3. MATERIALS AND METHODS

3.1. Test and Reference Items

Details of the reference items are presented in Figure 1 and Certificates of Analysis are presented in Appendix 1.

3.2. Principle of the Method

The concentration of the analytes was determined by LC-MS/MS. Control soil samples and fortified controls were extracted with a 100:0.5 methanol:acetic acid (v:v) solution followed by centrifugation. Residues were subsequently determined by LC-MS/MS, monitoring two ion transitions, the first transition for quantification and the second for confirmation.

The analytical flow chart is presented in Figure 3.

3.3. Reagents and Equipment

Details of reagents and equipment are presented in Appendix 2.

4. ANALYTICAL METHODOLOGY

4.1. Test System

The soil used in the study was a loamy sand from the Test Facility's stock of control soils. See Appendix 3 for detailed characteristics.

4.2. Fortification

A stock solution of the Pyridate reference item at a concentration of approximately 1000 µg/mL was prepared by dissolving approximately 5 mg in 5 mL of methanol + 0.5% Acetic Acid.

A separate stock solution containing the CL-9673 reference item at a concentration of approximately 1000 µg/mL was prepared by dissolving about 5 mg in 4.85 mL of methanol.

Working solutions containing the two reference items at 1.0 and 10.0 µg/mL were prepared by mixing and diluting appropriate amounts of the stock solution with a 100:0.5 methanol:acetic acid (v:v) mixture and used for fortification purposes. These working solutions were stored at 4°C and remained stable for up to 10 days.

4.3. Extraction – Ultra-Turrax

Samples of soil (10 g) were transferred into a plastic bottle, fortified at the LOQ (0.01 mg/kg) and at 10 x LOQ (0.10 mg/kg) with a solution containing each analyte and extracted with 100 mL of 100:0.5 methanol:acetic acid (v:v) solution by blending with an Ultra-Turrax macerator. The samples were centrifuged and an aliquot of the extract was then transferred into glass vials. Six replicate analyses at each fortification level were performed for each analyte and ion mass transition to verify accuracy and precision.

4.4. Extraction – Solvent Shake

As a comparison to extraction by Ultra-Turrax, three replicate control samples fortified at the LOQ and two controls fortified at 10 x LOQ were extracted with 100:0.5 methanol/acetic acid (v:v) mixture by shaking for twenty minutes followed by centrifugation. Residues in the solvent extracts were determined by LC-MS/MS.

4.5. LC-MS/MS Analysis

An aliquot of each sample was transferred to an autosampler vial for determination by LC-MS/MS. Two transitions were selected for each analyte. The primary transition was used for quantification and the secondary transitions for confirmation of residues.

4.5.1. Instrument Description

Pump	Agilent 1100 series Binary pump model number G1312A
Degasser	Agilent 1100 series model number G1379A
Column Oven	Agilent 1100 series model number G1316A fitted with switching valve
Autosampler	Agilent 1100 series model number G1313A
Detector	API 5000 LC-MS/MS System with Q Jet Ion Guide

4.5.2. Liquid Chromatography Conditions

Column	Phenomenex Aqua C18 5µm 125Å [50 x 2 mm]		
Mobile Phase A	99:1 v:v Water: Acetic Acid		
Mobile Phase B	99:1 v:v MeCN:Acetic acid		
Gradient	Time [min]	%A	%B
	0	90	10
	0.2	90	10
	1.0	10	90
	2.0	10	90
	2.65	90	10
	3.5	90	10
Flow Rate	1 mL/min direct into MS ion source.		
Injection Volume	10 µL		

The retention times of Pyridate and CL-9673 were ca. 2.0-2.2 and 1.2-1.5 min respectively.

4.5.3. Mass Spectrometry Conditions

Ion Source	Positive Ion Turbo Spray Ionisation			
Curtain Gas [CUR]	20 (arbitrary units)			
Temperature [TEM]	600 °C			
Ion Transfer Voltage [IS]	5500 V			
Collision Gas Cell [CAD]	5.00 (arbitrary units)			
GS1 Nebuliser Gas	45 (arbitrary units)			
GS2 Turbo Gas	60 (arbitrary units)			
Interface Heater [ihe]	On			
CEM [Electron Multiplier]	2000			
DF [Deflector]	-400			
Scan Type	MRM			
MRM Conditions	Pyridate Transition 1 $[M+H]^+$ to $[C_3HNO+H]^-$	Pyridate Transition 2 $[M+H]^+$ to $[C_4HN2_5]^+$	CL-9673 Transition 1 $[M+H]^+$ to $[C_7H_5N+H]^+$	CL-9673 Transition 2 $[M+H]^+$ to $[C_3HNO+H]^+$
Q1 m/z	379.05	379.05	207.07	207.07
Q3 m/z	77.20	68.10	103.97	67.97
Dwell Time	150	150	150	150
Resolution Q1	Unit	Unit	Unit	Unit
Resolution Q3	Low	Low	Low	Low
Declustering Potential [DP]	171	171	171	171
Entrance Potential [EP]	10	10	10	10
Collision Energy [CE]	81	71	31	45
Collision Cell Exit Potential	34	30	16	30

4.6. Extract Stability

Following the initial analysis of the fortified samples (section 4.3), the calibration solutions and the control and recovery extracts were stored at nominally -18°C for 5 days and re-analysed by LC-MS/MS.

5. CALIBRATION AND CALCULATION

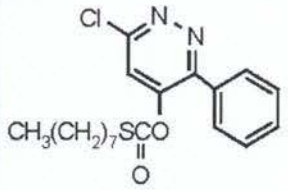
Calibration solutions were prepared in soil matrix for both analytes at seven concentrations ranging from 0.2 to 20 ng/mL, equivalent to residues of 0.002 to 0.2 mg/kg in soil.

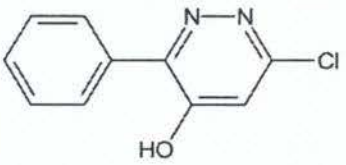
Multi-level calibration curves of the form $y = mx + c$ were obtained for each analyte. The calibration curves were constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression. The quantification of the analytes in the final sample extract was made by comparison to the calibration curve. The concentration in a sample extract and recovery efficiencies were calculated as detailed in Appendix 4.

Examples of LC-MS/MS chromatograms of calibration, control and fortified samples are presented in Figure 4 to Figure 7 with typical calibration curves in Figure 8.

10. FIGURES

Figure 1: Chemical Nomenclature, Structure and Batch Details of Reference Items

Common Name	Pyridate
Report Name	Pyridate
CAS and IUPAC Nomenclature	*O*-6-chloro-3-phenylpyridazin-4-yl *S*-octyl thiocarbonate
CAS Number	55512-33-9
Chemical Formula	$C_{19}H_{23}ClN_2O_2S$
Molecular Weight	378.9 g/mole
Structures	
Source	QMX Laboratories
Batch Number	80404
Purity	96.0 %
Expiry date	04 June 2012

Common Name	Pyridafol
Report Name	CL-9673
CAS and IUPAC Nomenclature	6-chloro-3-phenylpyridazin-4-ol
CAS Number	40020-01-7
Chemical Formula	$C_{10}H_7ClN_2O$
Molecular Weight	206.6 g/mole
Structure	
Source	QMX Laboratories
Batch Number	60105
Purity	98.0 %
Expiry date	01 January 2012

Appendix 6: Study Plan (continued)

1.5

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Study Director's Initials:

Study Plan

1. INTRODUCTION

Pyridate is a selective post-emergence contact herbicide, absorbed predominantly by the leaves, used as post-emergence control of annual broad-leaved weeds, especially *Solanum* spp., *Chenopodium* spp., *Galium aparine*, *Kochia scoparia* and *Amaranthus retroflexus*, and some grass weeds, in maize, sweet corn, oilseed rape, and vegetables. An analytical method to determine the residues of pyridate in soil therefore has to be developed to meet residue regulatory requirements.

2. OBJECTIVE

The objective of this study is to validate a method for the determination of residues of pyridate and its breakdown product, CL-9673 (pyridafol), in soil at an LOQ of 0.01 mg/kg.

3. JUSTIFICATION OF THE TEST SYSTEM

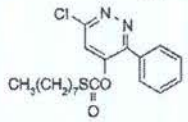
Control soil will be sourced from soil stocks held by the Test Facility and used for fortification purposes. The soil will be free from residues of pyridate and CL-9673.

4. PROPOSED TIMETABLE

Experimental start date:	August 2010
Experimental completion date:	September 2010
Draft report:	October 2010
Final report:	Within 3 weeks of receiving comments from sponsor.

5. TEST ITEMS

5.1 Active Substance

Common name:	Pyridate
Batch No.:	Will be recorded in raw data
Chemical name (IUPAC):	*O*-6-chloro-3-phenylpyridazin-4-yl *S*-octyl thiocarbonate
Molecular structure:	
CAS registry number:	55512-33-9
Molecular formula:	$C_{19}H_{23}ClN_2O_2S$
Molecular weight:	378.9
Physical form:	Colourless crystals
Storage Conditions	Ambient temperature, avoid direct sunlight and heat.

Appendix 6: Study Plan (continued)

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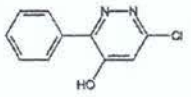
Study Director's Initials:

Study Plan

5.2 Metabolite

Code name:	CL-9673
Common name:	pyridafol
Batch no.:	Will be recorded in raw data
Molecular structure:	
Chemical name (IUPAC):	6-chloro-3-phenylpyridazin-4-ol
CAS RN:	40020-01-7
Molecular formula:	$C_{10}H_7ClN_2O$
Molecular Weight:	206.6
Storage Conditions	Ambient temperature, avoid direct sunlight and heat.

6. METHOD

The method will involve solvent extraction of residues of pyridate and CL-9673, one or more clean-up steps if required, followed by final determination by LC-MS/MS. Depending on the sensitivity of the detection system, sample extracts may require dilution or concentration prior to chromatographic injection. The analytical method will monitor at least two mass transitions for each compound. The limit of quantification (LOQ) is the lowest quantifiable level, which will be reported. The target LOQ is 0.01 mg/kg.

7. VALIDATION PARAMETERS

The analytical method shall be validated in terms of linearity, specificity, accuracy and precision.

The linearity will be investigated by analysing reference items (analytical standards), either duplicate determination at 3 or more concentrations or single determinations at 5 or more concentrations. The lowest point of the calibration curve shall correspond to 70% of the LOQ of the method or less, whilst the uppermost point will correspond to at least [10 x LOQ] + 20%. The equation of the calibration curve and the correlation coefficient will be reported.

A mass spectrum will be produced and two mass transitions selected. One mass transition will be used for quantification and the other will be used for confirmation purposes. No additional confirmatory chromatographic method is required.

Appendix 6: Study Plan (continued)

1-7

The accuracy and precision of the method will be will be assessed by spiking untreated samples of control soil with the test items as follows:

1. Two (unfortified) control samples per sample set
2. At least five replicate fortifications at the method LOQ (0.01 mg/kg)
3. At least five replicate fortifications at 10 times the LOQ (0.1 mg/kg)

The average recovery at each fortification level must be within the range of 70 to 110% of the known quantity of each analyte and the relative standard deviation (RSD) at each spiking level less than 20% for the validation set to be deemed acceptable. If the criteria are not obtained, an appropriate test (for example Dixon test) will be used to determine the outliers. A reagent blank will be included in one of the sequence sets.

Additional tests will be conducted to give an indication of the stability of the reference items in solution and in sample extracts.

8. MAINTENANCE OF RECORDS

All raw data and written records concerning the study will be part of study files established for the study. The study records will be maintained in accordance with Battelle UK's facility SOP for assembling study files and will include, but not necessarily be limited to, the following:

1. This study plan, and any study plan amendments or deviations
2. Test substance identification records, characterization records supplied by the sponsor, and receipt and inventory records
3. Study raw data
4. The final report and any amendments thereto

9. QUALITY ASSURANCE UNIT (QAU)

Battelle UK Quality Assurance Unit shall review the study plan, inspect the study and audit the final report.

10. GOOD LABORATORY PRACTICE

The study shall be conducted in accordance with the Principles of Good Laboratory Practice based on:

- Section II of Annex I to the European Parliament and Council Directive 2004/10/EC and Annex I to the European Parliament and Council Directive 2004/9/EC (Official Journal No. L 50) and embodied within:
- The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106) as amended by:
- The UK Good Laboratory Practice (Codification Amendments Etc.) Regulations 2004 (Statutory Instrument No 994)