2.0 INTRODUCTION

The objective of this study was to independently validate Belchim Crop Protection NV/SA Method OZ/10/012, entitled "Pyridate and CL-9673: Determination of Residues of Pyridate and the Metabolite CL-9673 in Soil" [1], in terms of linearity, specificity, accuracy, and precision. This study was designed to fulfill the requirements of the US EPA Test Guidelines OCSPP 850.6100 [2]. In addition, this study was conducted in compliance with US EPA FIFRA (40 CFR Part 160) GLP standards [3].

3.0 MATERIALS AND METHODS

3.1 Reference Items

Information on the pyridate and CL-9673 reference items is presented in Figure 1 and certificates of analysis are presented in Appendix 1.

3.2 Test System

The test system used for the validation was an untreated control (UTC) soil sample (sample ID DU-L, kUSA sample number GS-18-47-1) provided by Agvise Laboratories, Inc., 604 Highway 15 West, P.O. Box 510, Northwood, North Dakota, 58267-0510, USA. The control soil used in the study was classified as clay loam with an organic matter content of 6.5% (Walkley Black). See Appendix 2 for detailed soil characteristics. The sample was pre-processed by sifting through a No. 10 2.00-mm-opening sieve and stored refrigerated until needed for analysis.

3.3 Principle of the Method

Untreated control soil samples and fortified controls were extracted with a 100:0.5 methanol:acetic acid (v/v) solution followed by shaking and centrifugation. Untreated control soil samples and fortified controls were also extracted with a 100:0.5 methanol:acetic acid (v/v) solution followed by homogenization and centrifugation. The concentration of pyridate and CL-9673 residues were subsequently determined by LC-MS/MS monitoring two ion transitions, the first transition for quantification and the second for confirmation.

3.4 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in Belchim Crop Protection NV/SA Method OZ/10/012, Reagents and Equipment. Identical or equivalent equipment and materials were used.

3.4.1 Equipment

- Thermo Scientific 250-mL wide mouth bottles, HDPE
- Volumetric flasks, glass class A (assorted volumes)
- Eppendorf® Research® Pro electronic pipettes (1000 µL)
- VWR® manual pipettor (5000 µL)
- Transferpette® manual pipettors (assorted volumes)
- Millipore Direct-Q® 5 water purification system
- Burrell Scientific, LLC, Wrist Action® Shaker, Model 75
- Homogenizer (IKA ULTRA-TURRAX® T25)
- A&D Company, Ltd FX200i top-loading balance
- Mettler Toledo AT261 analytical balance
- Fisher Scientific sieve, No.10, 2.00-mm opening
- Beckman Coulter Allegra® X-22R centrifuge
- Nor-Lake Scientific refrigerator/freezer
- SO-LOW refrigerator
- Brown walk-in freezer (-20°C)
- Phenomenex Aqua C18 50 mm × 2.0 mm, 5 µm particle size
- LC-MS/MS—Shimadzu Nexera X2 UPLC coupled to an AB Sciex® API 6500™ tandem mass spectrometer with an electrospray ionization interface and Analyst® software version 1.6.2 data collection software (AB Sciex®)
- Various general laboratory glassware and utensils

3.4.2 Reagents

- Water (ultrapure)
- Acetonitrile (OmniSolv®, HPLC grade)
- Methanol (Pharmco, HPLC grade)
- Acetic acid, glacial (Fisher Scientific, ACS grade)
- 100:0.5 methanol:acetic acid (v/v)
- Mobile phase A—99:1 water:acetic acid (v/v)
- Mobile phase B—99:1 acetonitrile:acetic acid (v/v)

3.5 Establishment of the Method

Prior to performing the independent laboratory validation (ILV), the analyte retention times, instrument detection sensitivity, and linearity of instrument responses to a range of analyte concentrations were determined, and the test system was verified as free of interferences at appropriate retention times.

3.6 Analytical Methodology

The analytical set consisted of 15 samples: one reagent blank, two UTC soil samples, seven UTC soil samples fortified with the test substances at the LOQ (0.0100 mg/kg), and five UTC soil samples fortified with the test substances at 10× LOQ (0.100 mg/kg). Each sample analysis batch also included an extraction solvent blank. For the shaking extraction of both analytes and the homogenization extraction of pyridate, seven calibration standard solutions prepared in matrix at concentrations ranging from 0.200 to 15.0 ng/mL were used. For the homogenization extraction of CL-9673, eight calibration solutions prepared in matrix at concentrations ranging from 0.200 to 20.0 ng/mL were used.

3.6.1 Stock and Working Standard Solutions

A stock solution of the pyridate reference item at a concentration of 973 µg/mL (corrected for purity) was prepared by dissolving 24.57 mg in 25 mL 100:0.5 methanol:acetic acid (v/v) using a volumetric flask. A separate stock solution containing the CL-9673 reference item at a concentration of 1010 µg/mL (corrected for purity) was prepared by dissolving 25.33 mg in 25 mL methanol using a volumetric flask. The stock solutions were stored in a freezer (approximately -18°C) and given a 2-month expiration date [1].

Working standard solutions containing the two reference items at 1.00 and 10.0 µg/mL were prepared by mixing and diluting appropriate amounts of the stock solutions with 100:0.5 methanol:acetic acid (v/v) in 10-mL volumetric flasks. The working standards were used for fortification purposes and to prepare calibration standards. They were stored in a refrigerator (approximately 4°C) when not in use and given a 10-day expiration date [1].

3.6.2 Sample Fortification and Extraction

The fortification and extraction steps described below were followed for each sample.

1. Samples (10.00 ± 0.01 g) of UTC soil were weighed into 250-mL HDPE bottles. (A reagent blank sample was prepared using a soil-free 250-mL HDPE bottle.)
2. Samples were fortified at the LOQ level by pipetting 0.100 mL of the 1.00 µg/mL working standard solution onto the soil. Samples were fortified at the 10× LOQ level by pipetting 0.100 mL of the 10.0 µg/mL working standard solution onto the soil. A 0.100-mL aliquot of 100:0.5 methanol:acetic acid (v/v) was added to the reagent blank and UTC samples.
3. The solvent was allowed to evaporate before 100 mL of 100:0.5 methanol:acetic acid (v/v) solution was added to each sample.
4. One set of samples was shaken vigorously on a Wrist Action® Shaker for 20 minutes (shaking extraction) and a second set of samples was homogenized with a polytron for 5 minutes at high speed (homogenization extraction).
5. Samples were centrifuged for 10 minutes at 3600 rpm.
6. Aliquots of the extracts were then transferred into glass vials for analysis by LC-MS/MS.

3.6.3 Calibration Standard Solutions

Calibration standard solutions were prepared in UTC soil extraction solvent matrix at concentrations of 0.200, 0.500, 1.00, 2.00, 5.00, 7.50, 10.0, 15.0, and 20.0 ng/mL of pyridate and CL-9673 using the following dilution scheme. Except for the homogenization extraction of CL-9673, the 15.0 ng/mL standard was the highest standard used in the calibration curve for achieving the acceptable correlation coefficient value. Calibration standard solutions were stored in a freezer (approximately -18°C) and given a 5-day expiration date [1].

Calibration Standard Solution Preparation

Calibration Standard Solution ID	Conc. of Stock Solution Used for Dilution (ng/mL)	Stock Solution ID	Standard Amount (mL)	Final Volume (mL)	Diluent	Final Calibration Standard Solution Conc. (ng/mL)
CS-0331-08	1000	FS-0331-02	0.100	5.0	UTC soil extract	20.0
CS-0331-07	20.0	CS-0331-08	0.750	1.00	UTC soil extract	15.0
CS-0331-06	20.0	CS-0331-08	0.500	1.00	UTC soil extract	10.0
CS-0331-05	20.0	CS-0331-08	0.250	1.00	UTC soil extract	5.00
CS-0331-04	20.0	CS-0331-08	0.100	1.00	UTC soil extract	2.00
CS-0331-03	20.0	CS-0331-08	0.050	1.00	UTC soil extract	1.00
CS-0331-02	20.0	CS-0331-08	0.0250	1.00	UTC soil extract	0.500
CS-0331-01	20.0	CS-0331-09	0.010	1.00	UTC soil extract	0.200

3.7 LC-MS/MS Analysis

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

3.7.1 Instrument Description

Pump	Shimadzu 1200 LC-30AD
Degasser	Shimadzu DGU-20A5R
Column Oven	Shimadzu CTO-30A
Autosampler	Platform CTC Analytics HTS-xt
Detector	AB Sciex® API 6500™ LC-MS/MS tandem mass spectrometer
Software	AB Sciex® Analyst® software version 1.6.2

3.7.2 Liquid Chromatography Conditions

Column	Phenomenex Aqua C18, 50 mm × 2 mm, 5 μm particle size		
Column Temperature	Ambient		
Mobile Phase A	99:1 water:acetic acid (v/v)		
Mobile Phase B	99:1 acetonitrile:acetic acid (v/v)		
Gradient	Time (min.)	%A	%B
	0.00	90.0	10.0
	0.20	90.0	10.0
	1.00	10.0	90.0
	2.00	10.0	90.0
	2.65	90.0	10.0
	4.00	90.0	10.0
Flow Rate	1000 μL/min.		
Injection Volume	20.0 μL		

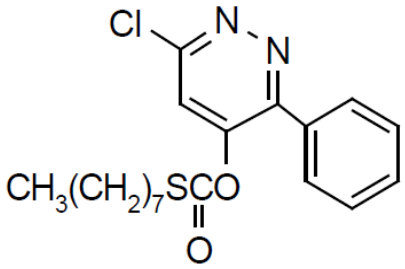
The retention times of pyridate and CL-9673 were approximately 1.74 and 0.93 minutes, respectively.

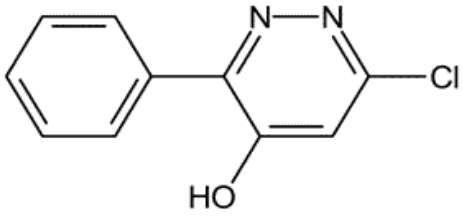
3.7.3 Mass Spectrometry Conditions

Ion Source	Turbo Spray (electrospray ionization)			
Polarity	Positive			
Curtain Gas (CUR)	30 (arbitrary units)			
Temperature (TEM)	600°C			
Ion Transfer Voltage (IS)	5500 V			
Collision Gas Cell (CAD)	12.0 (arbitrary units)			
Nebulizer Gas (GS1)	70 (arbitrary units)			
Turbo Gas (GS2)	60 (arbitrary units)			
Interface Heater (ihe)	On			
Scan Type	MRM			
MRM Conditions	Pyridate Transition 1 $[M+H]^+$ to $[C_3HNO+H]^+$	Pyridate Transition 2 $[M+H]^+$ to $[C_4HN_2]^+$	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	379.0	379.0	207.1	207.1
Q3 m/z	77.0	68.0	68.0	104.0
Dwell Time	100	100	100	100
Resolution Q1	Unit	Unit	Unit	Unit
Resolution Q3	Unit	Unit	Unit	Unit
Declustering Potential (DP)	55	55	55	55
Entrance Potential (EP)	10	10	10	10
Collision Energy (CE)	75	93	51	29
Collision Cell Exit Potential (CXP)	14	8	8	12

The transitions for CL-9673 are reversed based on the method provided in Appendix 7. However, satisfactory accuracy and precision data were achieved for both transitions using both extraction methods, thus demonstrating that either transition can be used for quantification or confirmation purposes with either method.

Figure 1 Analytical Reference Standard Details

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	378.92 g/mole
Reference Substance Lot No.	BCBV3044 (Sigma-Aldrich)
kUSA ID No.	18-CPS-Mar08-01
GLP Purity	99.0%
Expiration Date	April 2022
Storage Conditions	Refrigerated
Structure	

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
Reference Substance Lot No.	Cl-9673-1103001 (Ibacon)
CPS ID No.	15-CPS-Jul17-03
GLP Purity	99.7%
Expiration Date	September 2018
Storage Conditions	Stored for 5 days refrigerated then moved ambient temperature, under dark and dry conditions
Structure	

Appendix 3 Calculations

The concentrations of analytes 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:

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