EXPERIMENTAL

1.0 PROTOCOL AND METHOD

The protocol for this independent laboratory validation is included in Appendix 1. A copy of the method is included in Appendix 2.

2.0 MATERIALS

2.1. EQUIPMENT

A list of equipment used in the method validation is shown below. Similar equipment from other suppliers may also be used.

Autosampler vials, 1.8 mL, with Teflon®-lined septum caps (Agilent)
Balance, analytical, capable of weighing 0.0001 g (Sartorius)
Balance, top loading, capable of weighing to 0.01 g (Sartorius)
Büchner funnels, 9 cm diameter
Filter paper, 9 cm diameter, Whatman No. 1
Filter flask, 500-mL
Glass vials, 60-mL, with screw caps (Kimble OptiClear)
Glass vials, 40-mL, with screw caps (Kimble OptiClear)
Glass wool, Pyrex
Graduated cylinders, assorted volumes
Heated water bath, temperature <40°C
Rotary evaporator (Buchi Rotavapor-M)
Pipettes, Pasteur
Pipette, Volumetric; 25-mL, 10-mL, and 5-mL
Pipettor, adjustable, capable of accurately dispensing volumes of up to 0.25 mL
Pipettor, automatic, capable of accurately dispensing volumes of up to 2.5 mL
Reciprocating mechanical shaker (Erbach)
Refrigerator/Freezer
Round-bottom flasks, 500 mL
Ultrasonic bath
Vacuum manifold for SPE cartridges (Supelco)
Volumetric flasks, 100-mL and 50-mL
Waters Oasis® HLB 12 cc (500 mg) extraction cartridge

2.2 REAGENTS

Acetic acid, glacial, reagent grade
Acetone, pesticide quality
Acetonitrile (ACN), pesticide quality
Methanol, pesticide quality
Sodium ascorbate, reagent grade
Water, HPLC grade

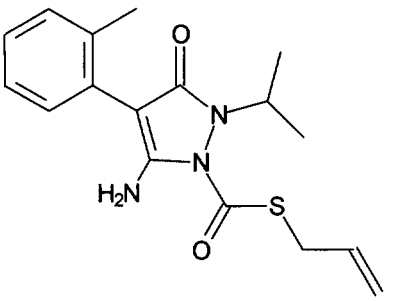
2.3 REAGENT SOLUTIONS

Acetonitrile/water, 1:1 (v/v)
Acetonitrile/water, 1:10 (v/v)
Methanol/water, 5:1 (v/v)
Sodium ascorbate solution, 1*M*
Water with 0.05% acetic acid (mobile phase "A")

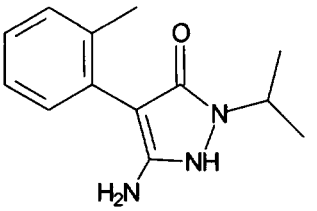
2.4 TEST SUBSTANCES / ANALYTICAL REFERENCE STANDARDS

The test substances/analytical reference standards were supplied by the Valent Technical Center in Dublin, CA. Copies of the certificates of quality are included in Appendix 3.

Common name:	S-2188 (Fenpyrazamine, V-10135)
Chemical name:	*S*-2-propen-1-yl-5-amino-2,3-dihydro-2-(1-methylethyl)-4-(2-methylphenyl)-3-oxo-1*H*-pyrazole-1-carbothioate
CAS number:	473798-59-3
Lot number:	AS 2177a
Stated purity:	99.4%
Expiration date:	June 12, 2009
Storage conditions:	Under nitrogen in freezer
Structure:	



Common name:	S-2188-DC
Chemical name:	5-amino-2,3-dihydro-2-(1-methylethyl)-4-(2-methylphenyl)-3-oxo-1*H*-pyrazole
CAS number:	NA
Lot number:	AS 2179b
Stated purity:	100%
Expiration date:	July 17, 2010
Storage conditions:	Under nitrogen in freezer
Structure:	



All single analyte stock standard solutions were stored in a freezer. The mixed fortification standards and the diluted standard solutions were also stored in a freezer.

2.5 LC/MS-MS INSTRUMENTATION

Analysis was performed using a high pressure liquid chromatograph with a tandem mass spectrometer (LC/MS-MS), operating in positive ion mode. The following equipment was used:

Surveyor autosampler
Surveyor pump system
Finnigan TSQ LC/MS-MS (ThermoElectron) triple quadrapole mass spectrometer, with electospray ionization (ESI) sample introduction
Excaliber software (version 1.4)

2.6 TEST SYSTEM AND SAMPLE STORAGE

The untreated control soil sample was a sub-sample of an untreated sample collected from a 2006 terrestrial field soil dissipation study of S-2188 on bare soil in California (Valent Project No. 30311). The soil sample was identified as V-30311-10U-1. The sample was stored frozen (nominally ≤-10°C) except when in use.

3.0 ANALYTICAL METHOD

3.1 PRINCIPLE OF THE METHOD

S-2188 and S-2188-DC residues were extracted twice from the soil with a methanol/water mixture (5:1, v/v), following addition of sodium ascorbate solution to the soil sample. The filtered extracts were rotary evaporated to remove methanol and obtain an aqueous mixture. The residues were loaded onto an Oasis HLB cartridge, washed with acetonitrile/water

(1:10, v/v), and then eluted with acetonitrile/water (1:1, v/v). The eluant was diluted 4-fold with acetonitrile/water (1:1, v/v) in an autosampler vial.

Extracts and standards were analyzed using a high pressure liquid chromatograph with a tandem mass spectrometer (LC/MS-MS). Five-point, 2nd order polynomial calibration curves (weighted relative to 1/concentration) were used to quantify S-2188 and S-2188-DC in the sample extracts. The primary transitions (most abundant product ions) for the analytes were used for quantitation.

3.2 LIMITS OF QUANTITATION (LOQ)

The LOQ in soil for both S-2188 and S-2188-DC was 0.02 ppm, µg/g (20 ng/g, ppb).

3.3 VALIDATION SAMPLE SETS

One trial was conducted with a validation set consisted of the following samples:

Instrument calibration standards
1 reagent blank
2 unfortified control samples
5 samples fortified with analytes at 0.02 µg/g, ppm (20 ng/g, ppb), LOQ
5 samples fortified with analytes at 0.20 µg/g, ppm (200 ng/g, ppb), 10x LOQ

An additional subset, consisting of one reagent blank, one control sample, two LOQ level fortifications, and two 10x LOQ level fortifications was also extracted.

3.4 PREPARATION OF STANDARD STOCK SOLUTIONS

3.4.1 1,000 µg/mL (ng/µL, ppm), single analyte, S-2188 and S-2188-DC standard stock solutions

For the calculations involved in the preparation of these standard solutions, see section 3.10.2.

For each neat analyte, 0.101 g – 0.104 g was weighed out (taking into account the percent purity) and brought to volume in a 100 mL volumetric flask with the acetonitrile. These 1,000 µg/mL standard solutions were stored in a freezer.

3.4.2 100 µg/mL (ng/µL, ppm), S-2188 and S-2188-DC standard stock solution

The 100 µg/mL mixed analyte standard stock solution (containing both S-2188 and S-2188-DC) was prepared by transferring 10.0 mL aliquots of the each of the 1,000 µg/mL standard stock solution (single analyte) into a 100 mL volumetric flask and filling the flask to the mark with acetonitrile. This standard was stored in a freezer.

3.4.3 10 µg/mL (ng/µL, ppm), S-2188 and S-2188-DC standard stock solution

The 10 µg/mL mixed analyte standard stock solution was prepared by transferring a 10.0 mL aliquot of the 100 µg/mL stock standard solution into a 100 mL volumetric flask and filling the flask to the mark with acetonitrile. This standard was stored in a freezer.

3.4.4 1.0 µg/mL (ng/µL, ppm), S-2188 and S-2188-DC standard stock solution

The 1.0 µg/mL mixed analyte standard stock solution was prepared by transferring a 10.0 mL aliquot of the 10 ng/µL stock standard solution into a 100 mL volumetric flask and filling the flask to the mark with acetonitrile. This standard was stored in a freezer.

3.5 PREPARATION OF INSTRUMENT CALIBRATION STANDARD SOLUTIONS

Five levels of working standards, at 0.100, 0.020, 0.010, 0.004, & 0.002 µg/mL (in acetonitrile) were prepared by serial dilution of a 1.0 µg/mL standard solution. These stock solutions were stored in the freezer.

The instrument calibration working standards were prepared in autosampler vials, by addition of an equal volume of HPLC-grade water to aliquots of each of the standards (in acetonitrile). Thus, the final concentrations were 0.050, 0.010, 0.005, 0.002, 0.001 µg/mL.

The instrument calibration working standards were prepared just prior to the analysis of each sample set. For the calculations involved in the preparation of these standard solutions, see section 3.10.3.

3.6 PREPARATION OF SAMPLES AND WEIGHING

The soil sample was mixed, and 10 g (± 0.05 g), of this prepared sample were weighed into 60-mL glass vials.

3.7 PREPARATION OF FORTIFICATION SAMPLES

An LOQ fortification at 0.02 µg/g (0.02 ppm; or 20 ng/g = 20 ppb) of a 10.0 g soil sample was prepared by adding 200 µL of the 1.0 ng/µL S-2188 and S-2188-DC standard solution *using a Gilson microman positive-displacement pipettor.*

A 10x LOQ fortification at 0.20 µg/g (0.20 ppm; or 200 ng/g = 200 ppb) of a 10.0 g soil sample was prepared by adding 200 µL of the 10.0 ng/µL mixed analyte fortification standard solution.

The calculations for fortification samples are presented in section 3.10.4.

3.8 EXTRACTION PROCEDURE

3.8.1 Extraction with methanol/water and rotary evaporation

A 5-mL aliquot of 1*M* sodium ascorbate solution was added to each soil sample, and the samples were allowed to stand for approximately 10 minutes. A 40-mL portion of methanol/water (5:1, v/v) was added to each vial, the vials were capped, and the samples were shaken on a mechanical shaker for about 1 hour.

For each sample, the sample mixture was filtered through a Whatman No. 1 filter paper, and the liquid was collected in a filter flask. The paper and soil were returned to the vial, and a second 40-mL portion of methanol/water (5:1, v/v) was added. The vial was capped, and the sample was shaken again on a mechanical shaker for about 1 hour.

The sample mixture was filtered through a Whatman No. 1 filter paper, combining the second extract with the first extract. The vial was rinsed with a 20-mL portion of methanol/water (5:1, v/v), the rinse was passed through the soil on the filter paper, and the rinse was combined with the filtrate.

For each sample, the combined filtrate was transferred into a 500-mL round-bottom flask. The round-bottom flask was place on a rotary evaporator and the methanol was removed (using a heated waterbath at a temperature of <40°C). The samples were rotary evaporated until the liquid trap (adjacent to the round-bottom flask) clouded with water vapor.

3.8.2 Oasis® HLB column cleanup

Preparation of the Waters Oasis® HLB cartridge: A Waters Oasis® HLB cartridge was placed onto a manifold and a plug of glass wool was placed at the bottom of the cartridge. The cartridge was preconditioned with a 5-mL rinse of acetonitrile and then (when the rinse was drained to the top of the frit) a 10-mL rinse with HPLC-grade water. The water was allowed to drain to the top of the frit.

For each sample, the aqueous extract was transferred onto the Oasis® HLB cartridge. Ten (10) mL of HPLC-grade water were added to the round-bottom flask and the flask was swirled to dislodge residues. After the extract in the cartridge had drained to the frit, the water rinse was added to the cartridge. The round-bottom flask was then rinsed with a 5-mL aliquot of acetonitrile/water (1:10, v/v) and, after the first rinse reached the frit, the second rinse was added to the cartridge. After the second rinse had drained to the frit, any accumulated eluant from loading and rinsing the cartridge was discarded.

A 25.0 mL aliquot of acetonitrile/water (1:1, v/v) was added to the flask, and a collection vial (40-mL glass vial) was placed under the cartridge. The residues were eluted from the cartridge by passing the acetonitrile/water (1:1, v/v) through the cartridge. Once the eluant had drained to the top of the cartridge, the collection vial was removed and capped.

For each sample, an autosampler vial was filled with 0.75 mL of acetonitrile/water (1:1, v/v) and then a 0.25 mL portion of the sample eluant was added (4x dilution). The autosampler vial was then capped and sonicated briefly to mix the sample. The diluted extract was analyzed by LC/MS-MS.

3.9 LC/MS-MS OPERATION PARAMETERS

3.9.1 Conditions

Column: YMC ODS-AM, 3 micron, 100 x 3 mm

Mobile phases: Acetonitrile (ACN)
Water, 0.05% acetic acid

Flow rate: 300 µL/min.

Injection volume: 20 µL

Gradient program:

Time (minutes from start of run)	% ACN	% Water (acidified)
0.0	20	80
1.0	20	80
3.0	80	20
10.0	80	20
12.0	20	80
15.0	20	80

Scan type: SRM

Polarity: Positive

Retention times: S-2188, ~6.8 min.
S-2188-DC, ~4.6 min.

3.9.2 Mass spectrometer mass calibration, optimization and operation

The sample was introduced into the mass spectrometer by electrospray ionization, operated in the positive ion mode. The mass spectrometer parameters were optimized for the each of the analytes prior to sample analysis.

3.9.3 Monitored transitions

Two transition ions were used for quantitation of each analyte. The transitions used were as follows:

S-2188, 332 amu to 189 and 230 amu
S-2188-DC, 232 amu to 190 and 145 amu

Copies of example chromatograms are included in Appendix 4.

3.9.4 Calibration procedures

Instrument calibration working standard solutions were prepared as described in section 3.5. Calibration consisted of injecting five instrument calibration standards throughout each run/sequence and calibrating (area counts versus concentration), using a 2nd order polynomial fit, with weighting relative to 1/concentration. The standard concentrations were 0.050, 0.010, 0.005, 0.002, and 0.001 µg/mL for each analyte (50, 10, 5, 2, and 1 ng/mL, respectively). The coefficient of determination (r^2) of the standard regression curves were required to be at least 0.995, and the coefficient of variation (CV) for the standard responses were required to be <15% for each analyte in each analytical set. The 2nd order polynomial fit was calculated using Excel, and the residues found were also calculated using Excel.

The method required that each sequence should begin and end with a continuing calibration standard. The mid-level standard used for the first analytical set was 0.010 µg/mL, and the mid-level standard used for the second analytical set was 0.005 µg/mL. There was no significant differences between the coefficient of variation (CV) for the continuing calibration standards in either set, with the CV's <10% for both analytical sequences.

3.10 CALCULATIONS

3.10.1 Threshold area counts

After decreasing the injection volume from 50 µL to 20 µL, the area reject threshold was still more than 10-fold less than the peak area of the lowest calibration standard (with an area count of approximately 2,000,000 for S-2188, and an area count of approximately 4,000,000 for S-2188-DC). Minor area counts between 18,000 and 44,000 were observed for S-2188 in the control sample and in the reagent blank. Minor area counts of approximately 200,000 for S-2188-DC were observed in one of the three control sample replicates. While average peak areas for the control samples were used for correction of the peak areas for the fortified samples (prior to calculation of percent recovery), these areas were recorded as "0 ppm" or "ND" in the validation recovery data tables.

3.10.2 Calculation of the amount of standard needed to set the amount weighed for the required concentration

Weight of standard (adjusted for purity) = Desired concentration x Volume of solvent
Example calculation:
Preparation of the 1,000 mg/L S-2188 standard stock solution:
Volume = 100 mL
Weight of S-2188 = 1000 mg/L x (100 mL x 1 L/1000 mL) = 100 mg = 0.100 g
S-2188, Percent purity = 99.4%
Weight of S-2188 standard = 0.100 g (100%/99.4%) = 0.10060 g

3.10.3 Calculations used in the preparation of instrument calibration standards and working standards

A mixed analyte standard stock solution was prepared by adding 10.0 mL of each single analyte 1000 µg/mL stock solution into a 100 mL volumetric flask. The flask was brought to 100 mL using acetonitrile, producing a 100 µg/mL S-2188 and S-2188-DC standard.

Example calculation:
For each standard, (1000 µg/mL x 10.0 mL) / 100 mL = 100 µg/mL

Dilutions of the mixed analyte stock solutions, using acetonitrile, were done to produce 10 µg/mL and 1.0 µg/mL mixed analyte standards.

Example calculations:
(100 µg/mL x 10.0 mL) / 100 mL = 10.0 µg/mL
(10 µg/mL x 10.0 mL) / 100 mL = 1.0 µg/mL

The preparation of the instrument calibration standards (in acetonitrile) was done by serial dilution of the 1.0 µg/mL S-2188 and S-2188-DC standard:
(1.0 µg/mL x 5.0 mL) / 50 mL = 0.100 µg/mL
(0.100 µg/mL x 10.0 mL) / 50 mL = 0.020 µg/mL
(0.020 µg/mL x 25.0 mL) / 50 mL = 0.010 µg/mL
(0.010 µg/mL x 20.0 mL) / 50 mL = 0.004 µg/mL
(0.004 µg/mL x 25.0 mL) / 50 mL = 0.002 µg/mL

Instrument calibration working standards were prepared from these by 1:1 dilution with HPLC-grade water in an autosampler vial (just prior to analysis).

Example calculation:
(0.004 µg/mL x 0.25 mL) / 0.50 mL = 0.002 µg/mL

3.10.4 Calculations used in the preparation of fortification samples

A 0.02 ppm (LOQ) fortification for both analytes on a 10.0 g soil sample was prepared by fortifying the sample with 200 µL of the 1.0 ng/µL mixed analyte standard solution:

(200 µL) (1.0 ng/µL) / (10.0 g) = 20 ng/g (ppb)

A 0.20 ppm (10x LOQ) fortification for both analytes on a 10.0 g soil sample was prepared by fortifying the sample with 200 µL of the 10.0 ng/µL (ppm) mixed analyte standard solution:

(200 µL) (10.0 ng/µL) / (10.0 g) = 200 ng/g (ppb)

3.10.5 Calculation of analyte concentrations

Calculations of the analyte concentrations were accomplished by entering the concentrations and peaks areas for the standards into Excel to calculate a 2nd order polynomial curve (with relative weighting of 1/concentration. The curve and the equation were displayed on an Excel graph, and the coefficients (a, b, and c) were copied into cells in the spreadsheet so that concentrations of both the standards and the samples could be calculated. The area counts were copied from the chromatograms into the spreadsheets (residue data sheets). Copies of the residue data sheets from both analytical sets are presented in Appendix 5. The results were transcribed to the tables of results, where they are reported as µg/g (ppm).

Analyte concentrations were calculated by the software using the following equation:

$$Y = (aX^2 + bX + c) \times 4$$

where:
- Y = concentration of analyte, µg/mL
- X = peak area (may be divided by 10^6 for simplicity)
- a = calibration coefficient
- b = calibration coefficient
- c = calibration coefficient (intercept)

Example calculation:
Concentration of S-2188 in sample F2, 0.020 µg/g (ppm), extracted 3/25/09 and analyzed 3/25/09:

The concentration of the sample extract includes the polynomial fit and the 4-fold dilution factor -

where
- X = 3.98
- a = 7.764×10^{-6}
- b = 4.496×10^{-4}
- c = 1.325×10^{-4}

$$Y = [\,(7.764 \times 10^{-6}) \times (3.98)^2 + (4.496 \times 10^{-4}) \times 3.98 + (1.325 \times 10^{-4})\,] \times 4$$

$$= 0.00818 \text{ µg/mL}$$

The concentration in the fortified soil sample is then

Y, µg/mL x (Final Extract Volume, mL) / (Sample Wt., g) = µg/g (ppm)

or 0.00818 µg/mL x 25.0 mL / 10.0 g = 0.0204 µg/g (ppm)

3.10.6 Calculation of method fortification percent recovery

The concentration in the fortified soil sample for percent recovery is calculated with correction for the area (or average area) in the untreated control sample(s):

Example calculation:
Corrected concentration of S-2188 in fortified sample F2, 0.020 µg/g (ppm), extracted 3/25/09 and analyzed 3/25/09:

Average area in the samples UTC1 and UTC2 = (0.0182 + 0.0373) / 2 = 0.0278

Corrected concentration in the sample extract =

$[(7.764 \times 10^{-6}) \times (3.98 - 0.0278)^2 + (4.496 \times 10^{-4}) \times (3.98 - 0.0278) + (1.325 \times 10^{-4})] \times 4$

= 0.00812 µg/mL

The corrected concentration in the fortified soil sample =

0.00812 µg/mL x 25.0 mL / 10.0 g = 0.0203 µg/g (ppm)

The method recovery is then calculated as

$$\text{Method recovery (\%)} = \frac{\text{Concentration found, µg/g}}{\text{Expected concentration, µg/g}} \times 100\%$$

Example calculation

$$\text{Percent Recovery} = \frac{0.0203 \text{ µg/g}}{0.0200 \text{ µg/g}} \times 100\%$$

= 101.5%

3.10.7 Calculation of standard deviation (s) and coefficient of variation (CV)

Sample standard deviations were calculated and expressed as an absolute percent value (CV) using the mean values:

$$\text{Coefficient of Variation, \%} = \frac{\text{standard deviation}}{\text{mean}} \times 100\%$$

Example calculation
Using the five replicates fortified at 0.02 ppm (LOQ) in the first analytical set, S-2188 fortification recoveries, where the mean recovery was 93% and the unrounded standard deviation was 5.6%:

$$CV, \% = \frac{5.6\%}{93\%} \times 100\%$$

$$= 6.1\%$$

3.11. STATISTICS STATEMENT

The mean percent recoveries, sample standard deviations, and coefficients of variation were calculated using Excel spreadsheets. The Excel spreadsheets also calculated the 2nd order polynomial calibration coefficients (weighted relative to 1/concentration) and the coefficient of determination (r^2) for each of the standard calibration curves. The coefficient of determination (r^2) values for the calibration standards in each analytical set were >0.995, and the coefficient of variation (CV) for the continuing calibration standards were <10% for each analyte in each analytical set.

5.5 SUGGESTED METHOD EDITS

Under the REAGENTS section, starting on page 1:

Oasis HLB Cartridges (misspelled as Oasis HBL Cartridges) is a consumable and could be relocated in the method to the EQUIPMENT section, starting on page 3.

Preparation of 1 *M* Sodium ascorbate probably does not require a 1 L volumetric flask. Consider just specifying the addition of 1 L of HPLC-grade water.

Under the STANDARD SOLUTIONS section, starting on page 2:

Change the line describing the Mixed Intermediate Standard Solutions to "five intermediate calibration standards" (rather than "linearity standards", as the calibration used is non-linear).

Change the Linearity Standard Solutions to Calibration Standard Solutions (as a non-linear calibration is used).

Change the Calibrating Standard Solution to either Continuing Calibration Standard Solution or Reference Standard Solution. And change the subsequent reference to the Linearity Standard Solution to reference the Calibration Standard Solution.

Under the EQUIPMENT section, starting on page 3:

Include balances - used for weighing standards, reagents, and samples.

Include a 25-mL volumetric pipet - used for measuring the final eluant/extract volume.

Specify Automatic pipettors (0.25 to 2.5 mL), 1-mL volumetric pipettes, 5-mL volumetric pipettes, and/or 10-mL volumetric pipettes - used for the preparation of standards and fortification of samples.

Specify a 10-mL graduated cylinder, automatic dispenser (set to 5-mL), and/or serological pipettes (5-mL or 10-mL). These volumes are used for adding sodium ascorbate to the samples, conditioning cartidges, and rinsing flasks/cartridges.

Change 250-mL to 500-mL round-bottom flask, as specified in the procedure

Include a low vaccum system – used for the filtration steps.

Under the ANALYTICAL PROCEDURE section, starting on page 4:

In the Extraction section, consider specifying that the samples were filtered through the Büchner funnel after the application of vaccum to the filter flask.

Relabel the section "3. Oasis HLB Cartridge Cleanup" as "2. Oasis HLB Cartridge Cleanup". Also relabel the subsequent LC/MS-MS Conditions and Calculations sections as 3 and 4, respectively.

In the LC/MS-MS Conditions section (starting on page 5), should refer to "calibration standards" instead of "linearity standards" as the calibration is non-linear (based on a 2^{nd} order polynomial fit - not on a linear fit). Also, this statement should mention that at least three continuing calibration standards (a mid-range calibration standard) are analyzed within each analytical set.

In the Calculations section (starting on page 6), should specify that the coefficient of determination (r^2) must be greater than 0.99 (or similar) for acceptance of the calibration curve.

In the Calculations section (starting on page 6), should specify that the coefficient of variation (CV) for the continuing calibration standards must be equal to or less than 10% (or similar) for acceptance of the analytical set. This specifies the expected precision for repetitive injections within the sequence.