1.0 PRINCIPLE AND SCOPE

Fluazinam and HYPA are extracted from soil using methanol and a digital sonifier. The extracts are then diluted 1:10 with UHP water. Analysis is performed by LC/MS/MS utilizing TurbolonSpray in the positive ionization mode. This assay uses 10 g sub-samples of soil. Soil should be stored at approximately -20°C.

2.0 SAFETY

Analysts must be acquainted with the potential hazards of the reagents and solvents employed in the laboratory. Such information may be obtained from the MSDS, literature, and other documents such as the laboratory chemical hygiene plan. Disposal of chemicals and reagents must comply with all federal, state, and local regulations.

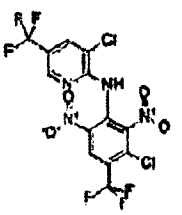
Methanol is a flammable reagent and formic acid is corrosive.

3.0 COMPOUNDS

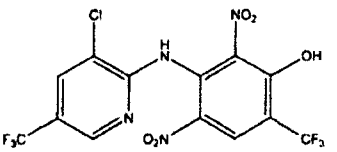
The following reference standards are stored frozen (≤ -20°C), protected from light:

- Fluazinam (IKF-1216)
- HYPA

4.0 CHEMICAL STRUCTURES



Fluazinam



HYPA

5.0 REAGENTS, CHEMICALS AND SUPPLIES

- Acetonitrile (HPLC Grade)
- Acetone (HPLC Grade)
- Methanol (HPLC Grade)
- Formic Acid
- Ultra High Purity Water or HPLC Grade Water

- Column: Synergi Polar-RP 4 µm, 50 × 2.0 mm
- Agilent 1.5 mL Capped Glass Autosampler Vials
- Glass Qorpak Tubes 27.5 × 95 mm with Teflon cap
- 100 mL glass mixing cylinders

6.0 EQUIPMENT

- Centrifuge Beckman Coulter Allegra X-12R (or equivalent)
- API 5000 Mass Spectrometer
- Symbiosis HPLC system
- Branson 450 digital sonifier with cup horn
- Calibrated adjustable pipettes
- Class A volumetric flasks, various sizes
- Class A volumetric pipettes, various sizes
- Analytical balance, 20 mg – 200 g
- Top loading balance, 1 g – 400 g

7.0 PREPARATION OF SOLUTIONS

UHP water (18-megaohm-cm or equivalent) or HPLC water is used wherever water is required. All solutions are mixed well.

10/90/0.02 Acetonitrile/Water/Formic Acid (v/v): Add 100 mL of acetonitrile to a 1000-mL class A volumetric flask and dilute to volume with water. Add 200 µL of formic acid and mix well.

0.1% Formic Acid in Water: Add 1 mL of formic acid to 1 L of UHP water. Mix well.

0.1% Formic Acid in Acetonitrile: Add 1 mL of formic acid to 1 L of acetonitrile. Mix well.

8.0 STABILITY

Per SOP 1.4005[1], all reagents, except 0.1% formic acid in water, have six months stability at ambient temperatures. An expiration of two weeks is given to the 0.1% formic acid in water.

9.0 PREPARATION OF ANALYTICAL SOLUTIONS

Analytical standard stock solutions are stored at < -10°C, in glass containers protected from light and equilibrated to ambient temperature before use. The solutions are remade every three months. The following charts indicate suggested standard solutions and concentrations.

Stock and Intermediate Solutions:

Solution Name	Preparation	Nominal Concentration
A1	*Weigh approximately 50 mg of fluazinam and dissolve in 50 mL acetone (correct for purity)	1000 µg/mL
A2	*Weigh approximately 50 mg of HYPA and dissolve in 50 mL acetonitrile (correct for purity)	1000 µg/mL

*Use an analytical balance capable of weighing to 0.1 mg
Use glass Class A pipettes and volumetric flasks

Fortification Solutions:

Fortification and calibration solutions are stored at < -10°C, in glass containers protected from light, and equilibrated to ambient temperature before use. The solutions are remade every three months. The following charts indicate suggested standard solutions and concentrations.

QC Sample	Preparation	Nominal Concentration
B1	Add 0.1 mL of A1 and A2 to a 100-mL class A volumetric flask and dilute to volume with acetonitrile	1.00 µg/mL
B2	Add 5 mL of B1 to a 50-mL class A volumetric flask and dilute to volume with acetonitrile	100 ng/mL

Use glass Class A pipettes and volumetric flasks
Note: Other dilution aliquots can be used as long as the nominal concentrations are maintained.

Calibration Solutions:

Solution Name	Preparation	Nominal Concentration
C1	Add 5 mL of B2 and dilute to 50 mL with 10/90/0.02 acetonitrile/water/formic acid	10.0 ng/mL
C2	Add 5 mL of C1 and dilute to 25 mL with 10/90/0.02 acetonitrile/water/formic acid	2.00 ng/mL
C3	Add 1 mL of C1 and dilute to 10 mL with 10/90/0.02 acetonitrile/water/formic acid	1.00 ng/mL
C4	Add 5 mL of C1 and dilute to 100 mL with 10/90/0.02 acetonitrile/water/formic acid	0.500 ng/mL
C5	Add 1 mL of C2 and dilute to 10 mL with 10/90/0.02 acetonitrile/water/formic acid	0.200 ng/mL
C6	Add 1 mL of C3 and dilute to 10 mL with 10/90/0.02 acetonitrile/water/formic acid	0.100 ng/mL
C7	Add 1 mL of C4 and dilute to 10 mL with 10/90/0.02 acetonitrile/water/formic acid	0.0500 ng/mL

Use glass class A pipettes and volumetric flasks
Note: Other dilution aliquots can be used as long as the nominal concentrations are maintained.

10.0 EXTRACTION PROCEDURE

1. Weigh 10 grams ± 0.5 g of soil into a glass 40 mL Qorpak tube.
2. Fortify any required laboratory QC samples at this time.
3. Add 30 mL of methanol to the samples and QC samples.
4. Shake by hand for a few seconds to disperse and wet the sample.
5. Subject samples to pulse sonication for 10 minutes using the Branson 450 digital sonifiers w/ cup horn, pulsed signal at 50 seconds on and 10 seconds off at 80% of capacity.
6. Centrifuge the samples at 2000 rpm for five minutes.
7. Without disturbing the sample at the bottom, decant the extract into a 100-mL graduated mixing cylinder.
8. Repeat Steps 4 through 7 with 20 mL of methanol. Be sure to break up the pellet at the bottom of the tube prior to sonication.

9. The 100-mL graduated mixing cylinders are brought to a final volume of 50 mL with methanol. Mix by inversion before aliquotting.

10. Take 0.100 mL of extract and aliquot it into an autosampler vial containing 900 µL of UHP water for a 1:10 dilution. Other dilution schemes may be used as necessary. Mix by vortexing prior to analysis.

11.0 ANALYSIS PROCEDURE

Typical HPLC Conditions

HPLC System:	Symbiosis Pharma
Column:	Synergi Polar-RP (4 µm, 50 × 2.0 mm)
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
Needle Wash:	Methanol

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

Typical Mass Spectrometer Conditions

Mass Spectrometer:	Applied Biosystems, API 5000
Interface:	Turbo IonSpray, 550°C
Polarity:	Positive
Scan Type:	MRM
Resolution:	Q1: Low, Q3: Unit

Compound	Transition (amu)	Dwell Time (ms)	DP
Fluazinam Quant	465.2→373.0	100	110
Fluazinam Conf	465.2→338.0	100	110
HYPA Quant	447.0→355.0	100	110
HYPA Conf	447.0→382.9	100	110

12.0 TYPICAL ANALYTICAL SEQUENCE

A validation batch's analytical sequence contains at a minimum a reagent blank, two untreated control (UTC) samples, five UTC samples fortified at the limit of quantitation (LOQ), 0.01 µg/g, and five UTC samples fortified at 10×LOQ, 0.1 µg/g. The six calibration standards are interspersed throughout the run.

13.0 SYSTEM SUITABILITY

The LC/MS system is considered ready for analysis when the signal-to-noise ratio, using peak-to-peak, of the low standard is greater than or equal to 5:1.

14.0 COLUMN PROCEDURES

Synergi Polar-RP columns, when not in use, should be stored in 100% acetonitrile.

15.0 QUANTITATION METHOD

A linear 1/x regression is used.

16.0 CALCULATIONS

Calibration standards can be embedded throughout the analytical batch. Instrument control and data collection are accomplished using the Analyst® 1.4.2 software program. The data are analyzed and the raw nanogram amounts calculated using the Analyst® 1.4.2 software program.

A calibration curve calculated by linear regression with 1/x weighting was determined to be a best fit of the data during method validation. From this curve, the concentration of analyte in the sample was calculated using the equation

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

where

y = the analyte peak area
x = the concentration of the analyte in ng/g

The coefficients, m and b, were calculated automatically by the validated Analyst® 1.4.2 software program. The software also corrects for the dilution factor or the amount by the initial extract was diluted to yield a concentration within the calibrated range.

The standard concentration in ng/g can be calculated as follows:

$$AC\,(ppb) = \frac{x_i \times V_f}{W} \qquad (2)$$

where

AC = Analyte concentration found
x_i = Standard concentration (ng/mL)
V_f = Final volume of extract (50 mL)
W = Nominal sample weight (10 g)

17.0 ACCEPTANCE CRITERIA

Individual recoveries should be between 70-120%. Calibration curves should exhibit a correlation coefficient of 0.995 or better. If one analyte passes and another fails, the passing analyte may be accepted.

18.0 REFERENCES

1. "Preparation and Labeling of Reagents and Solutions." Pyxant Labs Inc. SOP 1.4005. Rev 13, February 12, 2010.