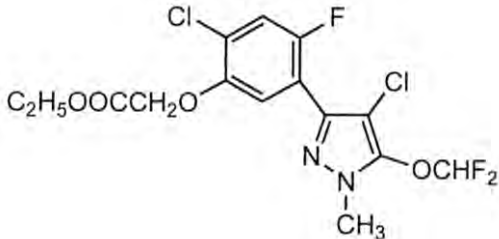
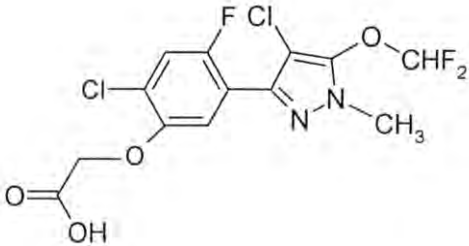
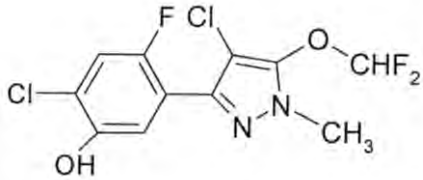


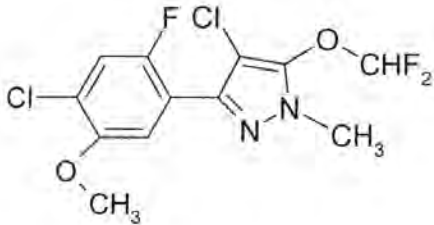
## 2 Reference Item Details

The following reference items were used in the study. The certificate's of analysis are shown in Appendix A.

Common name	Pyraflufen-ethyl
IUPAC Name	ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate
Empirical formula	C <sub>15</sub> H <sub>13</sub> Cl <sub>2</sub> F <sub>3</sub> N <sub>2</sub> O <sub>4</sub>
Molar mass	413.2 g/mol
Structure	
ResChem Lot. No.	RAL 019/2016
Batch Identification	3AM0058P
Purity	98.6%
Expiry date	16 February 2020

Common name	Pyraflufen-ethyl metabolite E-1
IUPAC Name	2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetic acid
Empirical formula	C <sub>13</sub> H <sub>9</sub> Cl <sub>2</sub> F <sub>3</sub> N <sub>2</sub> O <sub>4</sub>
Molar mass	385.1 g/mol
Structure	
ResChem Lot. No.	RAL 020/2016
Batch Identification	6AM4407S
Purity	98.6%
Expiry date	04 June 2022

Common name	Pyraflufen-ethyl metabolite E-2
IUPAC Name	2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenol
Empirical formula	C <sub>11</sub> H <sub>7</sub> Cl <sub>2</sub> F <sub>3</sub> N <sub>2</sub> O <sub>2</sub>
Molar mass	327.1 g/mol
Structure	 <p>The structure shows a central pyrazole ring with a methyl group on the nitrogen and a difluoromethoxy group at the 5-position. The pyrazole ring is substituted at the 3-position with a 4-chloro-5-difluoromethoxyphenyl group. The phenyl ring also has a fluorine atom at the 2-position and a hydroxyl group at the 4-position.</p>
ResChem Lot. No.	RAL 028/2017
Batch Identification	2AM0707S
Purity	99.8%
Expiry date	28 February 2021

Common name	Pyraflufen-ethyl metabolite E-3
IUPAC Name	4-chloro-3-(4-chloro-2-fluoro-5-methoxyphenyl)-5-difluoromethoxy-1-methylpyrazole
Empirical formula	C <sub>12</sub> H <sub>9</sub> Cl <sub>2</sub> F <sub>3</sub> N <sub>2</sub> O <sub>2</sub>
Molar mass	341.1 g/mol
Structure	 <p>The structure shows a central pyrazole ring with a methyl group on the nitrogen and a difluoromethoxy group at the 5-position. The pyrazole ring is substituted at the 3-position with a 4-chloro-2-fluoro-5-methoxyphenyl group.</p>
ResChem Lot. No.	RAL 022/2016
Batch Identification	1AM0308S
Purity	99.4%
Expiry date	29 April 2019

### 3 Test System Details

#### 3.1 Specimen Origin

The validation was undertaken on LUFA Speyer soils type 2.2 (loamy sand) and 6S (clayey loam).

### 4 Analytical Methodology

The analytical method involves extraction of sub-samples of each soil type using sequential extraction by shaking using acetonitrile/1M ammonium chloride solution and acetonitrile/1M hydrochloric acid solution. The extracts are combined and the volume adjusted prior to determination of residues of pyraflufen-ethyl, E-1, E-2 and E-3 by LC-MS/MS.

#### 4.1 Reagents and Materials Used

Reagent	Description	Supplier
Acetonitrile	HPLC Grade	Rathburns
Ammonium chloride	ACS Reagent Grade	Sigma Aldrich
Hydrochloric acid	S.G. 1.18 AR Grade	Fisher Scientific
Deionised Water	HPLC Grade	Rathburns
Acetic Acid	LC-MS Grade	Fisher Scientific
Deionised Water	LC-MS Grade	Romil
Acetonitrile	LC-MS Grade	Romil

#### 4.2 Equipment and Apparatus used

Item	Description
Laboratory Balance's	A&D GR-202, UWE HGS-300
Ultrasonic Bath	GT Sonic, 10 L Capacity
Adjustable Pipettes	Gilson P100, P200, P1000, P10mL
Centrifuge	Hettich Rotanta 460
Orbital Shaker	Gerhardt Laboshake
General Laboratory Supplies	Volumetric Flasks, Pipette's, Beakers, Autosampler Vials, Centrifuge tubes, Measuring Cylinders, HDPE bottles (150 mL) etc.
LC-MS/MS	AB Sciex 5500 Mass Spectrometer with an Agilent 1260 Binary HPLC Pump, Agilent 1260 Degasser, CTC Analytics HTC PAL Autosampler, Agilent 1260 Column Oven and a Peak Scientific ABN2ZA Gas Generator
HPLC Column	Zorbax SB-C3, 150 x 4.6 mm, 5.0 µm Particle Size, Agilent
HPLC Column	Luna Phenyl Hexyl, 150 x 4.6 mm, 5.0 µm Particle Size, Phenomenex

#### 4.3 Standard Preparation

Individual pyraflufen-ethyl, E-1, E-2 and E-3 stock solutions were prepared in acetonitrile with the aid of an ultrasonic bath, by dissolving 10 mg in 10 mL of solvent. The standards were allocated unique reference numbers i.e. PE-12/5/17, E1-12/5/17, E2-12/5/7 and E3-12/5/17.

The stock solutions were combined and further diluted for use as fortification standards in the procedural recovery process and for the preparation of intermediate standards for instrument set-up and matrix-matched standard preparation.

#### 4.3.1 Preparation of Fortification Solutions

Fortification standard solutions were prepared by serial dilution of the stock solutions using acetonitrile as listed below.

Standard Ref.	Standard Conc. (µg/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (µg/mL)	Standard Ref.
PE-12/5/17	1000	0.1			
E1-12/5/17	1000	0.1	10	10	MIX1-12/5/17
E2-12/5/7	1000	0.1			
E3-12/5/17	1000	0.1			
MIX1-12/5/17	10	1.0	10	1.0	MIX2-12/5/17
MIX2-12/5/17	1.0	1.0	10	0.1	MIX3-12/5/17
MIX3-12/5/17	0.1	1.0	10	0.01	MIX4-12/5/17

#### 4.3.2 Preparation of Intermediate Standard Solutions

Dilutions were performed in untreated clayey loam soil final extract as follows;

Standard Ref.	Standard Conc. (ng/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (ng/mL)	Standard Ref.
MIX2-12/5/17	1000	0.25	5.0	50	Calinter1-15/5/17
Calinter1-15/5/17	50	0.025	5.0	0.25	Calinter2-15/5/17

Dilutions were performed in untreated loamy sand soil final extract as follows;

Standard Ref.	Standard Conc. (ng/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (ng/mL)	Standard Ref.
MIX2-12/5/17	1000	0.25	5.0	50	Calinter1-18/5/17
Calinter1-18/5/17	50	0.025	5.0	0.25	Calinter2-18/5/17

#### 4.3.3 Preparation of Matrix-Matched Calibration Standard Solutions

Dilutions were performed in untreated clayey loam soil final extract as follows;

Standard Ref.	Standard Conc. (ng/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (ng/mL)	Standard Ref.
Calinter1-15/5/17	50	0.18	2.0	4.5	MMS1-15/5/17
Calinter1-15/5/17	50	0.08	2.0	2.0	MMS2-15/5/17
Calinter1-15/5/17	50	0.04	2.0	1.0	MMS3-15/5/17
Calinter1-15/5/17	50	0.02	2.0	0.5	MMS4-15/5/17
Calinter1-15/5/17	50	0.01	2.0	0.25	MMS5-15/5/17
Calinter2-15/5/17	0.25	0.8	2.0	0.1	MMS6-15/5/17
Calinter2-15/5/17	0.25	0.28	2.0	0.035	MMS7-15/5/17
Calinter2-15/5/17	0.25	0.08	2.0	0.01	MMS8-15/5/17

Dilutions were performed in untreated loamy sand soil final extract as follows;

Standard Ref.	Standard Conc. (ng/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (ng/mL)	Standard Ref.
Calinter1-18/5/17	50	0.18	2.0	4.5	MMS1-18/5/17
Calinter1-18/5/17	50	0.08	2.0	2.0	MMS2-18/5/17
Calinter1-18/5/17	50	0.04	2.0	1.0	MMS3-18/5/17
Calinter1-18/5/17	50	0.02	2.0	0.5	MMS4-18/5/17
Calinter1-18/5/17	50	0.01	2.0	0.25	MMS5-18/5/17
Calinter2-18/5/17	0.25	0.8	2.0	0.1	MMS6-18/5/17
Calinter2-18/5/17	0.25	0.28	2.0	0.035	MMS7-18/5/17
Calinter2-18/5/17	0.25	0.08	2.0	0.01	MMS8-18/5/17

For extract stability testing, matrix matched standards were freshly prepared (using freshly prepared intermediate standards) at the same concentration as those listed above.

#### 4.3.4 Preparation of Solvent Standard Solutions equivalent to the LOQ

Standard solutions were prepared for assessment of matrix effects (in acetonitrile/1M ammonium chloride/1M hydrochloric acid, 56/9/5, v/v/v) as described below.

For assessment of clayey loam matrix effects standards were prepared as follows;

Standard Ref.	Standard Conc. (ng/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (ng/mL)	Standard Ref.
MIX2-12/5/17	1000	0.25	5.0	50	Solvinter1-15/5/17
Solvinter1-15/5/17	50	0.025	5.0	0.25	Solvinter2-15/5/17
Solvinter2-15/5/17	0.25	0.28	2.0	0.035	Solventstd-15/5/17

For assessment of loamy sand matrix effects standards were prepared as follows;

Standard Ref.	Standard Conc. (ng/mL)	Volume Used (mL)	Final Vol. (mL)	Final Conc. (ng/mL)	Standard Ref.
MIX2-12/5/17	1000	0.25	5.0	50	Solvinter1-18/5/17
Solvinter1-18/5/17	50	0.025	5.0	0.25	Solvinter2-18/5/17
Solvinter2-18/5/17	0.25	0.28	2.0	0.035	Solventstd-18/5/17

#### 4.4 Soil Moisture Content Determination

90 g (3 x 30 g aliquots per soil type) of soil were weighed and left to dry in an oven at 105 °C. The samples were initially weighed after approximately 16 hours of drying. The samples were returned to the oven and re-weighed again after approximately 26.5 hours of drying. As both dry weights were the same (within a tolerance of  $\pm 0.1$  g) the soil samples were deemed to be dry. The final dry weight was used to determine the moisture content of the initial sample.

#### 4.5 Extraction

1. An aliquot of soil (50 g, dry weight) was dispensed into a HPDE extraction bottle (150 mL capacity).
2. Procedural recoveries were prepared by fortifying aliquots of untreated soil with the appropriate mixed analyte fortification solution as detailed in the table below.

Sample Weight (g) (dry weight)	Standard Reference	Standard Conc. ( $\mu\text{g}/\text{mL}$ )	Volume added (mL)	Fortification Level ( $\mu\text{g}/\text{kg}$ )
50.0	MIX4-12/5/17	0.01	0.25	0.05
50.0	MIX3-12/5/17	0.1	0.25	0.5
50.0	MIX2-12/5/17	1.0	0.25	5.0

3. Acetonitrile:1M ammonium chloride solution (4:1 v:v, 30 mL) was added and the soil extracted by mechanical shaking (15 mins).

Note: acetonitrile (24 mL) and 1M ammonium chloride (6 mL) was added separately.

4. The extraction bottles were placed in a centrifuge (4000 rpm, 2 mins) and the supernatant was decanted into a measuring cylinder (100 mL capacity).
5. The extraction procedure was repeated once more after ensuring that all centrifuged solids were freed. Acetonitrile:1M ammonium chloride solution (4:1 v:v, 15 mL) was added and the residual soil extracted by mechanical shaking (15 mins).

Note: acetonitrile (12 mL) and 1M ammonium chloride (3 mL) was added separately.

6. The extraction bottles were placed in a centrifuge (4000 rpm, 2 mins) and the supernatant was combined with the initial extract in the same measuring cylinder (100 mL capacity).
7. The extraction procedure was repeated one final time after ensuring that all centrifuged solids were freed. Acetonitrile:1M hydrochloric acid solution (4:1 v:v, 15 mL) was added and the residual soil extracted by mechanical shaking (15 mins).
8. The extraction bottles were then placed in a centrifuge (4000 rpm, 2 mins) and the supernatant was combined with the initial two extracts in the same measuring cylinder (100 mL capacity).
9. The volume of the sample was adjusted to 70 mL using acetonitrile:1M hydrochloric acid solution (4:1 v:v) and the sample was then transferred to an HPDE bottle (150 mL capacity).
10. The sample bottles were placed in a centrifuge (4000 rpm, 2 mins) and an aliquot was transferred to an HPLC vial prior to quantitation of pyraflufen-ethyl, E-1, E-2 and E-3 residues by LC-MS/MS.

#### 4.6 LC-MS/MS Conditions

An AB Sciex 5500 Mass Spectrometer with an Agilent 1260 Binary HPLC Pump, Agilent 1260 Degasser, CTC Analytics HTC PAL Autosampler, Agilent 1260 Column Oven and a Peak Scientific ABN2ZA Gas Generator was used for quantitation.

##### 4.6.1 Chromatography Parameters for Pyraflufen-ethyl, E-1, E-2 and E-3

Parameter	Description		
HPLC Column	Zorbax SB-C3, 150 x 4.6 mm, 5.0 µm Particle Size		
Column Temperature	50 °C		
Injection Volume	50 µL		
Retention Times (approximate)	Pyraflufen-ethyl 9.1 minutes, E-1 6.7 minutes, E-2 8.2 minutes, E-3 9.0 minutes		
Mobile Phase	A: 0.2% Acetic Acid in Water B: 0.2% Acetic Acid in Acetonitrile		
Flow Rate	0.75 mL/min		
Gradient	Time (minutes)	A (%)	B (%)
	0.0	90	10
	10.0	5	95
	11.0	5	95
	11.1	90	10
	12.5	90	10

##### 4.6.2 Chromatography Parameters for E-1 (confirmatory quantitation method)

Parameter	Description		
HPLC Column	Luna Phenyl Hexyl, 150 x 4.6 mm, 5.0 µm Particle Size		
Column Temperature	50 °C		
Injection Volume	50 µL		
Retention Time	E-1 4.4 minutes (approximate)		
Mobile Phase	A: 0.2% Acetic Acid in Water B: 0.2% Acetic Acid in Acetonitrile		
Flow Rate	1.0 mL/min		
Gradient	Time (minutes)	A (%)	B (%)
	0.0	90	10
	3.0	5	95
	5.0	5	95
	5.1	90	10
	6.5	90	10

**4.6.3 Mass Spectrometry Parameters for E-1**

Parameter	Description		
Ionisation Mode	TurboSpray (Electrospray)		
Probe Position	5 Horizontal, 5 Vertical		
Polarity	Negative		
Curtain Gas	40		
CAD Gas	12		
Gas 1	40		
Gas 2	50		
Source Temperature	700 °C		
Spray Voltage	-4500		
Declustering Potential	-90		
Entrance Potential	-10		
Mass Transition	Ions monitored ( <i>m/z</i> )	Collision Energy	Cell Exit Potential
E1	383 → 274	-43	-12

**4.6.4 Mass Spectrometry Parameters for Pyraflufen-ethyl, E-2 and E-3**

Parameter	Description				
Ionisation Mode	TurboSpray (Electrospray)				
Probe Position	5 Horizontal, 5 Vertical				
Polarity	Positive				
Curtain Gas	40				
CAD Gas	12				
Gas 1	40				
Gas 2	50				
Source Temperature	550 °C				
Spray Voltage	5500				
Declustering Potential	150 (pyraflufen-ethyl), 70 (E2) and 100 (E-3)				
Entrance Potential	10				
Mass Transitions	Ions monitored ( <i>m/z</i> )	Declustering Potential	Collision Energy	Cell Exit Potential	Primary / Confirmatory
Pyraflufen-ethyl	413 → 339	150	27	14	Primary
	413 → 289	150	40	14	Confirmatory
E-2	327 → 277	70	33	10	Primary
	329 → 279	70	33	10	Confirmatory
E-3	341 → 291	100	34	10	Primary
	341 → 276	100	45	10	Confirmatory



## 4.7 Quantitation

The quantitative determination of all analytical samples was carried out by external standardisation using calibration standards in matrix. Injections of calibration standards were interspersed throughout the sequence between injections of samples (not greater than 4).

Detector linearity was assessed by constructing a calibration curve of peak area versus analyte concentration. Linear regression with 1/x weighting at 8 different concentrations ranging from 0.01 ng/mL to 4.5 ng/mL was used and correlation co-efficients (r) above 0.995 were obtained for each analyte which meets the criteria of ResChem Analytical standard operating procedures.

Residues (R) in µg/kg were calculated according to the following equation:

$$R = (C_{\text{END}} \times \text{FV}) / \text{FW}$$

where:

R = Analyte residue, mg/kg

C<sub>END</sub> = Final concentration of analyte in sample extract in ng/mL.

FV = Final extract volume (70 mL)

FW = Final sample wt. (50 g)

Percent recovery from fortified samples was calculated as described below:

$$\text{Recovery (\%)} = \frac{(R_{\text{fortified}})}{F} \times 100$$

where:

R<sub>fortified</sub> = Residue determined in fortified sample (µg/kg)

F = Fortification rate (µg/kg)

### 4.7.1 Example Calculation

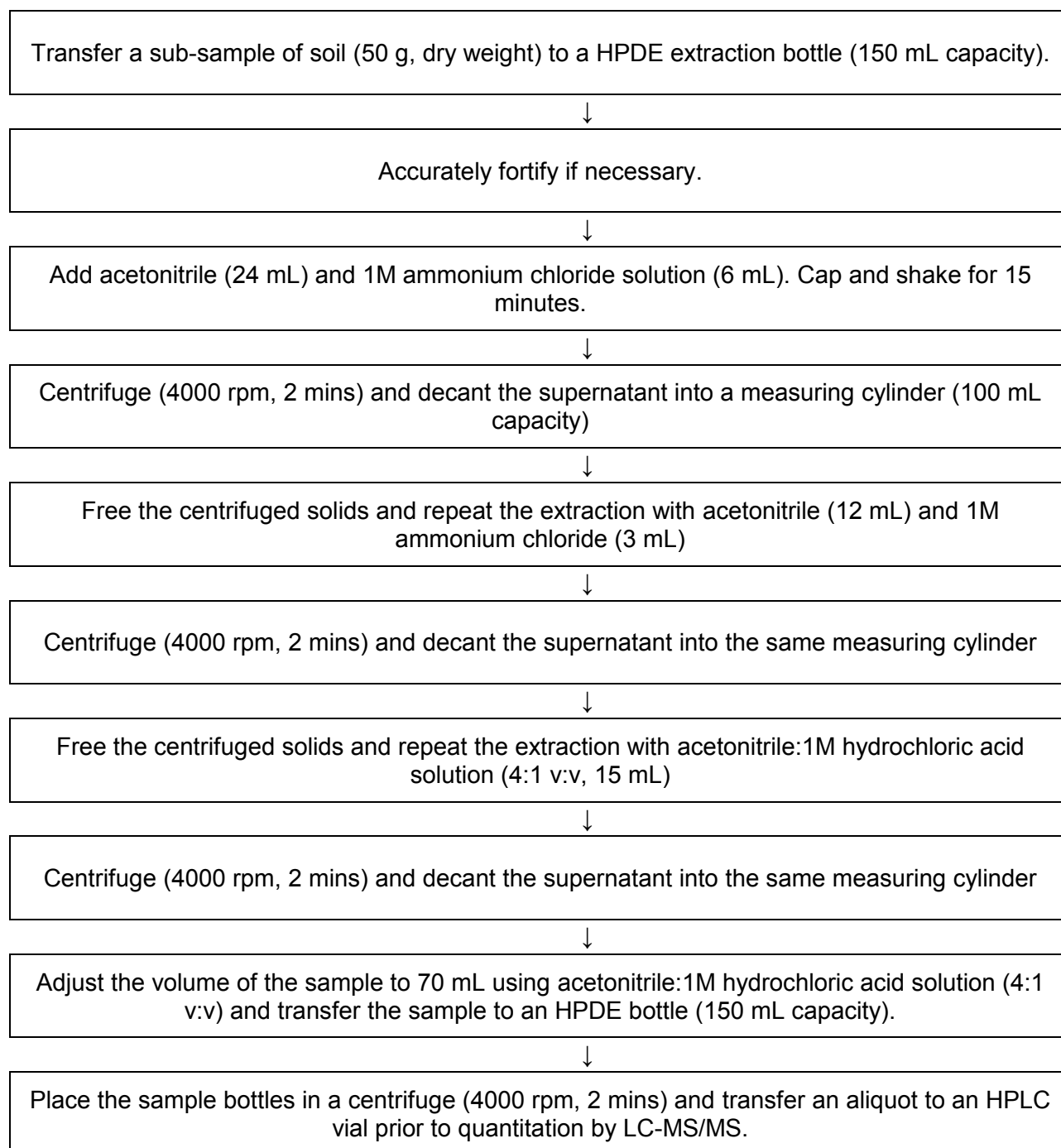
For a 0.05 µg/kg fortified loamy sand sample (sample no. 3576) the concentration of pyraflufen-ethyl found was calculated as follows:

$$\begin{aligned} R &= (0.035351 \text{ ng/mL} \times 70 \text{ mL}) / 50 \text{ g} \\ &= 0.04949 \text{ µg/kg} \end{aligned}$$

The percent recovery found was calculated as follows:

$$\begin{aligned} \text{Recovery (\%)} &= (0.04949 \text{ µg/kg} / 0.05 \text{ µg/kg}) \times 100 \\ &= 99.0 \% \end{aligned}$$

#### 4.8 Method Flow Chart



#### 4.9 Time Management

Preparation of standards and extraction of one sample set can be conducted in 1 working day (7.5 hours). Analysis of samples by LC-MS/MS can be conducted over approximately 20 hours.

Therefore, one matrix set, consisting of 21 samples (including 18 recoveries, 1 reagent blank, 2 control samples) and preparation of 8 calibration standards can be prepared, extracted and analysed within approximately 2 days i.e. extraction starts at beginning of day 1, submitted for quantitation at end of day 1 and quantitation completed by end of day 2.