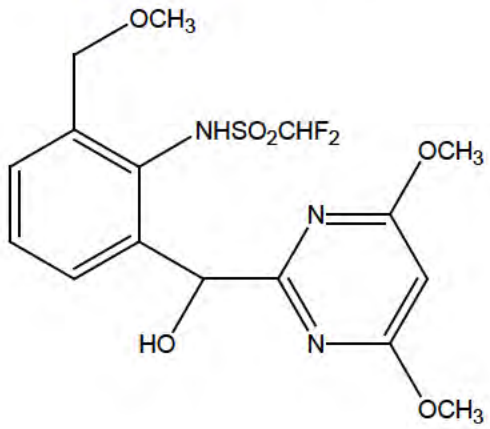
TEST SUBSTANCE/REFERENCE STANDARDS

- **Pyrimisulfan**

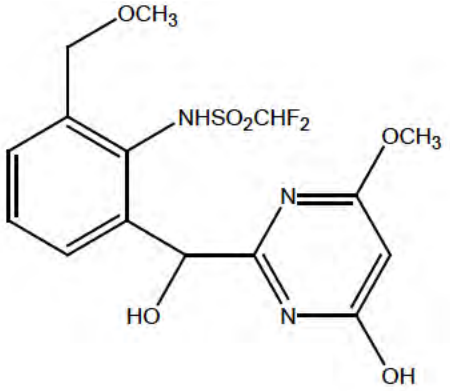
The test substance pyrimisulfan (two different shipments) was received from PBI Gordon on December 12 and 23, 2014. Upon receipt the test compound was stored at ambient condition. The samples were assigned the Ricerca ID of CS_20003 and CS_20017. The Certificate analysis is given in Appendix B.



Common Name:	Pyrimisulfan
Chemical Name:	(RS)-2'-(4,6-dimethoxypyrimidin-2-yl)hydroxymethyl-6'-methoxymethyl-1,1-difluoromethanesulfonamide
Molecular Formula:	$C_{16}H_{19}F_2N_3O_6S$
Ricerca Code:	CS_20003 and CS_20017
Lot Number:	13J016
Molecular Weight:	419.4 g/mole
Purity:	100%
Storage:	Ambient
Expiration Date:	April 24, 2017

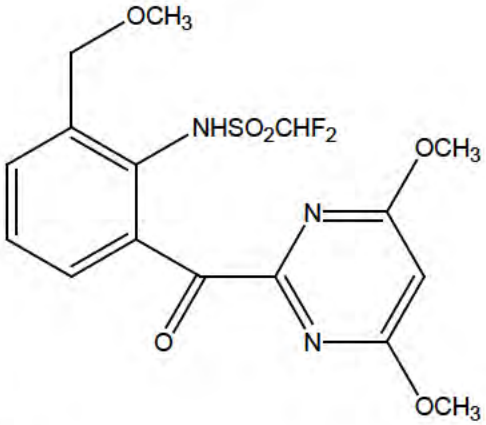
The reference standards M1, M15, and Imino M18 were supplied by Ricerca Biosciences on June 5, 2015, April 1, 2015, and June 16, 2015, respectively. The reference standards were stored in a refrigerator. The details of the reference standards are given below and the Certificate analysis is given in Appendix B.

- M1



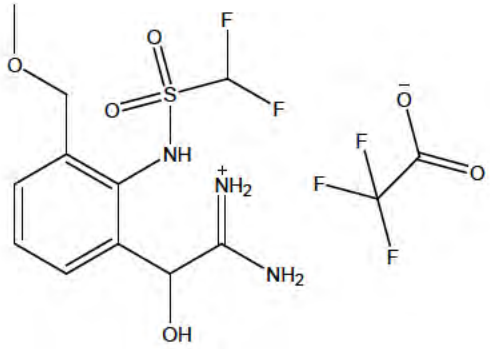
Common Names:	M1
Chemical Name:	(*RS*)-2'-(4-hydroxy-6-methoxypyrimidin-2-yl)hydroxymethyl-6'methoxymethyl-1,1-dilfuoromethanesulfonanilide
Molecular Mass:	405.37
Molecular Formula:	$C_{15}H_{17}F_2N_3O_6S$
Lot Number:	55269-7-36
Stated Chemical Purity:	100.0%

- M15



Common Names:	M15
Chemical Name:	2'-(4,6-Dimethoxypyrimidin-2-yl)carbonyl-6'-methoxymethyl-1,1-difluoromethanesulfonanilide
Molecular Mass:	417.38
Molecular Formula:	$C_{16}H_{17}F_2N_3O_6S$
Lot Number:	55231-12-16
Stated Chemical Purity:	96.6%

- Imino M18 TFA salt



Common Names:	Imino M18 TFA salt
Chemical Name:	2-[2-(difluoromethylsulfonamido)-3-(methoxymethyl)phenyl]-2-hydroxyacetamide
Molecular Mass:	324.3
Molecular Formula:	$C_{13}H_{16}F_5N_3O_6S$
Lot Number:	55357-16-35
Stated Chemical Purity:	93.6%

MATERIALS AND METHODS

REAGENTS AND SOLUTIONS

- Acetonitrile: Fisher or Sigma-Aldrich, Optima/HPLC Grade
- Water: Fisher Scientific or Sigma-Aldrich HPLC Grade
- Formic Acid: Sigma- Aldrich ACS Grade
- Mobile Phase A: 0.1% Formic Acid in water. For every liter prepared, 1000 mL of water was added to a glass bottle and added 1.0 mL of formic acid. The solution was mixed properly and stored ambient. The resulting solution expired after 2 weeks.
- Mobile Phase B: 0.1% Formic Acid in acetonitrile. For every liter prepared, 1000 mL of water was added to a glass bottle and added 1.0 mL of formic acid. The solution was mixed properly and stored ambient. The resulting solution expired after one month.
- Extraction Solvent Preparation: 80:20 Acetonitrile:water (v:v). For every liter prepared, 800 mL of acetonitrile was added to a glass bottle and added 200 mL of acetonitrile. The solution was mixed properly and stored at ambient conditions. The solution expired after one year. This solution was used to extract the soil and sediment samples.
- Dilution Solvent Preparation: 80:20 Water:acetonitrile. For every liter prepared, 800 mL of water was added to a glass bottle and added 200 mL of acetonitrile. The solution was mixed properly and stored at ambient conditions. The solution expired after one year.

Preparation of Test and Reference Substances Stock Solutions

Pyrimisulfan Stock Solution:
On an analytical balance, weighed 10.5 and 10.3 mg of the standard into seperate 10 mL Class A volumetric flasks and diluted to volume with acetonitrile. The concentration of the stock solutions was 1,050 and 1,030 µg/mL after correcting for purity. The stock solutions were identified as Replicate 1 and Replicate 2. One replicate was used to prepare the fortification solution and the second replicate was used to prepare the calibration soluions. Both replicates were stored in a freezer when not in use.

M1 Stock Solution:
On an analytical balance, weighed 10.2 and 10.3 mg of the standard into seperate 10 mL Class A volumetric flasks and diluted to volume with acetonitrile. The concentration of the stock solutions was 1,020 and 1,030 µg/mL after correcting for purity. The stock solutions were identified as Replicate 1 and Replicate 2. One replicate was used to prepare the fortification solution and the second replicate was used to prepare the calibration soluions. Both replicates were stored in a freezer when not in use.

M15 Stock Solution:
On an analytical balance, weighed 10.0 and 10.4 mg of the standard into seperate 10 mL Class A volumetric flasks and diluted to volume with acetonitrile. The concentration of the stock solutions was 966 and 1,005 µg/mL after correcting for purity. The stock solutions were identified as Replicate 1 and Replicate 2. One replicate was used to prepare the fortification solution and the second replicate was used to prepare the calibration soluions. Both replicates were stored in a freezer when not in use.

Imino M18 Stock Solution:
On an analytical balance, weighed 10.9 and 10.7 mg of the standard into seperate 10 mL Class A volumetric flasks and diluted to volume with acetonitrile. The concentration of the stock solutions was 1,020 and 1,002 µg/mL after correcting for purity. The stock solutions were identified as Replicate 1 and Replicate 2. One replicate was used to prepare the fortification solution and the second replicate was used to prepare the calibration soluions. Both replicates were stored in a freezer when not in use.

Preparation of Mixed Standard Stock Solutions of Pyrimisulfan, M1, M15, and Imino M18

Preparation of Mixed Standard Stock Solution of Replicate 1 (10 µg/mL)
The mixed standard stock solution 1 was prepared by transferring 95.2 µL, 98.0 µL, 103.6 µL, and 98.0 µL of pyrimisulfan (1,050 µg/mL), M1 (1,020 µg/mL), M15 (966 µg/mL) and Imino M18 (1,020 µg/mL) to a 10 mL Class A volumetric flask from replicate 1 of the stock solutions prepared above using a calibrated pipet. The flask was brought to volume with acetonitrile and the final concentration of each analyte was 10 µg/mL. The solution was mixed properly and transferred into a labeled 20 mL glass scintillation vial and stored in a freezer when not in use.

Preparation of Mixed Standard Stock Solution of Replicate 2 (10 µg/mL)
The mixed standard stock solution 2 was prepared by transferring 97.2 µL, 97.2 µL, 99.6 µL, and 99.8 µL of pyrimisulfan (1,030 µg/mL), M1 (1,030 µg/mL), M15

(1,005 µg/mL) and Imino M18 (1,002 µg/mL) to a 10 mL Class A volumetric flask from replicate 2 of the stock solutions prepared above using a calibrated pipet. The flask was brought to volume with acetonitrile and the final concentration of each analyte was 10 µg/mL. The solution was mixed properly and transferred into a labeled 20 mL glass scintillation vial and stored in a freezer when not in use.

Preparation of Fortification Solution (Replicate 1)

Pyrimisulfan, M1, M15, and Imino M18 Mixed Standard Fortification Solution (2,000 ng/mL):
This solution was used to fortify the soil, sediment, and water samples at 20 ppb level (10XLOQ).

A 2,000 ng/mL fortification solution was preapred by diluting 2.0 mL of 10 µg/mL stock solution (Replicate 1), into a 10 mL Class A volumetric flaks and diluting to volume with acetonitrile.

Pyrimisulfan, M1, M15,a nd Imino M18 Mixed Standard Fortification Solution (200 ng/mL):
This solution was used to fortify the soil, sediment, and water samples at 2 ppb level (LOQ).

A 200 ng/mL fortification solution was preapred by diluting 1.0 mL of 2,000 ng/mL fortification solution (Replicate 1), into a 10 mL Class A volumetric flaks and diluting to volume with acetonitrile.

The fortification solutions were stored in a freezer when not in use.

Preparation of Calibration Stock Solution (Replicate 2)

Pyrimisulfan, M1, M15,a nd Imino M18 Mixed Standard Calibration Stock Solution (100 ng/mL and 10 ng/mL):
This solution was used to prepare the calibration solutions.

A 100 ng/mL calibration stock solution was preapred by diluting 0.1 mL of 10 µg/mL stock solution (Replicate 2), into a 10 mL Class A volumetric flask and diluting to volume with acetonitrile.

A 10 ng/mL calibration stock solution was preapred by diluting 1.0 mL of 100 ng/mL calibration stock solution prepared above (Replicate 2), into a 10 mL Class A volumetric flask and diluting to volume with acetonitrile.

The calibration stock solutions were stored in a freezer when not in use.

Preparation of Calibration Standard Solutions

The calibration solutions prepared in this section were used to verify the concentration of the fortification solution (2000 ng/mL and 200 ng/mL) using the dilution solvent as discussed in the Reagents and Solutions section. The volume and the concentration of the solutions used in the calibration standard solutions were given below.

Standard Name	StandardSolution Used (ng/mL)	Aliquot Volume (mL)	Final Volume (mL)	Concentration of the Standard (ng/mL)
5.0 ng/mL std	100	0.50	10	5.0
3.0 ng/mL std	100	0.30	10	3.0
2.0 ng/mL std	100	0.20	10	2.0
1.0 ng/mL std	100	0.10	10	1.0
0.50 ng/mL std	10	0.50	10	0.50
0.20 ng/mL std	10	0.20	10	0.20
0.15 ng/mL std	10	0.15	10	0.15
0.10 ng/mL std	10	0.10	10	0.10

All calibration solutions were stored frozen (~ -20 ℃) when not in use.

CONCENTRATION VERIFICATION OF FORTIFICATION SOLUTION

Prior to the method validation part of the study, the concentration of the fortification solution was verified with the LC-MS method. The LC-MS/MS chromatogram and the data are presented in Appendix C.

ANALYTICAL METHODOLOGY

FORTIFICATION OF SOIL/SEDIMENT WITH PYRIMISULFAN, M1, M15, AND IMINO M18

1. Approximately 10 g of the soil/sediment samples were weighed into fourteen, 50 mL polypropylene centrifuge tubes. Two samples were used for control samples, 7 samples for the fortification at the 2 ppb level (LOQ) and 5 samples for the fortification at the 20 ppb level (10X LOQ). Two control samples per matrix (~10 g each, the exact weights shown in the tables below) were not fortified, as described in the tables below.

Sample Name	Soil weight (g)	Sediment weight (g)	Fortification with Pyrimisulfan, M1, M15, and Imino M18AQ in ACN (concentration, volume)
Control Rep 1	10.0431	10.03	NA
Control Rep 2	10.0810	10.07	NA
Fortified LOQ Rep 1	10.0868	10.25	200 ng/mL, 100 µL
Fortified LOQ Rep 2	10.0063	10.06	200 ng/mL, 100 µL
Fortified LOQ Rep 3	10.0670	10.13	200 ng/mL, 100 µL
Fortified LOQ Rep 4	10.0801	10.07	200 ng/mL, 100 µL
Fortified LOQ Rep 5	10.0911	10.03	200 ng/mL, 100 µL
Fortified LOQ Rep 6	10.0497	10.08	200 ng/mL, 100 µL
Fortified LOQ Rep 7	10.0625	10.05	200 ng/mL, 100 µL
Fortified 10X LOQ Rep 1	10.0981	10.04	2,000 ng/mL, 100 µL
Fortified 10X LOQ Rep 2	10.0491	10.08	2,000 ng/mL, 100 µL
Fortified 10X LOQ Rep 3	10.0881	10.05	2,000 ng/mL, 100 µL
Fortified 10X LOQ Rep 4	10.0574	10.04	2,000 ng/mL, 100 µL
Fortified 10X LOQ Rep 5	10.0565	10.08	2,000 ng/mL, 100 µL

2. 100 µL of the 200 ng/mL pyrimisulfan, M1, M15, and Imino M18 was added to the samples for the 2 ppb fortification level.
3. 100 µL of the 2,000 ng/mL pyrimisulfan, M1, M15, and Imino M18 was added to the samples for the 20 ppb fortification level.

Extraction of Pyrimisulfan, M1, M15, and Imino M18 from Soil and Sediment

4. Fifteen (15) mL of extraction solvent (80:20 (v:v) acetonitrile:water)was added to the samples using a 15 mL Class A volumetric pipet.
5. The samples were shaken for approximately one hour on a platform shaker.
6. The samples were centrifuged for 10 minutes at ~ 4,000 rpm.
7. The supernatant was transferred into a 50 mL polypropylene centrifuge tube.
8. The samples were re-extracted with additional 15 mL of extraction solvent and the extraction procedure was repeated by shaking and centrifugation.
9. The supernatants were transferred into the respective 50 mL polypropylene centrifuge tubes.
10. The volume of the final extract was adjusted to 30 mL with extraction solvent.

Preparation of Samples for LC-MS/MS Analysis

11. The LOQ samples were diluted four times with water (0.25 mL of the extract and 0.75 mL of water) and the 10XLOQ samples were diluted five times with water (0.20 mL of the extract and 0.80 mL of water) in a glass Autosampler vial and analyzed by LC-MS/MS.

FORTIFICATION OF SURFACE WATER AND NATURAL WATER WITH PYRIMISULFAN, M1, M15, AND IMINO M18

1. Approximately 10 g of the water samples were weighed into fourteen, 50 mL polypropylene centrifuge tubes. Two samples were used for control samples, 7 samples for the fortification at 2 ppb level (LOQ) and 5 samples for the fortification at 20 ppb level (10X LOQ). Two control samples per matrix (~10 g each, the exact weights shown in the tables below) were not fortified, as described in the tables below.

Sample Name	Soil Weight (g)	Sediment Weight (g)	Fortification with Pyrimisulfan, M1, M15, and Imino M18AQ in ACN (concentration, volume)
Control Rep 1	10.2037	10.0430	NA
Control Rep 2	10.0696	10.0708	NA
Fortified LOQ Rep 1	10.0326	10.0358	200 ng/mL, 100 µL
Fortified LOQ Rep 2	10.0360	10.0216	200 ng/mL, 100 µL
Fortified LOQ Rep 3	10.0219	10.0587	200 ng/mL, 100 µL
Fortified LOQ Rep 4	10.0405	10.0732	200 ng/mL, 100 µL
Fortified LOQ Rep 5	10.0574	10.0298	200 ng/mL, 100 µL
Fortified LOQ Rep 6	10.0630	10.0547	200 ng/mL, 100 µL
Fortified LOQ Rep 7	10.0860	10.0643	200 ng/mL, 100 µL
Fortified 10X LOQ Rep 1	10.0029	10.0351	2,000 ng/mL, 100 µL
Fortified 10X LOQ Rep 2	10.0033	10.0010	2,000 ng/mL, 100 µL
Fortified 10X LOQ Rep 3	10.0490	10.0810	2,000 ng/mL, 100 µL
Fortified 10X LOQ Rep 4	10.0573	10.0740	2,000 ng/mL, 100 µL
Fortified 10X LOQ Rep 5	10.0354	10.0123	2,000 ng/mL, 100 µL

2. 100 µL of the 200 ng/mL pyrimisulfan, M1, M15, and Imino M18 was added to the samples for the 2 ppb fortification level.
3. 100 µL of the 2,000 ng/mL pyrimisulfan, M1, M15, and Imino M18 was added to the samples for the 20 ppb fortification level

Extraction of Pyrimisulfan, M1, M15, and Imino M18 from Water

4. The fortified water samples were mixed properly by shaking by hand and a vortex mixer for one minute.

Preparation of Samples for LC-MS/MS Analysis

5. The LOQ samples were diluted 10 times and the 10XLOQ samples were diluted 100 times with 80:20 (v:v) water:acetonitrile (0.1 mL of LOQ sample and 0. 9 mL of dilution solvent and 0.1 mL of 10XLOQ sample and 9.9 mL of dilution solvent) and analyzed by LC-MS/MS.

LC-MS/MS Analysis

Samples were analyzed using the following LC-MS/MS system (controlled by the operating software Analyst™ version 1.6.2).

LC-MS/MS System

Shimadzu Nexera UHPLC Component List:

Component	Model	Serial No.
Autosampler	Sil-30acmp	L20645250096
Tray	Reservoir Tray	L20305247423
Pump	LC-30AD	L20555251698
Pump	LC-30AD	L20555251707
Column Oven	CTO-20AC	L20215252174
Degasser	DGU-20A5R	L20705364760
Controller	CBM-20A	L20235255613
Degasser	DGU20A3R	L20695261880
Tray	Reservoir Tray	L20305249147

Mass Spectrometer Component List

MDS-Sciex API 4000
Analyst (Version 1.6.2)

HPLC Method

Column:	Phenomenex Synergi Hydro RP, 100 Å, 2.5µm, 50 x 2.0 mm
Column Temperature:	Ambient
Injection volume:	10 µL
Run Duration:	7.0 minutes

Solvent System:

Mobile Phase:	A: 0.1% Formic Acid in Water B: 0.1% Formic Acid in Acetonitrile

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	% A	% B
0.0	0.650	98	2
1.0	0.650	98	2
4.5	0.650	20	80
4.6	0.650	5	95
5.6	0.650	5	95
5.7	0.650	98	2
7.0	0.650	98	2

The LC flow was diverted to the MS between 0.9 to 4.7 minutes, and to waste between 0.0 and 0.9 minutes and between 4.7 and 7.0 minutes.

MS Parameters:

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Ion Source Gas 1 (GS1):	50 psi
Ion Source Gas 2 (GS2):	60 psi
Curtain Gas (CUR):	30 psi
Collision Gas (CAD):	10 psi
Ion Spray Voltage (IS):	5000 V
Temperature (TEM):	500 °C
Declustering Potential (DP):	45 V
Entrance Potential (EP):	10 V

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Retention Time (min)	CE (V)	CXP (V)
Quantitation	PYM-370	420	370	4.20	29	15
Confirmation	PYM-255	420	255	4.20	39	15
Quantitation	M1-356	406	356	3.30	26	15
Confirmation	M1-241	406	241	3.30	38	15
Quantitation	M15-386	418	386	4.00	25	13
Confirmation	M15-272	418	272	4.00	34	8
Confirmation	M15-243	418	243	4.00	47	7
Quantitation	Imino-M18-292	324	292	2.60	21	15

MRM Transition	Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Retention Time (min)	CE (V)	CXP (V)
Confirmation	Imino-M18-161	324	161	2.60	31	15
Confirmation	Imino-M18-160	324	160	2.60	38	15

METHODS OF CALCULATION

Calibration and Linearity

A series of calibration standard solutions for pyrimisulfan, M1, M15, and Imino M18 from 0.10 ng/mL to 5.0 ng/mL were injected with the validation set to quantify that each analyte in the samples. These calibration standards generated a linear plot of the concentration of each standard versus area with 1/x weighting. The resulting linear plot of the calibration standards had to yield a correlation coefficient (r) of at least 0.99 for the analytical set to be acceptable.

Limits of Quantification and Detection (LOQ and LOD)

For this study the limit of quantification (LOQ) was defined as the lowest concurrent fortification that could be successfully processed through the method. The LOQ was 2 ppb for this study. The limit of detection (LOD) was determined using a statistical method by examining the standard deviation of seven QC sample recoveries fortified at the target LOQ. The method detection limit (MDL) for each analyte was calculated using the equation,

MDL (calculated) = (standard deviation × $t_{0.99}$).

For n-1 degrees of freedom, where n = 7, $t_{0.99}$ has a value of 3.143. The method detection limit (MDL) was calculated and given in the table below.

Analyte	m/z	Method Detection Limit (ng/g)			
		Soil	Sediment	Natural Water	Surface Water
Pyrimisulfan	370	1.22	0.23	0.19	0.26
Pyrimisulfan	255	1.36	0.54	0.60	0.40
M1	256	0.34	0.28	0.26	0.29
M1	241	0.41	0.62	0.55	0.45
M15	386	1.37	0.41	0.16	0.21
M15	272	1.34	0.38	0.29	0.48
M15	243	1.18	0.73	0.53	0.73
Imino M18	292	0.21	0.23	0.24	0.15
Imino M18	161	0.31	0.94	0.55	0.40
Imino M18	160	0.41	0.48	0.42	0.26

	Residue ppb	Solution Concentration upon LC-MS analysis
LOQ	2.0	0.167 ng/mL
10% LOD (area threshold)	0.030	0.01 ng/mL

Calculation of Residue

Residues were calculated in Microsoft Excel® spreadsheets. Spreadsheets presenting the raw data are presented in Appendix D.

Representative Chromatography

Example chromatograms of pyrimisulfan, M1, M15, and Imino M18 standards and the associated calibration curve graph from the method validation were presented in this report from Figure 1 to Figure 96.

Statistics

The software program Microsoft Excel®, a non-validated system, generated mean, range and standard deviation of analyte recovery data. Analyst version 1.6.2 software, a validated system, was used to acquire and process data for the LC-MS/MS to calculate regression and correlation of standard curves for residue quantification.

RESIDUE SAMPLE CALCULATIONS

Quantitation of pyrimisulfan residues was made by injecting a series of calibration standards with the samples. The response of the standards by area count was plotted against concentration. The sample concentration in µg/mL was determined from the first order 1/x weighted curve generated from the calibration standards.

The final concentration of pyrimisulfan in the sample in ng/g (ppb), were calculated based on weight and were calculated using the following formulas:

$$\text{ng/g (ppb)} = \frac{\text{ng/mL Analyte} \times \text{Final Sample Volume (mL)} \times \text{Dilution Factor}}{\text{Sample Weight (g)}}$$

Where:

ng/mL Analyte = ng/mL of analyte found from standard curve
Final Sample Volume = Volume of final LC-MS sample (30 mL)
Dilution Factor = 5 for 10XLOQ samples
Sample Weight 10.04 g

and

$$\text{ng/mL analyte} = \frac{(\text{Peak Area} - b)}{m}$$

Peak Area = Peak area of the analyte
b = Y-intercept of calibration curve
m = slope of calibration curve

$$\% \text{ Recovery} = \frac{\text{Analyte Residue Detected (ng/g)}}{\text{Analyte fortification level (ng/g)}} \times 100$$

An example calculation for pyrimisulfan residue in sediment (10X LOQ fortified Rep 1 at 20 ng/g) is shown below:

Linear Regression analysis (1/x weighting) of the pyrimisulfan standards gave a curve as calculated by the Analyst Software version for the quantitation ion transition 420 → 370 with the equation

y = 5.18e+004 x+1.69e+003 (r = 0.9992)

The ng/mL pyrimisulfan injected determined by this curve was:

$$\text{ng/mL pyrimisulfan} = \frac{(74{,}700 - 1{,}690)}{51{,}800} = 1.41 \text{ ng/mL}$$

Where:

Peak Area of pyrimisulfan = 74,700
Y-intercept of calibration curve = 1,690
Slope of calibration curve = 51,800

$$\text{The pyrimisulfan residue (ng/g)} = \frac{1.41 \text{ ng/mL} \times 30.0 \text{ mL} \times 5}{10.04 \text{ g}}$$

$$= 21.1 \text{ ng/g or ppb}$$

$$\text{\% Recovery} = \frac{21.1 \text{ (ng/g)}}{20.0 \text{ (ng/g)}} \times 100 = 105.5\%$$