1. EXECUTIVE SUMMARY

The objective of this study was to validate an analytical method for the determination of residues of pyraflufen-ethyl and its metabolites E-1, E-2 and E-3 in two types of soil to fulfil the requirements of guidance documents ENV/JM/MONO(2007)17, SANCO 825/00 rev 8.1 and OPPTS 860.1340.

Residues of pyraflufen-ethyl and metabolites were extracted from soil by sequential extractions with acetonitrile: 1M ammonium chloride and acetonitrile: 1M hydrochloric acid. Final determination was by liquid chromatography with tandem mass spectrometry (LC-MS/MS), monitoring at least two ion mass transitions per analyte. For E-1, the primary transition was analysed by two separate LC-MS/MS methods with chemically different stationary phases. This was due to an interference in the confirmatory transition which prevented reliable quantitation. The method was validated in terms of linearity, specificity, LOQ, accuracy, precision, matrix effects and stability.

The soil moisture for each soil type was determined prior to analysis of the validation samples.

Calibration curves were obtained from at least six matrix-matched calibration solutions containing pyraflufen-ethyl and metabolites, covering a range from at least 30% of the LOQ to at least 20% above the highest concentration level, with correlation coefficients (r) all greater than 0.99, demonstrating satisfactory linearity.

Interfering peaks in control samples that eluted at the same retention time as pyraflufen-ethyl and metabolites were either non-detectable or less than 30% of the limit of quantification (LOQ) demonstrating acceptable specificity.

2. OBJECTIVE

The objective of this study was to validate an analytical method for the determination of residues of pyraflufen-ethyl and metabolites E-1, E-2 and E-3 in soil to fulfil the requirements as per guidance documents ENV/JM/MONO(2007)17, SANCO 825/00 rev 8.1 and OPPTS 860.1340.

3. TEST ITEMS

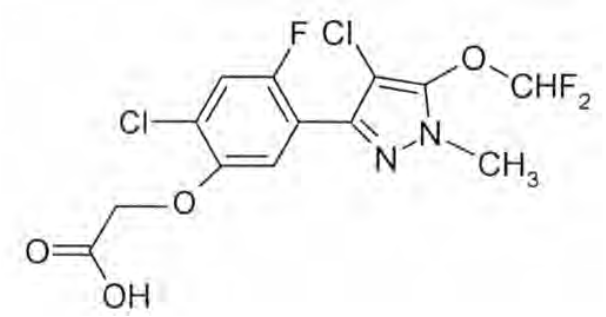
The certificates of analysis for pyraflufen-ethyl, E-1, E-2 and E-3 are presented in Appendix 4 to Appendix 7. In addition, full Q1 mass spectral and product ion scans to illustrate the ion mass transitions used for final determination are presented in Appendix 10 to Appendix 13.

3.1. Reference Items

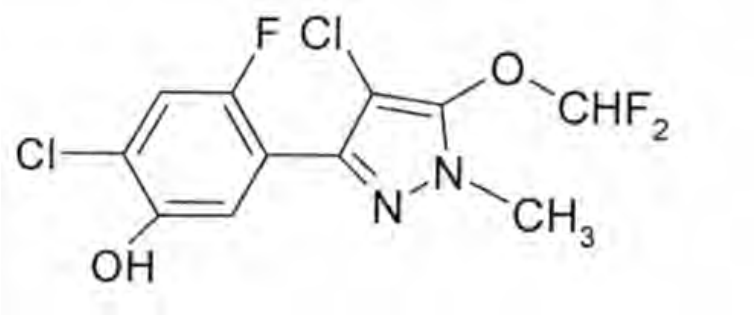
3.1.1. Pyraflufen-ethyl

Product Name:	Pyraflufen-ethyl standard
Common Name:	Pyraflufen-ethyl
IUPAC Name:	ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate
Empirical formula	$C_{15}H_{13}Cl_2F_3N_2O_4$
Molar mass	413
Structure:	
Batch Identification:	3AM0058P
Purity:	98.6%
Storage Conditions:	Refrigerator in dark conditions
Expiry Date:	16 Feb. 2020

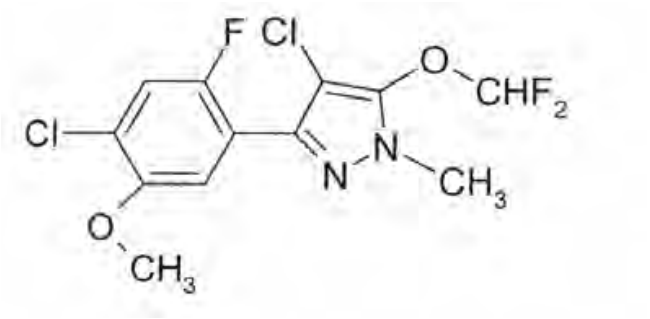
3.1.2. Pyraflufen-ethyl metabolite E-1

Product name	E-1 Standard
IUPAC Name	2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetic acid
Empirical formula	$C_{13}H_9Cl_2F_3N_2O_4$
Molar mass	385
Structure:	
Batch Identification:	6AM4407S
Purity:	98.6%
Storage Conditions:	Room temperature in dark conditions
Expiry Date:	4 June 2022

3.1.3. Pyraflufen-ethyl metabolite E-2

Product name	E-2 standard
IUPAC Name	2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenol
Empirical formula	$C_{11}H_7Cl_2F_3N_2O_2$
Molar mass	327
Structure:	
Batch Identification:	2AM0707S
Purity:	99.8%
Storage Conditions:	Refrigerator in dark conditions
Expiry Date:	28 February 2021

3.1.4. Pyraflufen-ethyl metabolite E-3

Product name	E-3 Standard
IUPAC Name	4-chloro-3-(4-chloro-2-fluoro-5-methoxyphenyl)-5-difluoromethoxy-1-methylpyrazole
Empirical formula	$C_{12}H_9Cl_2F_3N_2O_2$
Molar mass	341
Structure:	
Batch Identification:	1AM0308S
Purity:	99.4%
Storage Conditions:	Refrigerator and in dark conditions
Expiry Date:	29 Apr. 2019

4. TEST SYSTEMS

A clay soil and a sandy soil were taken from Battelle validation control stock samples. See Appendix 8 and Appendix 9 for the characterisation reports. The soil moisture for each soil type was determined prior to analysis.

5. METHOD VALIDATION

The method was validated in terms of linearity, specificity, accuracy and precision, matrix effects and stability.

5.1. Linearity

Matrix-matched calibration solutions containing pyraflufen-ethyl and metabolites were prepared in each soil type covering a nominal range from 0.00910 to 4.50 ng/mL and calibration curves constructed after injection of the solutions.

5.2. Specificity

Specificity was determined by assessing the method's ability to differentiate between chromatographic peaks that are a result of the presence of each analyte from those chromatographic signals that generated co-extractive peaks in untreated samples that eluted at the same retention time as each analyte. A reagent blank and duplicate control samples for each soil type were assessed.

5.3. Accuracy

Accuracy was assessed by conducting a series of recovery efficiency tests at the LOQ, 10xLOQ and 100 x LOQ.

5.4. Precision

The precision of the method was determined by measuring the relative standard deviation of the recovery efficiency data at each fortification level.

5.5. Matrix Effects

Matrix effects were investigated at the LOQ, 10 x LOQ and 100 x LOQ levels, by comparing peak areas of solvent standard solutions to peak areas of matrix-matched standard solutions for each soil type at equivalent concentrations. Experiments assessed whether matrix effects were significant (i.e. > 20% enhancement or suppression).

5.6. Extract Stability

All samples from the validation were stored under refrigerated conditions at a nominal temperature of 4°C. After 13 days (clay soil), 33 - 35 days (sandy soil) storage, an LOQ recovery sample from each soil type was re-vialled and injected within batches containing freshly prepared matrix matched standards.

5.7. Solvent Standard Stability

The stability of the solvent stock solution was assessed after storage under refrigerated conditions at a nominal temperature of 4°C. A solvent standard at 10 x LOQ was prepared in acetonitrile from the original stock solution after storage of 42 days. A freshly prepared solvent standard was also prepared at 10 x LOQ in acetonitrile from a newly weighed stock. Both stored and fresh standards were injected and the areas compared.

5.8. Limit of Detection

The limit of detection (LOD) for each soil type and each analyte was defined as the lowest quantifiable calibration standard.

6. EXPERIMENTAL

6.1. Principle of the Method

The method consisted of sequential extractions from soil with acetonitrile: 1M ammonium chloride solution (4:1 v: v) and acetonitrile: 1M hydrochloric acid solution (4:1 v: v) followed by quantification by LC-MS/MS monitoring at least two ion mass transitions.

The extraction flow chart is presented in Appendix 1.

6.2. Reagents and Equipment

Full details of all equipment, consumables and reagents are presented in Appendix 2 and Appendix 3.

6.3. Standards and Fortifications

6.3.1. Stock Solutions

Stock solutions were prepared by dissolving a known weight (mg) of each analyte, correcting for purity and dissolving in acetonitrile to produce a final concentration of 1000 µg/mL (with the exception of standards 7329 and 7237). Full stock solution preparation and final concentrations are detailed in the following table:

Analyte	Battelle (BUKL) Stock ID	Purity (%)	Actual Amount Weighed (mg)	Actual Volume Added (mL)	Concentration (µg/mL)
Pyraflufen-ethyl	7230	98.6%	10.37	10.224	1000
	7234	98.6%	10.13	9.988	1000
	7328	98.6%	10.12	9.978	1000
E-1	7233	98.6%	10.41	10.264	1000
	7232	98.6%	10.42	10.274	1000
	7329	98.6%	10.28	10.057	1008
E-2	7231	99.8%	10.13	10.109	1000
	7235	99.8%	10.37	10.349	1000
	7330	99.8%	10.23	10.209	1000
E-3	7236	99.4%	10.20	10.138	1000
	7237	99.4%	10.01	10.949	909
	7331	99.4%	10.14	10.079	1000

One stock was used for calibration standard preparation, one was used for recovery fortification preparation and one was used for stability testing.

6.3.2. Fortification Solutions

Three fortification solutions, containing pyraflufen-ethyl and metabolites at concentrations of 0.01, 0.10 and 1.0 µg/mL, were prepared by diluting appropriate amounts of the stock solution with acetonitrile.

Recovery efficiency samples were fortified as per the following tables:

Matrix	Reagent Blank Replicates	Untreated Control Replicates	Replicates at LOQ Fortification Level	Replicates at LOQ × 10 Fortification Level	Replicates at LOQ × 100 Fortification Level
Clay Soil	1	2	6 at 0.05 µg/kg	6 at 0.50 µg/kg	6 at 5.0 µg/kg
Sandy Soil	1	2	6 at 0.05 µg/kg	6 at 0.50 µg/kg	6 at 5.0 µg/kg *

*6 replicates were spiked however due to a laboratory issue, only 5 samples were analysed

Matrix	Sample Weight* (g)	Fortification Standard Concentration (µg/mL)	Fortification Volume (mL)	Fortification Level (µg/kg)
Clay Soil	50	0.01	0.25	0.05
	50	0.10	0.25	0.50
	50	1.0	0.25	5.0
Sandy Soil	50	0.01	0.25	0.05
	50	0.10	0.25	0.50
	50	1.0	0.25	5.0

* Dry weight of soil

6.3.3. Calibration Solutions

An intermediate solution containing pyraflufen-ethyl and metabolites at a concentration of 1000 ng/mL was prepared by diluting an appropriate amount of the stock solutions with acetonitrile. This intermediate solution was then used to prepare a secondary intermediate solution at a concentration of 50.0 ng/mL by dilution with control matrix. Matrix matched solutions in the range of 0.150 ng/mL to 4.50 ng/mL were then prepared from the 50 ng/mL solution. A 0.350 ng/mL solution prepared as part of the calibration curve was then used to prepare calibration solutions in the range of 0.00910 – 0.0350 ng/mL.

All standard solutions were stored in the refrigerator when not in use.

6.4. Soil Moisture Determination

90 g (3 x 30 g aliquots per soil type) of soil were weighed and left to dry in an oven at 105 ºC. The samples were initially weighed after approximately 4 hours of drying. The samples were returned to the oven and re-weighed again after approximately 18 hours further drying. As both dry weights were the same the soil samples were determined to be dry (within a tolerance of ± 0.5 g). The final dry weight for each aliquot was used to calculate the moisture of the initial sample.

The soil moisture was calculated as per the following equation:

$$\text{Moisture Content [\%]} = \frac{\text{Initial wet weight (g)} - \text{Final dry weight (g)}}{\text{Initial wet weight (g)}} \times 100$$

The mean moisture content for each soil type was calculated by averaging the three individual moistures.

6.5. Extraction Procedure

Aliquots of 50 g (dry weight) of soil were placed into 125 mL extraction bottles. For recovery efficiency tests, the control matrices were fortified with the appropriate spiking solutions at the LOQ, 10 x LOQ and 100 x LOQ levels.

For all samples, 30 mL of acetonitrile: 1M ammonium chloride solution (4:1 v: v) was added to each bottle and mechanically shaken for 15 minutes. After centrifugation (4000 rpm for 2 minutes), the supernatant was decanted into a suitable receptacle capable of accurately measuring 70 mL. The extraction process was repeated once more with 15 mL acetonitrile: 1M ammonium chloride solution (4:1 v: v). The extraction process was repeated a final time with 15 mL acetonitrile: 1M hydrochloric acid solution (4:1 v: v). After combining all supernatants, the volume was adjusted to 70 mL with acetonitrile: 1M hydrochloric acid solution (4:1 v: v). After a final centrifugation at 4000 rpm for 2 minutes, an aliquot was transferred to an HPLC vial. Final residue levels were determined by LC-MS/MS.

The extraction method is presented in the form of a flow chart in Appendix 1.

6.6. LC-MS/MS Analysis

All samples were analysed by liquid chromatography coupled with a tandem mass spectrometer (LC-MS/MS), monitoring at least two ion mass transitions for each analyte.

A summary of these conditions is presented below:

6.6.1. LC-MS/MS Conditions (XG17002)

HPLC Conditions					
Columns	Zorbax SB-C3, 150 x 4.6 mm, 5.0 um particle size				
Column Oven Temperature	50 °C				
Mobile Phase A	0.2% Acetic acid in water				
Mobile Phase B	0.2% Acetic acid in acetonitrile				
Method	Time	%A	%B		
	0.0	90	10		
	10.0	5	95		
	11.0	5	95		
	11.1	90	10		
	11.5	90	10		
Flow Rate	0.75 mL/min				
Injection Volume	100 µL				
Mass Spectrometer and General Instrument Conditions					
Instruments	API 5500 Triple Quadrupole Mass Spectrometer fitted with Turbo ion spray ion source (Used for all validation with the exception of the stock stability tests) API 6500 Triple Quadrupole Mass Spectrometer fitted with Turbo ion spray ion source (Used for stock stability tests only)				
Ion Source	Positive Mode (Pyraflufen-ethyl, E-2 and E-3) Negative Mode (E-1)				
Run Time	11.5 minutes Approximate retention times API 5500: Pyraflufen-ethyl = 9.9 minutes, E-1 = 8.9 minutes, E-2 = 8.8 minutes and E-3 = 9.8 minutes Approximate retention times API 6500: Pyraflufen-ethyl = 9.9 minutes, E-1 = 8.9 minutes, E-2 = 8.9 minutes and E-3 = 9.9 minutes				
Analyte	Dwell Time (msec)	Transition (m/z)	Declustering Potential	Collision Energy	Cell Exit Potential
Pyraflufen-ethyl	100	413/339	30	25	20
	100	413/289	30	40	15
E-1 *	100	383/274	-40	-45	-10
E-2	100	327/277	40	30	15
	100	329/279	40	30	20
E-3	100	341/291	40	30	10
	100	341/276	40	45	20
Curtain Gas	Positive Mode: 20 Negative Mode:15				
CAD Gas	-2				
Gas 1	40				
Gas 2	Positive Mode: 40 Negative Mode: 50				
Spray Voltage	Positive Mode: 4000 V Negative Mode: -4500 V				
Source Temperature	700 °C				
Entrance Potential	Positive mode: 10 Negative Mode: -10				
Notes:	*E-1 confirmatory conditions analysed in a separate LC-MS/MS method				

6.6.2. LC-MS/MS Conditions (E1 only (confirmatory conditions))

HPLC Conditions					
Columns	Luna Phenyl Hexyl, 150 x 4.6 mm, 5 µm				
Column Oven Temperature	50 °C				
Mobile Phase A	0.2% Acetic acid in water				
Mobile Phase B	0.2% Acetic acid in acetonitrile				
Method	Time	%A	%B		
	0.0	90	10		
	3.0	5	95		
	5.0	5	95		
	5.1	90	10		
	5.5	90	10		
Flow Rate	1.0 mL/min				
Injection Volume	100 µL				
Mass Spectrometer and General Instrument Conditions					
Instruments	API 5500 Triple Quadrupole Mass Spectrometer fitted with Turbo ion spray ion source				
Ion Source	Negative Mode				
Run Time	5.5 minutes (Approximate retention time of 4.6 minutes)				
Analyte	Dwell Time (msec)	Transition (m/z)	Declustering Potential	Collision Energy	Cell Exit Potential
E-1 *	100	383/274	-40	-45	-10
Curtain Gas	15				
CAD Gas	-2				
Gas 1	40				
Gas 2	50				
Spray Voltage	-4500 V				
Source Temperature	700 °C				
Entrance Potential	-10				
Notes:	*E-1 primary conditions analysed in a separate LC-MS/MS method				

6.7. Time Management

Preparation of standards and extraction of one sample set can be conducted in 1 working day (7.5 hours). Analysis of samples using two separate LC-MS/MS methods can be conducted over approximately 15 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 2.5 working days.

7. CALIBRATION AND CALCULATION

Matrix matched calibration solutions containing pyraflufen-ethyl and metabolites were prepared in the nominal concentration range of 0.00910-4.50 ng/mL.

A multi-point calibration curve was obtained from injections of calibration solutions by plotting peak areas versus the concentration in ng/mL. The curve was calculated by the method of least squares linear regression. A weighting factor of 1/x was applied to each curve to improve the accuracy. Correlation coefficients (r) were all greater than 0.99.

The quantification of the analyte in the samples was made by comparison to the calibration curve of the form $y = mx + c$. The amount of analyte in a given sample was calculated as follows:

Soil

$$\text{Compound [µg/kg]} = \frac{(A - C) \times V}{M \times W}$$

Where:

A	=	Area of analyte peak
M	=	slope of the calibration curve
C	=	intercept of the calibration curve
V	=	extraction volume (mL)
W	=	dry weight of soil (g)

The recovery efficiency in the fortified samples was calculated as follows:

$$\text{Recovery efficiency [\%]} = \frac{\text{Amount found (µg/kg)}}{\text{Amount spiked (µg/kg)}} \times 100$$

Example LC-MS/MS chromatograms of matrix matched calibration solutions, control samples and fortified samples are presented in Figure 1 to Figure 16 with example calibration curves presented in Figure 17 to Figure 24. Response factors for each soil type are presented in Figure 25 to Figure 32.

13. APPENDICES

Appendix 1: Flow Chart - Soil

EXTRACTION

- Weigh 50 g of homogenised sample into a 125 mL plastic bottle
- Fortify if necessary
- Add 30 mL of acetonitrile: 1M ammonium chloride solution (4:1 v: v) and place the sample in a mechanical shaker for 15 minutes
- Centrifuge sample for 2 minutes at 4000 rpm
- Decant into a vessel capable of accurately measuring 70 mL
- Repeat extraction of soil with 15 mL acetonitrile: 1M ammonium chloride solution (4:1 v: v) ensuring that all centrifuged solids are freed before shaking - combine the extracts
- Repeat extraction a final time with 15 mL acetonitrile: 1M hydrochloric acid solution (4:1 v: v) ensuring that all centrifuged solids are freed before shaking - combine the extracts
- Adjust the volume of the sample to 70 mL using acetonitrile: 1M hydrochloric acid solution (4:1 v: v)
- Decant into a 125 mL plastic bottle
- Centrifuge sample for 2 minutes at 4000 rpm

ANALYSIS

- Transfer an aliquot of the sample into a clean clear screw cap vial
- Analyse by LC-MS/MS
- Matrix standards used

The LOQ of the validated method was: 0.05 µg/kg

Appendix 2: Equipment List

EQUIPMENT	MANUFACTURER / SUPPLIER
Sample Extraction	
Shakers: - Stuart mechanical flask shaker SF1 Stuart Scientific Reciprocating Shaker SSL2	Fisher Scientific UK Ltd. Bishop Meadow Road Loughborough Leicestershire, UK
General Laboratory Equipment	
Dispensers: - Eppendorf Multipette Xstream®	Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG
Balances: - Sartorius LP 4200 (Balance 6) Sartorius U4100 (Balance 12) Sartorius MSU324S Cubis 2504 (Balance 16) Sartorius MSU225S Cubis 220-5 (Balance 18) A&D EK-6100i (Balance 19)	Sartorius Ltd. Longmead Business Centre Blenheim Road Epsom, Surrey, UK All Ohaus and A&D balances bought from European Instruments.
Centrifuge: - Varifuge 3.0R (Centrifuge no. 3) (Floor-standing)	Heraeus, Sepatech 9 Watesway, Brentwood, Essex, CM15 9TB, UK
Specialised Equipment	
Memmert Oven	Memmert GmbH + Co.KG, P.O. Box 17 20 D-91107 Schwabach, Germany
LC-MS/MS	
MDS Sciex API 5500 and MDS Sciex API 6500 Analyst 1.6.2 Software	Applied Biosystems Lingley House 120 Birchwood Boulevard, Warrington, WA3 7QH, UK
Agilent HPLC 1290 Series	Agilent Technologies UK Ltd, Lakeside, Cheadle Royal Business Park, Stockport, Cheshire, SK8 3GR, UK
Eksigent (Presearch) HTS-xt Autosampler	Presearch Ltd., System House, 59-61 Knowl Piece, Hitchin, Herts, SG4 OTY, UK
Nitrogen Generator	Peak Scientific Instruments Ltd., Fountain Crescent Inchinnan Business Park, Renfrewshire, PA4 9RE, UK

EQUIPMENT	MANUFACTURER / SUPPLIER
LC Columns	
Phenomenex Agilent	Phenomenex Hurdsfield Industrial Estate, Macclesfield, Cheshire, SK10 2BN, UK Agilent Technologies UK Ltd, Lakeside, Cheadle Royal Business Park, Stockport, Cheshire, SK8 3GR, UK

Appendix 3: Reagents

Chemical	Supplier
Acetonitrile HPLC grade Hydrochloric acid S.G. 1.16 (32%)	Fisher Scientific (Acros Organics, Fisons) Fisher Scientific UK Ltd Bishop Meadow Road Loughborough Leicestershire LE11 5RG
Ammonium Chloride AnalaR Normapur Acetic Acid 100% Ph. Eur	VWR international Ltd (BDH, MERCK Eurolab) Hunter Boulevard Magna Park Lutterworth LEICS LE17 4XN
HPLC grade water	Rathburn Chemicals Ltd. Walkerburn, Scotland