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. Details of the GLP-certification of the pyridate reference material are documented in the raw data.

3.2 Test System

The test system used for the validation was an untreated control (UTC) soil sample (sample ID RS295, CPS sample number GS-15-32-3) provided by Southeast Ag Research, Inc., 86 Jim Moore Road, Chula, Georgia. See Appendix 2 for detailed soil characteristics. The sample was pre-processed using a Hobart sample processor and stored frozen 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. 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 (various volumes)
- VWR® manual micropipettor (20 µL)
- Millipore Direct Q® 5 water purification system
- Burrell Scientific, LLC Wrist Action® Shaker, Model 75
- A&D Company, Ltd FX200i top-loading balance
- Mettler Toledo AT261 top-loading balance
- Hobart sample processor, Model 84145
- Beckman Coulter Allegra® X-22R centrifuge
- Thermo Scientific Heraeus® Megafuge® 11R centrifuge
- Desiccator
- Nor-Lake Scientific refrigerator/freezer
- Carroll Coolers Inc. walk-in freezer (-20 °C)
- Thermo Scientific ODS Hypersil™, 250 mm × 2.1 mm, 5 µm particle size
- Phenomenex Synergi™ Hydro-RP column, 50 mm × 2 mm, 4 µm particle size
- LC-MS/MS—Agilent Technologies 1200 series HPLC coupled to an API 4000™ tandem mass spectrometer with an electrospray ionization interface and Analyst® 1.6.2 data collection software (AB Sciex™)
- Various general laboratory glassware and utensils

3.4.2 Reagents

- Water (ultrapure)
- Acetonitrile (EMD Millipore, HPLC grade)
- Methanol (BDH Chemicals, HPLC grade)
- Methanol (EMD Millipore, LC-MS grade)
- Acetic acid, glacial (Fisher Scientific, ACS grade)
- 10:90 water:acetonitrile (v:v)
- 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 soil used in the study was a UTC soil sample (sample ID RS295, CPS sample number GS-15-32-3) provided by Southeast Ag Research, Inc., 86 Jim Moore Road, Chula, Georgia. The soil was pre-processed using a Hobart sample processor and stored frozen (approximately -18°C) until needed for analysis.

The analytical set consisted of 13 samples: one reagent blank, two UTC soil samples, five 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 and calibration standard solutions prepared in matrix at seven concentrations ranging from 0.200 to 20.0 ng/mL.

3.6.1 Stock and Working Standard Solutions

A stock solution of the pyridate reference item at a concentration of 1050 µg/mL (corrected for purity) was prepared by dissolving 26.52 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 1000 µg/mL (corrected for purity) was prepared by dissolving 25.28 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 as per CPS standard operation procedure (SOP).

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 100-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.

3.6.2 Sample Fortification and Extraction

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

1. Samples (10.00 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. Samples were shaken vigorously on a Wrist Action® Shaker for 20 minutes.
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, 10.0, and 20.0 ng/mL of pyridate and CL-9673 using the following dilution scheme. Calibration standard solutions were stored in a freezer (approximately -18°C) and given a 5-day expiration date.

Calibration Standard Solution Preparation

Calibration Standard Solution ID	Conc. of Native Standard Solution Used for Dilution (µg/mL)	Native Standard Solution ID	Native Standard Amount (mL)	Final Volume (mL)	Diluent	Final Calibration Standard Solution Conc. (ng/mL)
CS-1	1.00	WS-2 (working standard)	0.200	10.0	UTC soil solvent extract	20.0
CS-2	0.0200	CS-1	0.500	1.00	UTC soil solvent extract	10.0
CS-3	0.0200	CS-1	0.250	1.00	UTC soil solvent extract	5.00
CS-4	0.0100	CS-2	0.200	1.00	UTC soil solvent extract	2.00
CS-5	0.00500	CS-3	0.200	1.00	UTC soil solvent extract	1.00
CS-6	0.00200	CS-4	0.250	1.00	UTC soil solvent extract	0.500
CS-7	0.00100	CS-5	0.200	1.00	UTC soil solvent 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	Agilent 1200 series binary pump model number G1312A
Degasser	Agilent 1200 series model number G1379B
Column Oven	Agilent 1200 series model number G1316A
Autosampler	Agilent 1200 series model number G1367B
Detector	AB Sciex™ API 4000™ LC-MS/MS tandem mass spectrometer
Software	AB Sciex™ Analyst® version 1.6.2

3.7.2 Liquid Chromatography Conditions

Column	Phenomenex Synergi™ Hydro-RP, 50 mm × 2 mm, 4 µm particle size		
Column Temperature	27.00°C		
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	10.0 µL		

The retention times of pyridate and CL-9673 were approximately 2.7 and 1.6 min., respectively.

3.7.3 Mass Spectrometry Conditions

Ion Source	Turbo Spray (electrospray ionization)			
Polarity	Positive			
Curtain Gas [CUR]	30 (arbitrary units)			
Temperature [TEM]	550 °C			
Ion Transfer Voltage [IS]	5000 V			
Collision Gas Cell [CAD]	6.00 (arbitrary units)			
Nebulizer Gas [GS1]	55 (arbitrary units)			
Turbo Gas [GS2]	55 (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 $[C_4HN2]^+$	CL-9673 Transition 1 $[M+H]^+$ to $[C_7H_5N+H]^+$	CL-9673 Transition 2 $[M+H]^+$ to $[C_3HNO+H]^+$
Q1 m/z	379.3	379.3	207.3	207.3
Q3 m/z	77.2	68.1	104.1	68.0
Dwell Time	100	100	100	100
Resolution Q1	Unit	Unit	Unit	Unit
Resolution Q3	Unit	Unit	Unit	Unit
Declustering Potential [DP]	50	50	50	50
Entrance Potential [EP]	10	10	10	10
Collision Energy [CE]	70	70	28	45
Collision Cell Exit Potential [CXP]	10	10	22	16

3.8 Data Acquisition and Reporting

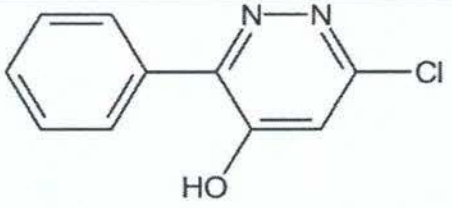
Peak integration was performed using Analyst® software version 1.6.2. The MS detector responses (peak areas) for various injected standard concentrations were used to generate an external calibration curve for each analyte. The calibration curves were constructed by plotting peak area of each level versus its concentration in ng/mL. The curve was calculated by the method of least squares linear regression.

The concentration in a sample extract was calculated by multi-point calibration curves of the form $y = mx + c$ (no weighting). The equations used for quantification and determination of recovery efficiency are presented in Appendix 3. A statistical treatment of the data included the calculation of means, standard deviations (SDs), relative standard deviations (RSDs) as percentages (%), and the 95% confidence intervals. All statistics were calculated using Microsoft® Excel® 2010.

Typical calibration curves are presented in Figure 2 to Figure 5, with example LC-MS/MS chromatograms of calibration, control, and fortified samples in Figure 6 to Figure 9.

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.	SZBC284XV
CPS ID No.	15-CPS-Jul17-02
GLP Purity	99.4%
Expiration Date	10 October 2017
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
CPS ID No.	15-CPS-Jul17-03
GLP Purity	99.2%
Expiration Date	September 2018
Storage Conditions	Stored for 5 days refrigerated then moved ambient temperature, under dark and dry conditions
Structure	

CPS Study Number: 15-CPS-014

1.0 EPA REQUIREMENTS

1.1 US EPA Test Guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation.

1.2 US EPA Test Guideline OPPTS (OCSPP) 860.1340(c)(6): Residue Analytical Method.

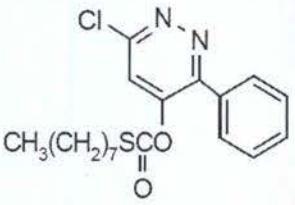
2.0 INTRODUCTION

2.1 The objective of this study is to perform an independent laboratory validation (ILV) of Belchim Method OZ/10/012- Pyridate and CL-9673: Determination of Residues of Pyridate and the Metabolite CL-9673 in Soil

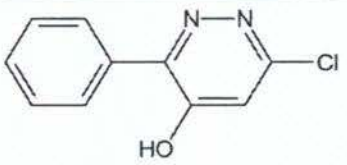
2.2 The laboratory personnel, including the Study Director, have had no prior laboratory experience with Belchim Method OZ/10/012- Pyridate and CL-9673: Determination of Residues of Pyridate and the Metabolite CL-9673 in Soil. This study is designed to fulfill the requirements of EPA OCSPP Guideline 850.6100. In addition, this study will be conducted in compliance with EPA FIFRA Good Laboratory Practice (GLP) Standards, 40 CFR Part 160.

3.0 TEST MATERIALS

3.1 Analytical Reference Standards

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
Reference Substance Lot No.	SZBC284XV
GLP Purity	To be determined. Will be recorded in raw data.
Expiration Date	10 Oct 2017
Storage Conditions	Refrigerated
Structure	

CPS Study Number: 15-CPS-014

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
GLP Purity	99.2%
Expiration Date	01 Sep 2018
Storage Conditions	Room temperature (20°C ± 5°C), under dark and dry conditions
Structure	

3.1.1 The reference standards will be supplied by Belchim Crop Protection. Methods of synthesis, fabrication, and/or derivation of the reference standards are maintained by Belchim Crop Protection.

3.1.2 Characterization data of this material will be archived at Belchim Crop Protection NV/SA, Technologielaan 7, B-1840 Londerzeel, Belgium. CPS may retain a copy of data for any standards characterized at CPS.

3.1.3 Any reference substances that have not been GLP-certified will be certified according to applicable CPS standard operating procedures (SOPs) and documented in the raw data.

3.1.4 A record of storage conditions, weights, and dilutions of the reference standards will be maintained and checked.

3.1.5 Safety data sheets (SDSs) or other information necessary for proper and safe handling, shipping, and storage of the test materials will be kept on file at CPS.

3.2 Reagents and Materials

3.2.1 All reagents and materials used will be the same as, or equivalent to, those specified in the method. Chemicals (grade and supplier) and materials will be documented in the raw data.

4.0 EXPERIMENTAL DESIGN

4.1 Establishment of the Method

4.1.1 Prior to performing the ILV, it will be necessary to establish the method (e.g., determine analyte retention times, instrument detection limits, and

CPS Study Number: 15-CPS-014

linearity of instrument responses to a range of analyte concentrations) and verify that the test system is free of interferences at appropriate retention times. In general, CPS will demonstrate that the method is under control before initiating the ILV.

4.1.2 Prior to the ILV trial, clarification of the method will be provided by the Sponsor/Study Monitor if requested by the Study Director. All contacts made during the establishment of the method will be documented and presented in the final report.

4.2 Test System

4.2.1 The test system used for the validation will be untreated soil provided by ARCADIS U.S., Inc. 3522 Thomasville Road, Suite 200, Tallahassee, FL 32309. Pre-processing (grinding and homogenization) of the soil sample will be conducted by CPS using a Hobart food cutter in the presence of dry-ice.

4.3 Sample Identification

4.3.1 Each portion of sample used for the validation will be assigned a unique code number/label within the analytical set during preparation and analysis. At minimum, documentation will also associate all samples with the study number and sample type.

4.4 Validation Set

4.4.1 One set of samples is defined as one reagent blank, two untreated control samples (UTCs), five UTCs fortified with each analyte at the limit of quantitation (LOQ: 0.01 mg/kg), and five UTCs fortified with each analyte at 10 × LOQ (0.1 mg/kg). Fortifications will be made by the addition of a diluted standard into the control matrix via pipette.

5.0 METHOD PERFORMANCE AND DATA EVALUATION

5.1 No changes or modifications to the method are permissible unless indicated in the method, this protocol, or brought about by clarification of the method with the Sponsor/Study Monitor. In case of a failed trial, the Sponsor/Study Monitor will be notified before another trial is attempted. Any modifications of the method after the first validation trial must be pre-approved by the Sponsor/Study Monitor and documented in the raw data and in the final report. The validation will be considered acceptable if the mean recovery at each spiking level at or above the LOQ is between 70% and 120%. The control matrix should be essentially free of any interference at the retention time of the pyridate and CL-9673.

5.2 The recoveries will be expressed as a percentage of the analyte concentrations determined relative to the concentrations added. The mean and individual values for recoveries, the standard deviations and relative standard deviations (RSDs) for fortified samples at each fortification level, and the 95% confidence intervals for the recoveries at each fortification level will be reported. The RSD of replicate measurements should not exceed 20% for each fortification level at or above the LOQ.

CPS Study Number: 15-CPS-014

5.3 For each test system, a maximum of three validation trials may be performed. Results of the first validation trial will be reviewed by the Study Director and the Sponsor/Study Monitor. If the results are determined to be acceptable, a final report will be written. If the validation trial is unsuccessful, the Study Director and the Sponsor/Study Monitor will consult with the Method Developer to clarify directions given in the method. This communication will be documented and presented in the final report. A second set of validation samples will then be analyzed.

5.4 If a second validation trial is conducted, the results will be reviewed by the same personnel. If the results are determined to be acceptable, a final report will be written. If the second validation trial is unsuccessful, the same process of consultation with the Method Developer will take place to further clarify directions given in the method. This communication will also be documented and presented in the final report. A third set of validation samples will then be analyzed.

5.5 If a third validation trial is conducted, the results will be reviewed by the same personnel. If the results are acceptable, a final report will be written. If the third validation trial is unsuccessful, method validation will be terminated, and a final report will be written.

6.0 GENERAL RECORDS AND REPORTING

6.1 Instruments and equipment that generate raw data or significantly impact the validity and results of the study should be maintained in proper working order. A logbook will be used to record maintenance, inspection, cleaning, and calibration of such equipment.

6.2 The Sponsor/Study Monitor will be informed of progress at appropriate intervals.

6.3 The draft study report will be submitted to the Sponsor/Study Monitor for review prior to finalization.

6.4 The final study report will be completed in EPA PRN 11-03 format.

6.5 The final study report will be submitted following the quality assurance audit.

7.0 DATA RETENTION

7.1 A copy of the protocol and final report will be retained in the CPS company archives.

7.2 All records necessary to support the study and to allow for reconstruction of the study, including the original report and all primary data and study records, will be shipped to Belchim Crop Protection and will include, but not be limited to the following.

- A record of all standard operating procedures (SOPs) and protocol deviations.
- A list of all personnel involved with the study conduct.
- An exact copy or original of all applicable correspondence.
- An exact copy or original of the analytical method.
- Reference substance purity and preparation records.
- All laboratory raw data, including coversheets, chromatograms, and spreadsheets.

CPS Study Number: 15-CPS-014

7.3 Laboratory-specific or site-specific raw data such as personnel files and equipment records will be retained at CPS.

8.0 QUALITY ASSURANCE

8.1 This study will be conducted in accordance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160.

8.2 Unless otherwise advised, all procedures will be performed in accordance with CPS SOPs.

8.3 Conduct, study records, and final report audits will be performed.

8.4 Changes to the protocol will be made by amendments approved by the Study Director and Sponsor/Study Monitor.

8.5 Deviations from company and departmental procedures will be documented in the study records and will be approved by the Study Director and Sponsor/Study Monitor.

8.6 An official copy of the protocol will be archived by the CPS Quality Assurance Department.