INTRODUCTION

The objective of this study was to independently validate the Ricerca BioSciences, LLC residue method 024119-2 for the determination of fenazaquin, 2-oxy-fenazaquin and 4-hydroxyquinazoline (4-OHQ) in soil. The method was found to be suitable for the determination of fenazaquin in soil over the concentration range 0.01 µg/g to 0.10 µg/g with a validated limit of quantitation (LOQ) of 0.01 µg/g. This independent laboratory validation was conducted to satisfy the requirements of the European Council Directive 91/414/EEC, as amended by European Commission Directive 96/46/EC, and the European Commission Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 7 (2, 3, 4). The study was also conducted to satisfy the requirements of U.S. EPA Guideline OPPTS 860.1340 (5), and PR Notice 96-1 (6). This validation report presents the results of the independent laboratory validation for fenazaquin, 2-oxy-fenazaquin and 4-OHQ in soil.

The independent laboratory, the Study Director, and the analyst chosen to conduct the ILV were unfamiliar with the method, both in its development and subsequent use in analyzing soil samples. The independent laboratory used all of its own equipment and supplies, so that there was no common link between the Sponsor or Ricerca BioSciences, LLC and the Study Director or analyst. Throughout the conduct of the study, any communications between the Sponsor or Ricerca BioSciences, LLC and the Study Director and/or the analyst were logged for inclusion in the report. No one from the Sponsor or Ricerca BioSciences, LLC was allowed to visit the independent laboratory during the ILV trial to observe, offer help, or assist the chemists or technicians. These steps successfully maintained the integrity of the ILV study.

ANALYTICAL

Sample Receipt, Labeling and Storage

One shipment of untreated control sample was received on July 1, 2010 from Ricerca BioSciences, LLC, frozen and in good condition. Upon receipt, the control sample was assigned unique master logbook (MLB) number 21716925-01 and stored frozen (approximately -20°C).

Preparation of Solutions and Standards

The analytical reference standards/test substances utilized during the independent laboratory method validation (ILV) are summarized below. The reference standard was received from the Sponsor and stored as indicated. The Certificate of Analysis is included in Appendix A.

Standard	**Percent Purity**	**Recertification Date**	**Lot Number**	**Storage Conditions**
Fenazaquin	99.92	May 2017	H29803 0416	Ambient
2-Oxy- Fenazaquin	100	23Sep2010	092209	Frozen
4-Hydroxyquinazoline (4-OHQ)	100	23Sep2011	10116012	Ambient

Standard solutions and calibration standard solutions were prepared as described below and stored frozen (approximately -20°C) when not in use.

Stock solutions were prepared in acetonitrile at a nominal concentration of 500 and 1000 µg/mL:

Name	Solution Type	Solution Lot Number	Weight [g]	Dissolve In [mL]	Obtain [µg/mL]*
Fenazaquin	Stock	N691P22	0.0532	50.0	1060
2-Oxy-Fenazaquin	Stock	N691P11-A	0.0247	50.0	494
4-OHQ	Stock	N691P11-B	0.0495	50.0	990

*Resulting concentrations after correcting for purity

Fenazaquin intermediate and fortification solutions were prepared in acetonitrile:

From Solution Lot Number	Conc. [µg/mL]	Pipette [mL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N691P22	1060	0.944	100	10.0	N691P23-A
N691P22	1060	0.472	100	5.00	N691P23-B
N691P23-A	10.0	1.000	100	0.100	N691P24-A
N691P23-A	10.0	5.000	100	0.500	N691P18-B

Fenazaquin calibration standards were prepared in acetonitrile:

Concentration of Stock Solution [µg/mL]	Aliquot of Stock Solution [mL]	Final Solution Volume [mL]	Calibration Solution Final Concentration [ng/mL]
0.100	20.0	200	10.0
0.100	10.0	200	5.00
0.100	4.00	200	2.00
0.100	2.00	200	1.00
0.100	1.00	200	0.500

2-oxy-fenazaquin intermediate and fortification solutions were prepared in acetonitrile:

From Solution Lot Number	Conc. [µg/mL]	Pipette [mL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N691P11-A	494	2.025	100	10.0	N691P17-A
N691P11-A	494	1.012	100	5.00	N691P17-B
N691P17-A	10.0	1.000	100	0.100	N691P18-A
N691P17-A	10.0	5.000	100	0.500	N691P18-B

2-oxy-fenazaquin calibration standards were prepared in acetonitrile:

Concentration of Stock Solution [µg/mL]	Aliquot of Stock Solution [mL]	Final Solution Volume [mL]	Calibration Solution Final Concentration [ng/mL]
0.100	20.0	200	10.0
0.100	10.0	200	5.00
0.100	4.00	200	2.00
0.100	2.00	200	1.00
0.100	1.00	200	0.500

4-OHQ intermediate and fortification solutions were prepared in acetonitrile:

From Solution Lot Number	Conc. [µg/mL]	Pipette [mL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N691P11-B	1010	1.010	100	10.0	N691P12-A
N691P11-B	1010	5.050	100	50.0	N691P12-B
N691P12-B	50.0	5.000	50.0	5.00	N691P16-B
N691P12-A	10.0	1.000	100	0.100	N691P13-A
N691P12-A	10.0	5.000	100	0.500	N691P13-B

4-OHQ calibration standards were prepared in acetonitrile:

Concentration of Stock Solution [µg/mL]	Aliquot of Stock Solution [mL]	Final Solution Volume [mL]	Calibration Solution Final Concentration [ng/mL]
10.0	20.0	200	1000
10.0	10.0	200	500
10.0	4.00	200	200
10.0	2.00	200	100
10.0	1.00	200	50.0

Fortification of Recovery Samples

The ILV trial of the method was performed for fenazaquin, 2-oxy-fenazaquin and 4-OHQ in soil. The trial was comprised of one set, which consisted of the following samples:

1 (one) reagent blank (containing no matrix or analyte)

1 (one) reagent blank spike at the LOQ level

2 (two) unfortified control samples

5 (five) control samples fortified at 0.01 µg/g, the LOQ of the method

5 (five) control samples fortified at 0.10 µg/g, or 10×LOQ

For preparation of recovery control specimens, appropriate volumes of the fortification standards were added as indicated below:

Specimen Portion	Nominal Target Fortification Level	Aliquot of Fortification Solution [mL]	Fortification Solution Concentration [µg/mL]
50 g	0.01 µg/g	1.00	0.500
	0.10 µg/g	1.00	5.00

Sample Extraction, Purification and Analysis

The ILV trial was conducted as described in the Ricerca BioSciences, LLC residue analytical method entitled: "Analytical Method Report for the Analysis of Fenazaquin, 2-Oxy-Fenazaquin and 4-OHQ in Soil," method number 024119-2 (1).

The samples (50 g) were extracted with acetonitrile followed by a second extraction with 50/50 acetonitrile/0.1N NaOH (v/v).

The sample extract for the analysis of fenazaquin and 2-oxy-fenazaquin was either analyzed directly by HPLC employing mass spectrometric detection (LC/MS/MS) or diluted as needed.

For the analysis of 4-OHQ, a 160-mL aliquot (equivalent to 20 g soil) of the pooled filtered extract was concentrated to the aqueous layer (approximately 40 mL) using rotary evaporation. The aqueous layer was adjusted to a pH of 5-6 with concentrated HCl, and then 5 mL of a K-PO4 buffer solution (pH 7.0) was added to achieve a pH of approximately 7.0. The sample was transferred to a 250-mL separatory funnel containing 2.5 g NaCl. The sample was shaken to dissolve the NaCl and then partitioned once with 100 mL of methylene chloride (DCM) followed by a second partitioning with 50 mL DCM. The DCM layers were pooled and then dried over Na_2SO_4. The combined dry DCM extract was concentrated to dryness by rotary evaporation. The sample residue was dissolved in 2 mL of acetonitrile and then diluted as needed or analyzed by LC/MS/MS.

For more specific details, refer to the analytical method (1).

Analytical Instrumentation and Equipment

Prior to initiation of the first ILV trial, the independent laboratory conducted preliminary studies necessary for establishing acceptable performance of the extraction and chromatographic instrumentation supplied by the method. These preliminary studies established that adequate retention times of the analytes and detector sensitivity could be achieved. The prepared standards that were used were also used throughout the remainder of the study. The following instruments and equipment were utilized in the conduct of the independent laboratory validation of the residue analytical method:

Instrumentation, Fenazaquin and 2-Oxy Fenazaquin

Typical HPLC Conditions

Instrument:	MDS/Sciex API 3000 LC/MS/MS System
Column:	Phenomenex Synergi Hydro RP 80, 4 µm, 2.0 × 50 mm
Temperature:	Ambient
Injection Volume:	10 µL
Run Time:	7.0 minutes
Mobile Phase:	A: Water with 0.1% formic acid
	B: Acetonitrile with 0.1% formic acid
Flow Rate:	350 µL/min

Gradient:

Time, min	Solvent A, %	Solvent B, %
0.0	90	10
0.6	5	95
5.0	5	95
5.1	90	10
7.0	90	Stop

Typical MS Conditions

Mass Spectrometer: Applied Biosystems API 3000 Mass Spectrometer

Detector Mode: Positive-ion electrospray

Source Temperature: 450°C

Ions Monitored:

Analytes:	Precursor Ion Q1	Product Ion Q3	Declustering Potential V	Collision Energy V	Retention Time (+/- 0.3min.)
Fenazaquin	307.3	161.3	30	22	2.20
2-Oxy Fenazaquin	323.4	161.2	50	20	3.20

Instrumentation, 4-OHQ

Typical HPLC Conditions

Instrument: MDS/Sciex API 3000 LC/MS/MS System

Column: Phenomenex Synergi Hydro RP 80, 4 µm, 2.0 × 150 mm

Temperature: Ambient

Injection Volume: 1 µL

Run Time: 7.5 minutes

Mobile Phase: A: Water with 0.025% acetic acid

B: Acetonitrile with 0.025% acetic acid

Flow Rate: 350 µL/min

Gradient:

Time, min	Solvent A, %	Solvent B, %
0.0	90	10
1.1	5	95
4.0	5	95
4.1	90	10
7.5	90	Stop

Typical MS Conditions

Mass Spectrometer: Applied Biosystems API 3000 Mass Spectrometer
Detector Mode: Negative-ion electrospray
Source Temperature: 550°C
Ions Monitored:

Analytes:	Precursor Ion Q1	Product Ion Q3	Declustering Potential V	Collision Energy V	Retention Time (+/- 0.3min.)
4-OHQ	145.0	102.0	-90	-30	3.30

Equipment

Top Loading Balance, Sartorius, model number Basic BA2100S, serial number 20303446
Analytical Balance, Sartorius, model number AC 120S, serial number 20103137
Cavitator ultrasonic cleaner (sonicator), model 4.6, serial number 85M16531
Centrifuge, Beckman, model number X12R, serial numbers ALX04DE6
Reciprocating shaker, model number 3520, serial number 208
Vortexer, Multi-Tube, model number VWR VX-2500, serial number 5130
Rotary Evaporator, model number R110, Buchi
Rotary Evaporator Water Bath model number 1142365,178374,143516, Buchi
Thermometer

Materials

Volumetric flasks, various sizes
Glass Class A pipettes, various sizes
Adjustable Pipettes, various sizes
250ml HDPE bottles
pH indicator strips, pH 0-14
500 and 250mL Boiling Flasks
250mL Separatory Funnels
500mL graduated mixing cylinders

Chemicals

Acetonitrile, HPLC grade, lot number PB002544ACN, Pharmco-AAPER
Acetonitrile, HPLC grade, lot number PB002974ACN, Pharmco-AAPER
Formic Acid, HPLC grade, lot number 82000, Fluka
Sodium Hydroxide, ACS Grade, lot number H21K51, JT Baker
Sodium Sulfate, USP grade, lot number 63811, VWR
Sodium Chloride, ACS Grade, lot number 46354704, EMD
Methylene Chloride, HPLC grade, lot number PB002693HPLC, Pharmco-AAPER
Potassium Phosphate Monobasic (KH_2PO_4) ACS Grade, lot number E16R040 Alfa Aesar
Potassium Phosphate Dibasic (K_2HPO_4) ACS Grade, lot number 10114579 Alfa Aesar
Hydrochloric Acid, ACS Grade, lot number 47016, EMD
Water, HPLC Grade, lot number 50090

Calculations

Calculations were not modified from the original analytical method. Using the calibration curve calculated by linear regression with 1/x weighting, the calculated analyte concentration in the sample extracts in ng/mL was calculated using Equation 1:

$$y = (CC)(m) + b \quad (1)$$

Where:

CC	=	Calculated analyte concentration in ng/mL
y	=	Analyte response, peak area
m	=	Slope, calculated by the Analyst® software program
b	=	y-intercept, calculated by the Analyst ® software program

Equation (1) was rearranged to solve for the analyte concentration:

$$CC = \frac{(y - b)}{m} \quad (2)$$

The calculated fenazaquin and 2-oxy fenazaquin concentrations found in the sample extracts in ng/mL were converted to sample analyte concentrations in µg/g using Equation 3:

$$AC = \frac{(CC \times V_i \times DF)}{W} \times \frac{1\,\mu g}{1000\,ng} \quad (3)$$

Where:

AC	=	Fenazaquin or 2-oxy fenazaquin concentration in µg/g
CC	=	Calculated concentration in ng/mL
V_i	=	Volume of initial extract (400 mL)
DF	=	Dilution Factor
W	=	Weight (g)

The calculated 4-OHQ concentration found in the sample extracts in ng/mL was converted to sample analyte concentration in µg/g using Equation 4:

$$AC = \frac{(CC \times V_f \times V_i \times DF)}{(W \times V_e)} \times \frac{1\,\mu g}{1000\,ng} \quad (4)$$

Where:

AC	=	Analyte concentration in µg/g
CC	=	Calculated analyte concentration in ng/mL
V_f	=	Volume of final partitioned extract (2.00 mL)
V_i	=	Volume of initial extract (400 mL)
DF	=	Dilution Factor
W	=	Weight (g)
V_e	=	Volume of extract partitioned (160 mL)

The percent recovery of the fortified samples was calculated using Equation 5:

$$\%\,\text{Recovery} = \frac{(\text{AC} - \text{Average AC}_{\text{UTC}})}{\text{FC}} \times 100 \qquad (5)$$

Where:

AC = Analyte concentration in µg/g
AC_{UTC} = Analyte concentration in µg/g in the UTC sample
FC = Concentration fortified (µg/g)

As an example, the LOQ quality control sample, Pyxant ID P2173B01-005 (Figure 13) resulted in a fenazaquin recovery of 93.4%. The calculations for this sample are demonstrated below as a representative example of how all the sample results for fenazaquin and 2-oxy fenazaquin were calculated for this study.

The linear regression analysis of the calibration curve used in the analysis of fenazaquin residues in soil samples from Trial 1 was determined to have the following regression coefficients: m = 39770 and b = 1623.3 (Table 4, Figure 1). The analyte peak area (y) was 47564; therefore the concentration of fenazaquin in the final extract of this sample was calculated using Equation 2:

$$\text{CC} = \frac{(47564 - 1623.3)}{39770} = 1.155\ \text{ng/mL} \qquad (2)$$

The concentration of fenazaquin found in the sample in µg/g was calculated using Equation 3:

$$\text{AC} = \frac{(1.155 \times 400\ \text{mL} \times 1)}{50.00\ \text{g}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = 0.00924\ \mu\text{g/g} \qquad (3)$$

The percent recovery of the sample was calculated using Equation 5:

$$\%\,\text{Recovery} = \frac{0.00924\ \mu\text{g/g}}{0.0100\ \mu\text{g/g}} \times 100 = 92.4\% \qquad (5)$$

As another example, the LOQ quality control sample, Pyxant ID P2173B01-005 (Figure 21) resulted in a 4-OHQ recovery of 100%. The calculations for this sample are demonstrated below as a representative example of how all the sample results for 4-OHQ were calculated for this study.

The linear regression analysis of the calibration curve used in the analysis of 4-OHQ residues in soil samples from Trial 1 was determined to have the following regression coefficients: m = 142.41 and b = 2449.1 (Table 6, Figure 3). The analyte peak area (y) was 16758;

therefore the concentration of 4-OHQ in the final extract of this sample was calculated using Equation 2:

$$CC = \frac{(16758 - 2449.1)}{142.41} = 100.48\ \text{ng/mL} \quad (2)$$

The concentration of 4-OHQ found in the sample in µg/g was calculated using Equation 4:

$$AC = \frac{(100.48\ \text{ng/mL} \times 2.00\ \text{mL} \times 400\ \text{mL} \times 1)}{(50.00\ \text{g} \times 160\ \text{mL})} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = 0.01005\ \mu\text{g/g} \quad (4)$$

The percent recovery of the sample was calculated using Equation 5:

$$\%\ \text{Recovery} = \frac{0.01005\ \mu\text{g/g}}{0.0100\ \mu\text{g/g}} \times 100 = 100\% \quad (5)$$

Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the "AVERAGE" function of the Microsoft Excel spreadsheet computer program, which divides the sum of the selected cells by the number of determinations. The standard deviation of the recoveries for a sample was calculated using the "STDEV" function of the same spreadsheet program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, %RSD, was calculated by dividing the standard deviation by the mean, and then multiplying by 100.