2. INTRODUCTION

The objective of this study was to validate a method in terms of linearity, specificity, accuracy and precision for the determination of residues of Pyridate and CL-9673 in soil according to EU Directive 96/46/EC amending Directive 91/414/EEC, Annex II, Part A, Section 4 and EU Guidance Document SANCO/825/00 rev. 8.1 of 16/11/2010 and SANCO 3029/99 rev. 4 of 11/07/2000.

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 4ºC 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 $[C_7H_5\ N+H]^+$	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

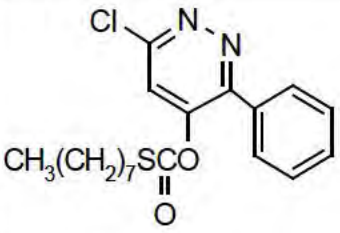
Calibration solutions were prepared in soil matrix for both analytes at seven concentrations ranging from 0.2 to 20 ng/mL, equivalent to residues of 0.002 to 0.2 mg/kg in soil.

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

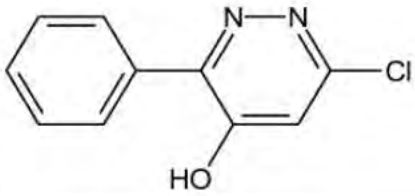
Common Name	Pyridafol
Report Name	CL-9673
CAS and IUPAC Nomenclature	6-chloro-3-phenylpyridazin-4-ol
CAS Number	40020-01-7
Chemical Formula	$C_{10}H_7ClN_2O$
Molecular Weight	206.6 g/mole
Structure	
Source	QMX Laboratories
Batch Number	60105
Purity	98.0 %
Expiry date	01 January 2012

Figure 3: Analytical Method Flow Chart

Ultra-Turrax method

EXTRACTION

- Weigh 10 g of homogenised sample into a 250 mL extraction bottle
- Fortify if necessary (allow solvent to evaporate)
- Add 100 mL of Methanol:Acetic Acid (100:0.5 v/v)
- Blend the sample with an Ultra-Turrax for 3 minutes (approx speed 11)
- Centrifuge the sample at 3000 rpm for 5 minutes

ANALYSIS

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

Shake Method

EXTRACTION

- Weigh 10 g of homogenised sample into a 250 mL extraction bottle
- Fortify if necessary (allow solvent to evaporate)
- Add 100 mL of Methanol:Acetic Acid (100:0.5 v/v)
- Shake the sample for 20 mins
- Centrifuge the sample at 3000 rpm for 5 minutes

ANALYSIS

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

Appendix 4: Calculation Formula

The concentration of analyte in fortified samples were calculated as follows:

$$\text{Analyte concentration} = \frac{[A - c] \times D \times V}{m \times W \times 1000} \text{ [mg/kg]}$$

Where:

A = analyte peak area
m = slope of the calibration curve
c = intercept of the calibration curve
D = dilution factor
V = extraction volume (mL)
W = sample weight (g)

The recovery efficiencies in the fortified samples were calculated as follows:

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