1. INTRODUCTION

The objective of this study was to independently validate the Pyxant Labs Inc. residue method STM2360 for the determination of fluazinam (IKF-1216) in sediment. The independent validation (ILV) was conducted using untreated control samples of sediment. Sediment is considered representative for the intent of the method.

The method was found to be suitable for the determination of fluazinam in sediment over the concentration range 0.01 µg/g (ppm) 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. 8.1 (2, 3, 4). The study was also conducted to satisfy the requirements of U.S. EPA Guidelines OPPTS 850.7100 (5), OPPTS 835.6200 (6) and PR Notice 96-1 (7). This validation report presents the results of the independent laboratory validation for fluazinam in sediment.

Although this study was conducted in the same laboratory as where the method was developed, the ILV was performed in a different department with a Study Director and analyst who have no prior experience or knowledge of the procedures. Hence the integrity of the ILV was maintained and confirmed that an independent group having no prior experience with the method can achieve results meeting the requirements of the U.S. EPA and the European Commission for General Health and Consumer Protection. These steps successfully maintained the integrity of the ILV study.

2. ANALYTICAL

2.1 Sample Receipt, Labeling and Storage

The control sediment was acquired by the independent laboratory from Agvise Laboratories by request of the Sponsor. The control sample was assigned unique master logbook (MLB) number 22757376-01 and stored frozen (approximately -20°C). The pertinent physical characteristics are summarized in the following table. Sediment samples were characterized at Agvise Laboratories (Northwood, ND). Characterization records are maintained at Pyxant Labs Inc.

MEASUREMENT	WYOMING SEDIMENT
Percent Sand	77%
Percent Silt	10%
Percent Clay	13%
USDA Textural Class	Sandy Loam
Bulk Density	1.07 gm/cc
Cation Exchange Capacity	14.5 meq/100 g
% Moisture at 1/3 Bar	12.8%
% Organic Matter	0.98%
pH in 1:1 sediment:water	8.1
Base Saturation Data	
Cation	Percent / ppm
Calcium	65.8 / 1910
Magnesium	20.7 / 361
Sodium	5.2 / 173
Potassium	1.7 / 95
Hydrogen	6.6 / 10

2.2 Preparation of Solutions and Standards

The analytical reference standard utilized during the independent laboratory method validation is summarized below. The reference standard was received from the Sponsor, assigned unique a MLB number, and stored frozen, protected from light. The Certificate of Analysis is included in Appendix B.

Standard	MLB Number	Percent Purity	Recertification Date	Lot Number
Fluazinam (IKF-1216)	00007302	99.98%	April 12, 2012	Y010920

Standard solutions and calibration standard solutions were prepared with both fluazinam and one metabolite, HYPA (lot number 0205); however, in this study, samples were only analyzed for fluazinam. Solutions were prepared in glass containers as described below and stored frozen (≤ -10°C), protected from light, when not in use.

The following stock solution was prepared in acetone to obtain a nominal concentration of 1.00 mg/mL:

Analyte	Solution Type	Solution Lot Number	Weight [mg]	Dissolve In [mL]	Obtain [µg/mL]*
Fluazinam	Standard	N748P01-1	11.9	10	1190

*Resulting concentration after correcting for purity

The following stock solution was prepared in acetonitrile to obtain a nominal concentration of 1.00 mg/mL:

Analyte	Solution Type	Solution Lot Number	Weight [mg]	Dissolve In [mL]	Obtain [µg/mL]*
HYPA	Standard	N748P01-2	10.8	10	1080

*Resulting concentration after correcting for purity

Mixed fortification solutions were prepared in acetonitrile:

From Solution Lot Number	Concentration [µg/mL]	Pipette [µL]	Dilute To [mL]	Obtain Total [µg/mL]	Final Solution Lot Number
N748P01-1	1190	84.0	100	1.00	N748P02-1
N748P01-2	1080	92.6			
N748P02-1	1.00	5000	50	0.100	N748P02-2

Mixed calibration standards were prepared in 10/90/0.02 acetonitrile/water/formic acid:

From Solution Lot Number	Concentration of Stock Solution [ng/mL]	Aliquot of Stock Solution [mL]	Final Solution Volume [mL]	Calibration Solution Final Concentration [ng/mL]	Final Solution Lot Number
N748P02-2	100	5	50.0	10.0	N748P03-1
N748P03-1	10.0	5	25.0	2.00	N748P03-2
N748P03-1	10.0	1	10.0	1.00	N748P03-3
N748P03-1	10.0	5	100.0	0.500	N748P03-4
N748P03-2	2.00	1	10.0	0.200	N748P03-5
N748P03-3	1.00	1	10.0	0.100	N748P03-6
N748P03-4	0.500	1	10.0	0.0500	N748P03-7

2.3 Fortification of Recovery Samples

The ILV trial of the method was performed for fluazinam in sediment. The trial was comprised of one batch, which consisted of the following samples:

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

2 (two) unfortified control samples

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

5 (five) control samples fortified with fluazinam 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 [µg/g]	Aliquot of Fortification Solution [mL]	Fortification Solution Concentration [µg/mL]
10 g	0.01	0.100	1.00
	0.1	1.00	1.00

2.4 Sample Analysis

The ILV trial was conducted as described in the Pyxant Labs Inc. residue analytical method STM2360 (1).

Sediment samples were extracted using methanol and a digital sonifier. The extracts were diluted with water. Analysis was performed by liquid chromatography with electrospray tandem mass spectrometry (LC/MS/MS).

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

2.5 Analytical Instrumentation and Equipment

Prior to initiation of the first ILV trial, the independent group 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. Confirmatory ion transitions were monitored. The following instruments and equipment were utilized in the conduct of the independent laboratory validation of the residue analytical method:

2.5.1 Instrumentation

Typical HPLC Conditions

HPLC System:	Applied Biosystems API 5000 LC/MS/MS System (System 16) equipped with Shimadzu 10AD Autosampler for glass vials and pumps
Column:	Synergi Polar-RP (4 µm, 50 × 2.0 mm), P/N 00B-4336-B0
Guard Column:	Phenomenex C18 (optional)
Column Temperature:	Ambient
Injection Volume:	35 µL
Mobile Phase:	Solvent A: 0.1% formic acid in water Solvent B: 0.1% formic acid in acetonitrile
Flow Rate:	0.500 mL/minute

Gradient:	Time (min)	% A	% B
	0:00	90	10
	1:02	90	10
	5:02	0	100
	5:07	90	10
	7:02	90	10

Typical MS Conditions

Mass Spectrometer: Applied Biosystems API 5000 Mass Spectrometer
Detector Mode: Positive-ion electrospray
Source Temperature: 550°C
Ions Monitored:

	Transition amu	Declustering Potential V	Collision Energy eV	Dwell Time ms	Retention Time (+/- 0.3 min.) Quant. Ion	Conf. Ion
Fluazinam	465.2 → 373.0 465.2 → 338.0	110	35	100	5.20	5.20

2.5.2 Equipment

Top loading, Sartorius, model number BA 2100S, serial number 20303446 (EQ36)
Analytical balance, Sartorius, model number AC 120S, serial number 20103137 (EQ37)
Digital sonifier with cup horn, Branson, model number S-450D, serial number BBT 11053161A (EQ170)
Centrifuge, Beckman, model number TJ-6, serial number 14659 (EQ74)

2.5.3 Materials

Class A Volumetric pipettes, various sizes
Class A Volumetric flasks, various sizes
Glass Qorpak tubes with Teflon cap, 27.5 × 95 mm
Glass mixing cylinders, 100 mL
Amber glass autosampler vials, capped, 1.5 mL

2.5.4 Chemicals

Acetone, HPLC grade, lot number 50337, Burdick and Jackson
Acetonitrile, HPLC grade, lot number DE497, Burdick and Jackson
Methanol, HPLC grade, lot number DD034, Burdick and Jackson
Formic acid, lot number SZBA3280V, Fluka Analytical
HPLC grade water, lot number DE742-B, Burdick and Jackson
UHP water, in house

2.6 Calculations

Calculations were not modified from the original analytical method except that the analyte concentration was calculated in ng/mL by the Analyst ® software program and was then converted to ng/g in Excel. 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:

$$y = mx + b \quad (1)$$

Where:

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

Equation 1 was rearranged as Equation 2 to solve for the analyte concentration.

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

The analyte concentration found in the final extract (µg/g), calculated by Excel, is given by Equation 3:

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

Where:

AC = Analyte concentration found in final extract (ng/mL)
x = Analyte concentration in ng/mL
DF = Dilution factor (10)
V = Volume of final extract (30 mL)
W = Sample weight (10 g)

The percent recovery of the fortified samples was calculated using Equation 4:

$$\% \text{ Recovery} = \frac{x}{FC} \times 100 \quad (4)$$

Where:

x = Analyte concentration in ng/mL
FC = Concentration fortified in ng/mL (0.333 ng/mL at the LOQ and 3.33 ng/mL at 10×LOQ)

As an example, the 10×LOQ quality control sample, Pyxant ID P2366B01-014 (Table 1, Figure 8) resulted in a fluazinam recovery of 94%. The calculations for this sample are demonstrated below as a representative example of how all the sample results were calculated for this study.

The linear regression analysis of the calibration curve used in the analysis of fluazinam residues in sediment samples from Trial 1 was determined to have the following regression coefficients: m = 7.62E+04 and b = 488 (Figure 1). The analyte peak area (y) was 2.40E+05; therefore the concentration of fluazinam in the final extract of this sample was calculated using Equation 2:

$$x = \frac{(2.40E+05 - 488)}{7.62E+04} = 3.14 \text{ ng/mL} \quad (2)$$

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

$$AC = \frac{(3.14 \text{ ng/mL} \times 10 \times 30 \text{ mL})}{10 \text{ g}} \times \frac{1 \text{ µg}}{1000 \text{ ng}} = 0.0942 \text{ µg/g} \quad (3)$$

The percent recovery of the sample was calculated using Equation 4:

$$\% \text{ Recovery} = \frac{3.15 \text{ ng/mL}}{3.33 \text{ ng/mL}} \times 100 = 94\% \quad (4)$$

2.7 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 (or %CV), was calculated by dividing the standard deviation by the mean, and then multiplying by 100.