MS for determination of fluroxpyr-DCP and fluroxpyr-MP, injecting the calibration standards interspersed with the samples throughout the run.

10. If any sample concentrations exceed 80% of the highest standard on the calibration curve, reanalyze the sample by performing a dilution to bring the sample within the linear range of the calibration curve. Combine an aliquot of the prepared sample from Step 7 with a known proportional volume of methanol/water (50:50) solution containing 0.1% acetic acid and place in an 8-mL vial. Cap the vial, vortex mix and transfer the diluted sample to a 96-deep well plate for reinjection.

11. Calculate the percent recovery for each fraction as described in the Calculations section.

A typical elution profile is illustrated in Figure 37. If the elution profile differs from that shown, adjust the volume of acetonitrile to be collected in Step 5.i. of the Analysis Procedure section for fluroxypyr-DCP and fluroxypyr-MP.

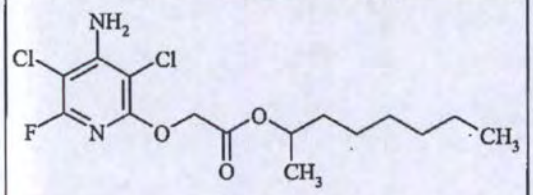
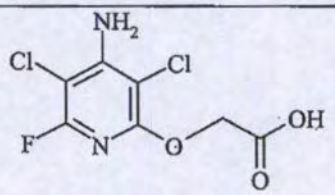
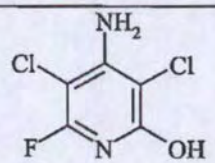
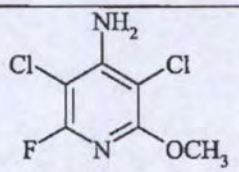
Supplemental Notes

1. Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available.

2. Electronic pipets are used only for pipetting aqueous solutions. If they are used for pipetting non-aqueous solutions, the pipets should be calibrated following the manufacturer's instruction manual and Standard Operating Procedures (9).

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

Table 1. Identity and Structure of Fluroxypyr 1-Methylheptyl Ester, Fluroxypyr, Fluroxypyr-DCP and Fluroxypyr-MP

| Identifying Information | Structure and CAS Name |
|---|---|
| Fluroxypyr 1-methylheptyl ester<br>Molecular Formula: $C_{15}H_{21}Cl_2FN_2O_3$<br>Formula Weight: 367.25<br>Nominal Mass: 366<br>CAS Number: 81406-37-3 |  <br>[(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]-, 1-methylheptyl ester |
| Fluroxypyr<br>Molecular Formula: $C_7H_5Cl_2FN_2O_3$<br>Formula Weight: 255.03<br>Nominal Mass: 254<br>CAS Number: 69377-81-7 |  <br>[(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid |
| Fluroxypyr-DCP<br>Molecular Formula: $C_5H_3Cl_2FN_2O$<br>Formula Weight: 197.0<br>Nominal Mass: 196<br>CAS Number: 94133-62-7 |  <br>(4-amino-3,5-dichloro-6-fluoro-2-pyridinol) |
| Fluroxypyr-MP<br>Molecular Formula: $C_6H_5Cl_2FN_2O$<br>Formula Weight: 211.02<br>Nominal Mass: 210<br>CAS Number: 35622-80-1 |  <br>(4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine) |